RHODES UNIVERSITY
Where leaders learn

A molecular investigation of stem-galling *Tetramesa* Walker (Hymenoptera: Eurytomidae) on African grasses: applications to biological control

By

Clarke J. M. van Steenderen

A thesis submitted in fulfilment of the requirements for the degree
of Doctorate of Philosophy in Entomology

Department of Zoology and Entomology
Centre for Biological Control

January 2023

ORCID iD: 0000-0002-4219-446X

This thesis is dedicated to my loving mother, Thea

Abstract

South Africa is a larger donor than receiver of alien grasses, where approximately 15% (± 165 spp.) of the country's native grass species have become naturalised elsewhere. Many of these grasses have become serious invaders, causing significant damage to native species, habitat structure, and ecosystem functioning. Biological control is a sustainable and cost-effective method for the control of invasive weeds, but its application to invasive grasses has been approached with trepidation in the past due to the fears of a lack of host-specific herbivores that may cause non-target damage to agriculturally-important crops.

The *Tetramesa* Walker genus (Hymenoptera: Eurytomidae) is one of three genera in the family that feed exclusively on grasses, and have a record of being host-specific to a particular species, or complex of closely-related congeners. There are over 200 described *Tetramesa* species, but this taxonomic effort has occurred almost exclusively in the Northern Hemisphere. Only about 2% of the described species are from Africa, with none from southern Africa despite the high diversity of grasses in the region. The low morphological variability between many *Tetramesa* groups has made identification difficult, where there may in fact be multiple undiscovered cryptic species.

This thesis generated genetic sequence data (mitochondrial COI and nuclear 28S) that revealed at least eight native southern African *Tetramesa* taxa that are new to science, focusing particularly on the assemblages associated with *Eragrostis curvula* Nees (African lovegrass) and *Sporobolus pyramidalis* Beauv. and *S. natalensis* Steud. (giant rat's tail grass) which are alien invasive pests in Australia. Approximately 200 eurytomid wasps were collected and sequenced from 19 grass species across six South African provinces. Additionally, 27 grass species were sequenced using four gene regions (rps16-trnK, rps16, rpl32-trnL, and ITS), which were added to existing sequence data to build a dataset comprising over 700 sequences. Field host ranges and the use of host grass genetic proxies were important in making inferences about the host-specificity of eurytomid wasps of interest.

Nine *Tetramesa* groups appeared to be host-specific to a single grass species, while six *Tetramesa* were associated with multiple species in a single grass genus. Since *S. pyramidalis*, *S. natalensis*, *S. africanus*, *Hyparrhenia hirta*, *E. trichophora*, and *Andropogon gayanus* are weeds elsewhere, there are at least six potential *Tetramesa* biological control agents that have been identified.

A high diversity of *Tetramesa* on grasses within the *Eragrostis* genus was reported, with at least four taxa associated with *E. curvula.* It is currently uncertain whether these taxa represent different cryptic species or intraspecific populations that are the result of geographic sub-structuring. No-choice host-specificity testing using *Tetramesa sp. 4* on *E. curvula* revealed that the wasp could complete its lifecycle on two non-target African grasses; namely *E. plana* and *E. planiculmis.* The wasp was also recorded on other *Eragrostis* species in the field (namely *E. biflora* and *E. capensis*). Using grass genetic sequences obtained in this study, it was found that there are four native Australian *Eragrostis* species that are more closely related to target *E. curvula* than to the non-target *E. plana* and *E. planiculmis.* This suggests that *Tetramesa sp. 4* may not be suitably host-specific for use as a biological control agent. Further host-specificity testing on these native Australian species is required, however, before this insect is ruled out completely. The *Tetramesa* on *S. pyramidalis* (*Tetramesa sp. 1*), and the unidentified *Sporobolus* species presumed to be *S. africanus*, were suitably host-specific to be used as biological control agents.

Since it was unclear whether some phylogenetic clades were true species or intraspecific populations, which is essential to understand when selecting agents for biological control, a new piece of software, “SPEDE-sampler”, was developed. It offers users of the Generalised Mixed Yule Coalescent (GMYC) species delimitation model a means of assessing the degree to which sampling effects such as data size and parameter choice can influence species diversity estimates. When applied to the *Tetramesa* data set, the software assisted in identifying which groups may contain cryptic species, uncovering that the COI marker is affected more by singletons than the 28S marker (i.e. species diversity tends to be overestimated), and confirming putative *Tetramesa* taxa that could be useful for biological control programmes going forward.

This thesis has provided evidence that South Africa contains a diverse assemblage of

Tetramesa and other eurytomids that are closely associated with their grass hosts, and that many of these taxa hold promise for grass biological control. This work has also highlighted the importance of integrative taxonomy in the discovery of novel taxa, and that biological control practitioners need to be aware of the caveats of each line of evidence used in the delimitation of putative species.

Research outputs

Publications

1. Sutton, G.F., **van Steenderen, C.J.M.**, Yell, L.D., Canavan, K., McConnachie, A., and Paterson, I.D. First record of an African grass-feeding wasp (*Tetramesa*; Eurytomidae) on the invasive grass *Eragrostis curvula* (African lovegrass; Poaceae) in Australia. 2023. *BioInvasions Records*. In press.
2. **van Steenderen, C.J.M.** and Sutton, G.F. 2022. SPEDE-sampler: an R Shiny application to assess how methodological choices and taxon-sampling can affect Generalised Mixed Yule Coalescent (GMYC) output and interpretation. *Molecular Ecology Resources* 20(2) doi: 10.1111/1755-0998.13591

Conference and Poster Presentations

1. **van Steenderen, C.J.M.**, Sutton, G.F., Yell, L., Canavan, K., Chari, L.D., McConnachie, A., and Paterson, I.D. The *Tetramesa* (Hymenoptera: Eurytomidae) as biological control agents of invasive African grasses: a molecular investigation. XVI International Symposium on Biological Control of Weeds. Puerto Iguazú, Argentina. Session 1: Taxonomy and biological control. 7 - 12 May 2023.
2. **van Steenderen, C.J.M.**, Paterson, I.D., Sutton, G.F., and Canavan, K. A genetic investigation of the native stem-galling *Tetramesa* Walker (Hymenoptera: Eurytomidae) in South Africa, and their potential use as biological control agents. 22nd Hybrid Congress of the Entomological Society of Southern Africa (ESSA). 28 June - 1 July 2021.
3. Sutton, G.F., **van Steenderen, C.J.M.**, Canavan, K., Yell, L., and Paterson, I.D. South Africa is a hotspot for previously unknown stem-boring wasps of grasses (*Tetramesa*; Eurytomidae). Grassland Society of Southern Africa, 56th Annual Congress. July 26 - 30 2021.

4. **van Steenderen, C.J.M.** and Sutton, G.F. 2022. SPEDE-sampler: An R Shiny application to assess how methodological choices and taxon-sampling may affect GMYC species delimitation. International Statistical Ecology Conference (ISEC). 27 June - 1 July 2022.
5. Sutton, G.F., Yell, L.D., **van Steenderen, C.J.M.**, McConnachie, A., Harvey, K., and Paterson, I.D. Biological control of African Lovegrass (*Eragrostis curvula*): native-range surveys in Africa. 22nd Australasian Weeds Conference. 25 - 29 September 2022.

Declaration

This thesis has not been submitted, either in the same or different form, to this or any other university for a degree, and it represents my own work. The content of this document, save for that which is properly acknowledged, is my own. All of the associated R code and input files for the data analyses presented in this thesis are available on the GitHub repository: `https://github.com/clarkevansteenderen/PhD_files`.

The ethics clearance number for this project is 2022-3842-6516.

Clarke van Steenderen

January 2023

Acknowledgements

This doctoral thesis is the product of a highly challenging, but rewarding journey. It is built upon the support and assistance from the following individuals and organisations:

The Centre for Biological Control (CBC), Rhodes University, and the Department of Forestry, Fisheries and the Environment (DFFE) for the provision of funding. It is a pleasure and honour to be part of the CBC group, which is not only a global leader in biological control research, but also a welcoming, supportive family. Rhodes University, too, has been my home for the last nine years, and I am incredibly grateful for the opportunities that it has afforded me; both academically and personally. My university experiences have been invaluable, and have shaped me into the person I am today.

My supervisors, Prof. Iain Paterson and Dr. Guy Sutton, for their valuable feedback, assistance, and encouragement throughout the course of this degree. I am very thankful to have had the opportunity to work with these excellent researchers, and have learnt so much from them. I am especially lucky to have them as both mentors and as friends. Thanks also to Guy for all the chats about statistics and coding, for sharing scripts and coding ideas with me, and for his enthusiasm and willingness to always go the extra mile.

The three external examiners who reviewed this thesis: Dr John Gaskin (United States Department of Agriculture), Dr Robert Plowes (Brackenridge Field Laboratory, University of Texas at Austin, USA), and Dr. Carla Lambertini (Department of Biosciences of the University of Milan, Italy). I wish to thank them for the time they took in providing me with invaluable feedback.

The Grass Gang: Kim Canavan, Pippa Muskett, Liam Yell, and Lenin Chari, for collecting specimens and sharing their ideas with me, and for their friendship.

Prof. Martin Hill, for all his support and good humour since my undergraduate years at Rhodes University. The Entomology Department is incredibly lucky to have someone as resourceful and passionate as he is. My original intention was to complete a B.Sc degree in software development, but after attending Martin's Entomology lectures in my first year, my

career path turned from predominantly computer bugs to six-legged bugs and wasps! I am grateful that this PhD has allowed me to blend my interests in software development and Entomology, and that the CBC has facilitated and encouraged this work.

The students and staff at the CBC and the Department of Zoology and Entomology at Rhodes, with a special thanks to Jeanne van der Merwe and Yvain Erasmus for their assistance in organising payments and other administrative work. Also thanks to Kim Weaver for organising conferences and other related events. Prof. William Froneman and Prof. Nicole Richoux (HODs) are thanked for all the hard work that they have put into the Department to keep it running smoothly. Thanks also to Prof. Shelley Edwards for the use of the molecular laboratory facilities.

Dr. Helen James and Mr. Musa Mlambo at the Albany museum for the use of their microscope and imaging software, and Abigail Kirkaldy for showing me some of the photographing tricks up her sleeve, and for the chats about phylogenetics.

Prof. Martin Villet for sharing his ideas and valuable reading material with me, for encouraging me to produce my best work, and for teaching me something new with each conversation we have.

Dr. Brendan Wilhelmi for all the squash coaching and kindness that he has shown over the years. His passion for coaching and giving of himself has been truly inspiring.

My family, and friends; particularly Anthony Evlambiou, Evans Mauda, Abigail Kirkaldy, Joshua van Staden, Megan Reid, Zilla Brown, and Rosemary Batchelor (in no particular order). Good friendships are invaluable, and I thank you all for making my path that much easier to tread. I also write this in remembrance of my aunty Anne Brimelow, who passed away less than a month before the submission of this thesis. She was always so proud of me, and I wish that she could have shared in the celebration of the completion of this work.

My father, Ronald, for sharing his interest in science and mathematics with me, and for showing me that determination, hard work, and logical thinking are the building blocks of a successful career.

My mother, Thea, for her unconditional love, for always encouraging me, and for assuring me that my best is enough. Thank you for being my biggest support system, and for being the best mother a child could ask for. I am especially proud that I have sparked your interest

in insects and the other little creepy crawlies with whom we share the Earth.

My loving partner, Elisa, for supporting me through the good and tough times, and for always believing in me. Thank you for all the music we have played, laughs we have shared, and memories we have made together. I treasure them all.

Abbreviations, Symbols, and Units

Abbreviations

ABGD	Automatic Barcode Gap Discovery
AMOVA	Analysis Of Molecular Variance
ANOVA	Analysis Of Variance
ARC-PPRI	Agricultural Research Council - Plant Protection Research Institute
ASAP	Assemble Species by Automatic Partitioning
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BI	Bayesian Inference
BIC	Bayesian Information Criterion
BIN	Barcode Index Number
BOLD	Barcode of Life Database
bp	Base Pairs
BPP	Bayesian Phylogenetic sand Phylogeography
bPTP	Bayesian Poisson Tree Processes
bt	Bootstrap Support
C:N	Carbon to Nitrogen Ratio
CBC	Centre for Biological Control
CI	Confidence Interval

COI	Cytochrome c Oxidase Subunit I
COII	Cytochrome c Oxidase Subunit II
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
EC	Eastern Cape
EL	East London
ESS	Effective Sample Size
FS	Free State
FST	Fixation Index
GBIF	Global Biodiversity Information Facility
GHT	Grahamstown/Makhanda
GMYC	Generalised Mixed Yule Coalescent
GPS	Global Positioning System
GR	Growth Regulator
GTR	General Time-Reversible
GUI	Graphical User Interface
HAD	Host-Associated Differentiation
HPD	Highest Posterior Density
HSTU	Host-Specific Taxonomic Unit
IAP	Invasive Alien Plant
ISSR	Inter-Simple Sequence Repeat

ITS	Internal Transcribed Spacer
ITS2	Internal Transcribed Spacer II
K2P	Kimura 2-Parameter
K80	Kimura 80
KZN	KwaZulu-Natal
lnL	Log-Likelihood
LP	Limpopo
ltt	Lineage Through Time
MCC	Maximum Clade Credibility
MCMC	Markov Chain Monte Carlo
MDPL	Mondplaas
ML	Maximum Likelihood
MOTU	Molecular Operational Taxonomic Unit
MP	Mpumalanga
MPR	Maximum Parsimony Reconciliation
MSA	Multiple Sequence Alignment
mtDNA	Mitochondrial DNA
NC	Northern Cape
nDNA	Nuclear DNA
NH	Northern Hemisphere
NPNS	Non-Pronotal Spot

NTA	Non-Target Attack
numt	Nuclear Mitochondrial DNA
ORF	Open Reading Frame
PACo	Procrustean Approach to Cophylogeny
PCR	Polymerase Chain Reaction
PE	Port Elizabeth/Gqeberha
PNS	Pronotal Spot
pp	Posterior Probability
PTP	Poisson Tree Processes
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RTU	Recognisable Taxonomic Unit
SA	South Africa
SH	Southern Hemisphere
SNP	Single Nucleotide Polymorphism
TBE	Tris-borate-EDTA
TD-PCR	Touchdown PCR
USA	United States of America
VOC	Volatile Organic Compound
WCPM	Wapshere Centrifugal Phylogenetic Model

ZIM	Zimbabwe

Phylogenetic Clades

ADON	*Arundo donax (Tetramesa romana)*
AGAYA	*Andropogon gayanus*
EBI	*Eragrostis biflora*
ECUR	*Eragrostis curvula*
EPLAN	*Eragrostis plana*
ERIG	*Eragrostis rigidior*
ETEF	*Eragrostis tef*
ETRICH	*Eragrostis trichophora*
HHIR	*Hyparrhenia hirta*
MIX	Mixed; multiple host plant species
NPNS	Non-Pronotal Spot
PNS	Pronotal Spot
SPOR	*Sporobolus*
SPYR	*Sporobolus pyramidalis*
U	Unknown

Symbols and Units

Γ	Gamma
μ	Micro
AU$	Australian Dollar

df	Degrees of Freedom
g	Gram
ha	Hectare
km	Kilometre
L	Litre
M	Molar
m	Metre
$M^{2}{}_{XY}$	Global Goodness-of-Fit Statistic
min	Minute
mm	Millimetre
my	Million Years
mya	Million Years Ago
n	Nano
PSV	Phylogenetic Species Variability
S_{TD}	Taxonomic Host Specificity
US$	United States Dollar
V	Volt
°C	Degrees Celsius

Contents

1 General Introduction 1

1.1 Introduction 1

1.2 The Poaceae 1

1.3 Impacts of invasive grasses 3

1.3.1 Biodiversity loss and habitat degradation 3

1.3.2 Fire cycles 4

1.3.3 Water dynamics 6

1.3.4 Agriculture 7

1.3.5 Hybridisation 7

1.3.6 Vectors of disease and spread of pathogens 7

1.4 Graminivorous insects 9

1.5 Invasive grass control methods 10

1.5.1 Manual 10

1.5.2 Chemical 12

1.5.3 Biological Control: a general overview 13

1.5.4 Native range surveys 15

1.5.5 Identification of target weeds and their agents 16

Cryptic species 16

1.5.6 Agent selection and prioritisation 20

1.5.7 Host specificity testing 20

1.5.8 Mass-rearing, release, and post-release evaluations 21

1.6 Control of Invasive Grasses 22

1.7 The *Tetramesa* wasps 27

1.7.1 Overview 27

1.7.2 Taxonomy . . . 27

1.7.3 *Tetramesa* as biological control agents . . . 29

1.7.4 Extent of species discovery to date . . . 30

1.7.5 Molecular tools and taxonomy . . . 32

1.8 Research Objectives . . . 34

2 *Tetramesa* Phylogenetics 36

2.1 Introduction . . . 36

2.1.1 Native-Range Surveys . . . 36

2.1.2 Taxonomy and Molecular Species Delimitation . . . 38

2.1.3 Aims . . . 42

2.2 Materials and Methods . . . 42

2.2.1 Sample Collection . . . 42

2.2.2 Sample preservation . . . 45

2.2.3 Wasp genetics . . . 46

DNA extraction . . . 46

Polymerase Chain Reaction (PCR) . . . 46

2.2.4 Nucleotide Sequencing . . . 47

2.2.5 Sequence Alignment . . . 48

2.2.6 Phylogenetic Analyses . . . 49

2.2.7 Species delimitation . . . 51

Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP) . . . 52

Generalised Mixed Yule Coalescent model (GMYC) . . . 52

Haplowebs . . . 53

Possion Tree Processes (PTP) and Bayesian Poisson Tree Processes (bPTP) . . . 53

2.2.8 Genetic divergence across geographic space . . . 53

2.3 Molecular clock dating . . . 55

2.4 Results . . . 56

2.4.1 COI data . . . 63

Species delimitation . . . 65

2.4.2 28S data . . . 67

Species delimitation . . . 69

2.4.3 Pronotal spot *Eragrostis curvula Tetramesa* wasps: genetic divergence across geographic space . . . 70

2.4.4 Nuclear clock dating . . . 72

2.5 Discussion . . . 76

2.5.1 Southern African *Tetramesa* phylogenetics . . . 76

2.5.2 Radiation patterns and unresolved clades . . . 79

2.5.3 Biological control outlook . . . 81

Eragrostis curvula . . . 82

Sporobolus pyramidalis . . . 84

Hyparrhenia hirta and *Andropogon gayanus* . . . 85

2.6 Conclusion . . . 85

3 Cophylogenetic analyses **87**

3.1 Introduction . . . 87

3.2 Materials and Methods . . . 91

3.2.1 Sample collection and sourcing . . . 91

3.2.2 Grass DNA extraction . . . 92

Grass Polymerase Chain Reaction . . . 93

3.2.3 Cophylogenetic analyses . . . 93

Genetic distances . . . 95

3.3 Results . . . 95

3.3.1 Phylogenetic placements . . . 95

3.3.2 Cophylogenetic relationships . . . 100

3.3.3 Event-based cophylogenetics . . . 103

3.4 Discussion . . . 105

3.4.1 Conclusion . . . 110

4 SPEDE-sampler software 111

4.1 Introduction 111

4.2 Materials and Methods 113

4.2.1 Workflow 113

4.2.2 Calculation of GMYC metrics 117

4.2.3 Sampling effects on *Tetramesa* species delimitation 121

4.2.4 Host-specificity indices 121

4.3 Results 122

4.3.1 Nuclear 28S 122

4.3.2 Mitochondrial COI 123

4.4 Discussion 126

4.4.1 Sample size and population structure 126

4.4.2 Singletons 127

Sampling effects 129

4.4.3 Relevance to grass biological control 129

Delimited genetic groups 130

4.4.4 Conclusion 132

5 General Discussion 135

5.1 Are the *Tetramesa* suitable biological control agents of invasive southern African grasses? 136

5.1.1 Herbivorous eurytomids on African grasses 136

5.2 Taxonomic and phylogenetic challenges 139

5.2.1 Identification 139

5.2.2 Evolutionary history 142

5.3 Sampling effects in phylogenetics and species delimitation 145

5.4 Conclusion 146

References 147

6 Supplementary Section 198

List of Figures

1.1 A summary of the main impacts of invasive grasses on an ecosystem. See the references in Section 1.3. 8

1.2 (A) burning gamba grass (*Andropogon gayanus*) in northern Australia (credit: CRC for Weed Management), (B) Saguaro cacti damaged by fire in the Saguaro National Park (credit: Perry Grissom), (C) invasive *Arundo donax* along the Rio Grande River, USA (credit: John Goolsby), and (D) African lovegrass (*Eragrostis curvula*) infestation in New South Wales, Australia (credit: Andrew McConnachie). 9

1.3 Examples of cryptic species, where groups are morphologically similar but genetically distinct. (A) and (B) *Eccritotarsus catarinensis* male and female, respectivley, (C) and (D) *E. eichhorniae* male and female, respectively (Henry, 2017). 19

1.4 The Centre for Biological Control's (CBC) (A) Waainek water weeds and (B) Uitenhage cactus mass rearing facilities for biological control agents. (C) The release of *Dactylopius opuntiae* 'stricta' lineage on *Opuntia stricta* in the Eastern Cape, South Africa. (D) The release of boxes of *Tetramesa romana* over the Rio Grande River, USA, from a Cessna aircraft. Images A, B, and C: credit to the CBC; image D taken from Racelis *et al.* (2010). 23

1.5 Synonyms used for the *Tetramesa* Walker genus since its first description in 1832. Data were sourced from the Natural History Museum Universal Chalcidoidea Database (`https://www.nhm.ac.uk/our-science/data/chalcidoids/database`). 28

1.6 (A) *Tetramesa romana* pupal stages and mature adult (adult females measure $\sim$ 5.4 mm in length (Moran and Goolsby, 2009)), (B) gall formation (blue arrow) and adult wasp exit hole (red arrow) in *Arundo donax*, (C) multiple exit holes in *A. donax*, (D) an adult *T. romana* female ovipositing in the stem of *A. donax*, and (E) the endophagous feeding of a *Tetramesa* larva. Figures A and B taken from Jiménez *et al.* (2017), Figure C credit: Quentin Paynter, Figures E and E credit: John Goolsby. 31

1.7 The global distribution of recorded and described *Tetramesa* species, as sourced from the literature (see the file in the 'maps' folder in `https://github.com/clarkevansteenderen/PhD_files`, compiled by Guy Sutton). 32

2.1 The top ten invasive plant families (A) most represented on a global scale (adapted from (Pyšek *et al.*, 2017)), and (B) originating from South Africa (adapted from Pyšek *et al.* (2020)). The percentages in plot (A) are the proportions of invasive species relative to the total number of described species in the family. 40

2.2 Distributions of some problematic African grasses in their native ranges, and invaded ranges in Australia. Data were sourced from the GBIF (Global Biodiversity Information Facility) database using the R spocc (SPecies OCCurrence) package (`https://github.com/ropensci/spocc`). 41

2.3 A) *Andropogon gayanus*, B) *Eragrostis curvula*, C) *Megathyrsus maximus*, and D) *Sporobolus pyramidalis*. Images taken from Plants of the World Online (POWO) (`http://www.plantsoftheworldonline.org/`). Facilitated by the Royal Botanic Gardens, Kew. 43

2.4 An elevation map of South Africa showing the collection sites of *Tetramesa* specimens used in the present study. 44

2.5 Counts of the wasp specimens collected across 19 host grasses. The bars represent the distribution of specimens collected, where *Eragrostis curvula* had the greatest sampling effort due to its prioritisation as a target for biological control. EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, LP = Limpopo, MP = Mpumalanga, NC = Northern Cape, ZIM = Zimbabwe. All species, except *Arundo donax*, are native South African grasses. 44

2.6 A) PNS *Tetramesa* (KCP 2021 00194) and B) NPNS eurytomid specimen (KCP 2021 00245). The prominent pronotal spot in (A) is shown with a red arrow. Scale bars represent 1 mm. 45

2.7 An example of a poly-T region at the start of a COI read, sequenced in the forward direction using primer FWPTF1 for *Tetramesa* sample GFS-2019-00079. The presence of this homopolymer often disrupts the sequencing process, causing the slippage pattern and double peaks visible here. 48

2.8 An elevation map of South Africa showing the localities of PNS *Tetramesa* specimens collected on *E. curvula* host plants. EC = Eastern Cape, FS = Free State, MP = Mpumalanga, NC = Northern Cape. 54

2.9 Bayesian inference phylogeny for the COI mitochondrial gene region. Coloured boxes next to sequence names indicate collection localities, as per the legend. Solid rectangular bars to the far right are the results of a suite of species delimitation tests. Samples that share the same coloured bar within each column belong to the same MOTU. (1) Blue text *Tetramesa* are sequences obtained from the BOLD database, (2) green text next to sample names indicate the host plant from which the sample was collected, (3) dark brown text indicates taxonomic identifications, (4) red and orange filled circles denote posterior probability values greater than or equal to 0.9, and between 0.8 and 0.95, respectively. Numbers above and below branches are BI posterior probability and ML bootstrap support values, respectively. Species delimitation results for the non-target polytomy groups are deliberately not displayed. See the list of phylogenetic clade abbreviations on page xv. 59

2.10 Histograms A - H are interspecific 28S and COI p-distance plots (K2P, 1000 bootstrap repeats) for *Tetramesa sp. 1* (PNS, *Sporobolus pyramidalis*), *Tetramesa sp. 2* (NPNS, *Sporobolus pyramidalis*), *Tetramesa sp. 4* (PNS, *Eragrostis curvula*), and *Tetramesa sp. 5* (NPNS, *Eragrostis curvula*) relative to the other major groups in the associated phylogenies. PNS = pronotal spot, NPNS = non-pronotal spot. 60

2.11 Bayesian inference phylogeny for the 28S nuclear gene region. Individually-coloured boxes next to sequence names represent host grass species as per the legend. Solid rectangular bars to the far right are the results of a suite of species delimitation tests. Samples that share the same coloured bar within each column belong to the same MOTU. (1) Brown triangles denote *Tetramesa* sequences obtained from GenBank, (2) green text next to sample names indicate the host plant from which the sample was collected, (3) dark brown text indicates taxonomic identifications, (4) red text marks samples that the GMYC analysis grouped as a separate MOTU, (5) red and orange filled circles denote posterior probability values greater than or equal to 0.9, and between 0.8 and 0.95, respectively. Numbers above and below branches are BI posterior probability and ML bootstrap support values, respectively. Species delimitation results for the non-target polytomy groups are deliberately not displayed. Genus names are shortened as: *C* = *Cynodon*, *E* = *Eragrostis*, *Eu* = *Eustachys*, *P* = *Panicum*, *S* = *Sporobolus*. See the list of phylogenetic clade abbreviations on page xv. 62

2.12 The unique gall formed on *Eragrostis trichophora*. Image: Guy Sutton. . . . 64

2.13 TCS Haplotype networks (28S gene region) for the pronotal spot (PNS, n = 91) and non-pronotal spot (NPNS, n = 59) clades marked with the vertical gold and grey bars in Figures 2.11 and 2.11. Hash marks denote the number of nucleotide differences between haplotypes, and the different colours represent the host grass species shown in the figure legend. 68

2.14 Genetic K2P distances plotted against geographic distance (km) as pairwise matrices, for *Tetramesa sp. 4* collected on *Eragrostis curvula* host plants. . 71

2.15 A COI TCS haplotype network for the Eastern Cape *Tetramesa* collected on *Eragrostis curvula*. The numbers between nodes indicate the number of mutations between haplotypes. 72

2.16 A Bayesian time-calibrated phylogeny for the nuclear 28S rRNA data set, showing important divergence events and points of origin, and the radiation of the *Tetramesa* (green shaded region). The horizontal blue lines through each node represent 95% HPD confidence intervals. See the associated text for more details. The lineage through time (ltt) plot shows the number of lineages (N) over millions of years. 75

2.17 A schematic diagram illustrating how mitonuclear discordance can occur between two gene trees. The arrows denote climatic cycles that can lead to changes in the degree of gene flow between taxa, where mitochondrial markers often overestimate sequence divergences while nuclear markers more readily reveal renewed gene flow. Diagram adapted from Després (2019). 79

3.1 A hypothetical cophylogenetic scenario, showing the relationship between a herbivore (green dashed line) and host (black solid line) phylogeny. Adapted from Charleston and Perkins (2006). 89

3.2 Summary phylogenetic relationships for selected *Sporobolus* species, particularly those closely related to *S. pyramidalis*. (A) nuclear ITS gene (Peterson *et al.*, 2014), (B) Chloroplast genes rpl32-trnL + rps16-trnK + rps16 (Peterson *et al.*, 2014), (C) RAPD markers (Shrestha *et al.*, 2005), and (D) ITS gene region (Shrestha *et al.*, 2003). 92

3.3 Maximum Likelihood phylogenies for the Eurytomidae (left; COI) and their host grasses (right; ITS). The coloured lines linking sequences represent herbivore-host associations. The clades marked “E” and “S” in the grass phylogeny are the clades that the pronotal spot *Eragrostis* and *Sporobolus* wasps might be able to utilise and should be prioritised for host-specificity testing. The text colour of the grass sequences of interest represent their native ranges (sourced from the Kew Plants of the World Online database: `https://powo.science.kew.org/results?`); green = Africa, purple = Asia and the Pacific, blue = Australia, red = North America, and orange = South America. 97

3.4 Maximum Likelihood phylogenies for the Eurytomidae (left; COI) and their host grasses (right; chloroplast rpl32-trnL, rps16-trnK, and rps16). The coloured lines linking sequences represent herbivore-host associations. The clades marked “E” and “S” in the grass phylogeny are the clades that the pronotal spot *Eragrostis* and *Sporobolus* wasps might be able to utilise and should be prioritised for host-specificity testing. The text colour of the grass sequences of interest represent their native ranges (sourced from the Kew Plants of the World Online database: `https://powo.science.kew.org/results?`); green = Africa, purple = Asia and the Pacific, blue = Australia, red = North America, orange = South America, black = unknown, widespread or cosmopolitan. 98

3.5 Extracts of the *Eragrostis* clades of interest from Fig. 3.4 (the sections marked “E”). (A) *Eragrostis* ITS, (B) *Eragrostis* chloroplast gene trees. The yellow highlights show the grass specimens included from the present study, and the arrows and green triangles indicate the position of target *E. curvula*. Specimen names are coloured by their native range, as indicated in the key. Values above the branches are bootstrap support values. 99

3.6 Genetic ITS p-distances (K2P, 1000 bootstrap repeats) for all the *Eragrostis* species shown in Figure 3.5 (A) relative to *Eragrostis curvula*. Species names are coloured according to their native range. The black and red bars indicate additional host plants that *Tetramesa sp. 4* can utilise based on field range observations and no-choice host-specificity testing. The arrows indicate that *Tetramesa sp. 4* may be able to utilise all the hosts above *E. plana* and *E. planiculmis*. 100

3.7 A cophylogenetic plot (grass chloroplast on the left (rpl32-trnL, rps16-trnK, and rps16), eurytomid COI phylogeny on the right) resulting from the R `PACo` analysis, where the thickness of the lines (thicker lines = smaller residuals) represent the relative strength of the association derived from residuals, where the thicker lines denote stronger support for phylogenetic congruence. The thickest line between *E. biflora* and its wasp represents a residual value of 0.0095, while the thinnest line between *Sporobolus pyramidalis* and its wasp represents a residual value of 0.052. 101

3.8 Residual plot for the cophylogenetic interactions shown in Figure 3.7. The smaller the residual value, the greater the support for phylogenetic congruence. The blue lines = *Eragrostis* wasps, the black line = *Tetramesa romana* on *Arundo donax*, and the red lines = *Sporobolus pyramidalis* wasps. PNS = pronotal spot, and NPNS = non-pronotal spot. 102

3.9 A residual box plot for the PNS and NPNS eurytomid interactions with *Eragrostis* hosts. The greater residual values in the NPNS group indicates lower support for phylogenetic congruence. 102

3.10 A Maximum Parsimony Reconciliation (MPR) phylogeny from eMPRess, showing the mapping of the eurytomid phylogeny into the grass phylogeny. Values shown at event-nodes indicate the probability of that event occurring. 103

3.11 A p-value histogram for the grass-eurytomid relationships, where the red line indicates the optimal reconciliation cost of the coevolution trees, and the grey columns represent the optimal cost of the same trees constructed with tip associations permuted at random 1000 times. 104

3.12 Distributions of *Eragrostis curvula*, *E. plana*, and *E. rigidior* in South Africa, with the bottom right map showing an overlap of the distributions. Data were sourced from the GBIF (Global Biodiversity Information Facility) database using the R spocc (SPecies OCCurrence) package (`https://github.com/ropensci/spocc`). 109

4.1 Illustrated workflow for the SPEDE-sampler R Shiny application. Resampling of FASTA sequences →creation of BEAST phylogenies →GMYC analyses →results →plotting. Figure created with BioRender.com 114

4.2 A detailed diagrammatic explanation of how SPEDE-sampler determines cases of merges, splits, exact matches, and singletons in a hypothetical example of one GMYC analysis (i.e. one BEAST phylogeny input). A merge occurs when the user has overestimated the number of groups, and the GMYC has lumped them into one (merge type I). A merge is recorded even if one group is a singleton (for example spW in merge type II). A match can take two forms; (1) a split, where the user has underestimated the number of groups, or (2) an exact match, where a user-defined group and a GMYC species delimitation corroborate exactly. Singletons occur when there is one sequence representing a GMYC species. Figure created with BioRender.com 119

4.3 SPEDE-sampler results for 93 28S sequences. (A1 and A2) The number of clusters and entities across subsetted data set sizes. The light blue band represents a 95 confidence interval. The dotted lines at y = 14 is the number of predefined morphospecies. (B) A boxplot of the splitting ratios across dataset sizes, including (blue) and excluding (red) singletons. The dotted line at y = 1 indicates the expected ratio if no splitting occurred. (C) A boxplot of the percentage of singletons across data set sizes. (D) The mean splitting ratios of morphospecies groups that exceeded a ratio of 1 (dotted horizontal line). (E) A boxplot of the percentage matches between predefined morphospecies and GMYC species, including (blue) and excluding (red) singletons. White triangles represent means. 124

4.4 SPEDE-sampler results for 91 COI sequences. (A1 and A2) The number of clusters and entities across subsetted data set sizes. The light blue band represents a 95% confidence interval. The dotted lines at y = 14 is the number of predefined morphospecies. (B) A boxplot of the splitting ratios across dataset sizes, including (blue) and excluding (red) singletons. The dotted line at y = 1 indicates the expected ratio if no splitting occurred. (C) A boxplot of the percentage of singletons across data set sizes. (D) The mean splitting ratios of morphospecies groups that exceeded a ratio of 1 (dotted horizontal line). (E) A boxplot of the percentage matches between predefined morphospecies and GMYC species, including (blue) and excluding (red) singletons. White triangles represent means. 125

4.5 Distribution ranges for each proposed *Tetramesa* GMYC species, based on their collection sites. 133

5.1 An image plate showing the morphological similarities between different wasp species, and the ease of misidentification. (A) *Tetramesa sp. 1A* from *Sporobolus pyramidalis* and (B) *Tetramesa sp. 1B* from *Sporobolus africanus* (present study), (C) *Anagyrus callidus* female, (D) *A. kamali* female, (E) *A. callidus* male, and (F) *A. kamali* male. Images C - F taken from Foster *et al.* (2021). 140

5.2 A diagram showing the divergence of species through time, where species delimitation becomes easier with older lineages that have reached a state of monophyly. Adapted from De Queiroz (1998). 144

S1 A - Q: microscope images of reference specimens appearing in the COI and 28S phylogenetic trees (Fig. 2.11 and Fig. 2.9). Sample codes and clade IDs are shown at the top and bottom of each image, respectively. 207

S2 Maximum likelihood phylogeny (IQTREE) for *Sporobolus* ITS sequences from Peterson *et al.* (2014), with the unknown *Sporobolus* samples SNAT01 and SNAT02 shown in blue text. *Sporobolus africanus*, *S. natalensis*, and *S. pyramidalis* are in green text. The best-fit BIC model was SYM + I + Γ, and the log-likelihood of the consensus tree was -21198.414. Three *Eragrostis* samples served as an outgroup (sourced from Peterson *et al.* (2010)). 208

S3 Maximum likelihood phylogeny (IQ-TREE) for *Sporobolus* chloroplast (trnL and trnK) sequences from Peterson *et al.* (2014), with the unknown *Sporobolus* samples SNAT01 and SNAT02 shown in blue text. *Sporobolus africanus*, *S. natalensis*, and *S. pyramidalis* are in green text. The best-fit BIC models for trnL and trnK was TPM3 + F + Γ and TPM3u + F + Γ, respectively, and the log-likelihood of the consensus tree was -7792.919. Three *Eragrostis* samples served as an outgroup (sourced from Peterson *et al.* (2010)). 209

S4 A maximum likelihood phylogeny for representative 28S sequences, showing that the pronotal spot (PNS) *Eragrostis Tetramesa* wasps were not resolved. The best-fit BIC model for this tree was TNe + I (log-likelihood of the consensus tree was -1011.932) 210

S5 Between-group p-distance matrix for the COI mitochondrial region (500 bootstrap repeats with pairwise deletion, applying the K2P model) showing the average number of base substitutions per site (n = 255 nucleotide sequences, 545 bp length). See Fig. 2.9 for acronyms. Values are displayed as percentages. Values above each vertical column are intraspecific divergence values. Black borders surround the PNS clades. 211

S6 Between-group p-distance matrix for the 28S nuclear region (500 bootstrap repeats with pairwise deletion, applying the K2P model) showing the average number of base substitutions per site (n = 230 nucleotide sequences, 605 bp length). Values are displayed as percentages. Values above each vertical column are intraspecific divergence values. Black borders surround the PNS clades. . 212

S7 Genetic distance plots for the 28S gene region, showing the pairwise p-distances between groups at threshold intervals of (A) 1%, (B) 1.5%, (C) 2%, and (D) 2.5%. The groups labelled in red in each vertical column are considered to belong to the same taxonomic group as the corresponding phylogenetic clade on the x-axis. GS = PNS, NGS = NPNS. 213

S8 Genetic distance plots for the COI gene region, showing the pairwise p-distances between groups at threshold intervals of (A) 3%, (B) 4%, (C) 5%, and (D) 6%. The groups labelled in blue in each vertical column are considered to belong to the same taxonomic group as the corresponding phylogenetic clade on the x-axis. GS = PNS, NGS = NPNS. 214

List of Tables

1.1 South African grasses that are confirmed invaders (as per the definition by Richardson *et al.* (2000)) around the world. Species marked with an asterisk (*) are endemic to South Africa, and those marked with a plus (+) are major ecosystem transformers in their invaded range. Adapted from Pyšek *et al.* (2017). 5

1.2 A list of all the agents released on invasive Poaceae from 1997 - 2019, extracted from Winston *et al.* (2021). 26

2.1 List of primers used for *Tetramesa* samples in this study. 47

2.2 Analysis of molecular variance (AMOVA) results of COI data from the *Tetramesa* collected on *Eragrostis curvula*. The AMOVA for provincial comparisons included *Tetramesa* from the Eastern Cape (n = 21), Free State (n = 12), Mpumalanga (n = 2), and the Northern Cape (n = 2), and the AMOVA for populations within the Eastern Cape were for *Tetramesa* collected from Grahamstown (n = 6), Port Elizabeth (n = 10), East London (n = 2), and Mondplaas (n = 3). Df = degrees of freedom. 71

2.3 Pairwise fixation index (FST) value comparisons (K2P model) for pronotal spot *Tetramesa* collected on *Eragrostis curvula* at A) a provincial level, and B) within the Eastern Cape. Significant comparisons are shown in bold ($p < 0.05$). EC = Eastern Cape, FS = Free State, MP = Mpumalanga, NC = Northern Cape, GHT = Grahamstown, PE = Port Elizabeth, EL = East London, MDPL = Mondplaas. 72

2.4 Divergence time estimates in millions of years (median ages) for the nodes shown in Figure 2.16, with 95% HPD confidence intervals. 74

3.1 The primer pairs used to amplify target plant gene regions. 93

3.2 Summary of the field host range and no-choice host specificity tests of *Tetramesa sp. 4* (Guy Sutton unpublished data). 95

4.1 The number of genetic sequences per morphospecies for the nuclear 28S and mitochondrial COI genes, used as input for SPEDE-sampler. 122

4.2 SPEDE-sampler summary results from the full datasets (100% sequence data) for the PNS *Tetramesa* 28S and COI sequences. SR = splitting ratio, + singletons = including singletons, - singletons = excluding singletons. Standard deviations are shown where appropriate. 128

4.3 A summary table of *Tetramesa* GMYC species delimitations. Host SpR = host species richness, Host S_{TD} and Host PSV_t = an index of taxonomic host specificity and phylogenetic species variability, respectively (Abram *et al.*, 2021). The map panel index numbers refer to Fig. 4.5, the clade names and numbers refer to those used in Chapter 2 Fig. 2.9, and the images refer to the reference microscope images in Fig. S1. Province acronyms: EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, LP = Limpopo, MP = Mpumalanga, NC = Northern Cape, ZIM = Zimbabwe. 134

S1 Sample information for all the wasp sequences (28S and COI) included in this work. Unshaded cells in the COI and 28S columns denote missing data. GenBank codes beginning with AY317 were sourced from (Chen *et al.*, 2004). PNS = pronotal spot, NPNS = non-pronotal spot, NA = unknown, SA = South Africa, EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, MP = Mpumalanga, GHT = Grahamstown, PE = Port Elizabeth, EL = East London, f = female, m = male. 198

S2 Divergence values (K2P), as percentages, for subsets of the 28S sequence data. Bold values along the diagonal are intraspecific distances. Bruch. = *Bruchophagus*, PNS = pronotal spot, NPNS = non-pronotal spot. Cells in grey represent putative conspecific groups, with divergenc values < 3.5%. . . 204

S3 Sample information for all the grass sequences (rpl32-trnL, rps16-trnK, rps16, and ITS) included in this work. Unshaded cells in the gene columns denote missing data. 215

S4 GMYC species assignments on the full 28S data set. EC = Eastern Cape, FS = Free State, KZN = Kwa-Zulu Natal, LP = Limpopo, MP = Mpumalanga. 217

S5 GMYC species assignments on the full COI data set. EC = Eastern Cape, FS = Free State, KZN = Kwa-Zulu Natal, LP = Limpopo, MP = Mpumalanga. 218

Chapter 1

General Introduction

1.1 Introduction

This chapter covers a literature review of the Poaceae grass family, the spread of invasive grasses, and South Africa as a major donor of invasive alien grass species to other parts of the world. It then introduces the concept of biological control as a management option for controlling these invasive alien grass species, and the importance of correctly identifying candidate agents and the implications of cryptic species. The potential use of the *Tetramesa* Walker wasps (Hymenoptera: Eurytomidae) as biological control agents for invasive African grasses is then discussed, followed by the importance of molecular identification tools and how they can be applied to *Tetramesa* taxonomy, species discovery, and host-specificity screening. The chapter ends with a list of research objectives.

1.2 The Poaceae

It is estimated that approximately 1% of naturalised plant species become invasive (Williamson and Fitter, 1996). These invaders can cause severe damage to ecosystem services, including, but not limited to, primary production, nutrient cycles, hydrology, soil stabilisation, fire regimes, native biodiversity and endemic species, and agricultural and horticultural practices (Pejchar and Mooney, 2009; Vilà *et al.*, 2011; Lockwood *et al.*, 2013; Vilà and Hulme, 2017).

The grass family (Gramineae, Poaceae) radiated during the late Cretaceous period (70 - 80 million years ago (Mya)) (Prasad *et al.*, 2005), and today comprises over 11 000 species (Pyšek *et al.*, 2017; Clayton *et al.*, 2021). It is arguably the most successful angiosperm family in the modern world (Linder *et al.*, 2018). Different grass species have adapted to survive in nearly every corner of the globe; from Antarctica to the Saharan Desert (Linder *et al.*, 2018). Grass-dominated ecosystems cover between 31 - 43% of the Earth's land surface (where the global coverage of C_3 and C_4 grasses is approximately 87.4 and 18.8 million km^2, respectively (Still *et al.*, 2003)), and their contribution to global primary productivity is estimated at 33% (Gibson, 2009) .

Multiple introductions of non-native grass species on a large scale has promoted invasion (D'Antonio and Vitousek, 1992; Williams and Baruch, 2000; Gaertner *et al.*, 2014; Godfree *et al.*, 2017; Hui and Richardson, 2017; Linder *et al.*, 2018). Numerous grass species have been intentionally spread around the world through human activity over the last few hundred years; mainly due to their use in improving the quality of pastures, as animal fodder, building materials, essential oils, ornamentals, and as soil and dune stabilisers (Cook and Dias, 2006; van Klinken and Friedel, 2018). As a result, this plant family currently has 4807 recorded weedy, and 1267 naturalised species worldwide - second only to the Asteraceae (comprising over 32 000 species) with 5094 weedy and 1343 naturalised species (Pyšek *et al.*, 2017; Randall *et al.*, 2017) (Fig. 2.1 A). The term "naturalised", as used here, means that the plant has established outside its native range, but is currently not considered invasive.

South Africa has been a major donor of invasive grasses to other parts of the world (Byrne *et al.*, 2022) (Table 1.1) due to the perception that African C_4 grasses were more palatable to livestock and resistant to grazing than native grasses (Visser *et al.*, 2016). Approximately 15.1% ($\sim$ 165 spp.) of the grass species native to South Africa have become naturalised elsewhere, and represent the largest donor plant family followed by the Asteraceae and Fabaceae (Pyšek *et al.*, 2017) (Fig. 2.1 B). Most of these species belong to the Paniceae (86 spp.), Chloridoideae (79 spp.), and Andropogoneae (47 spp.) clades, as reported in Visser *et al.* (2016). Contrastingly, only about 0.8% of the global pool of naturalised grass species have naturalised in South Africa (Visser *et al.*, 2016); making it a disproportionately larger donor than recipient. Many African grasses have subsequently become invasive and highly damaging due to the combination of (1) favourable traits (for example adaptations to fire, resistance to disturbance and to a diverse assemblage of large herbivores, and physiological traits such as photosynthetic type, life-history, and growth form), (2) multiple introduction events (reducing potential genetic bottlenecks and resulting founder effects) (D'Antonio and Vitousek, 1992; Daehler and Carino, 1998; Flanders *et al.*, 2006; Foxcroft *et al.*, 2010; Damasceno *et al.*, 2018) and (3) high propagule pressure (i.e. the number of individuals in each introduction event) (Firn *et al.*, 2009).

1.3 Impacts of invasive grasses

Once an exotic grass species has invaded and dominated an area, it can (1) out-compete native flora and cause biodiversity loss (D'Antonio and Vitousek, 1992; Grice *et al.*, 2013), (2) drive habitat degradation and alter ecosystem processes and fire regimes (D'Antonio and Vitousek, 1992; Setterfield *et al.*, 2010; Fusco *et al.*, 2019), (3) reduce pasture productivity (Witt and McConnachie, 2004; Firn *et al.*, 2009) and water resources (Seawright *et al.*, 2009; Gowda *et al.*, 2011), (4) hybridise with native species (Ayres *et al.*, 2004), and (5) facilitate the transmission of pathogens and vectors of disease (Racelis *et al.*, 2012; D'Urso *et al.*, 2017) (Fig. 1.1).

1.3.1 Biodiversity loss and habitat degradation

Invasive grasses are often better competitors than native species for resources such as light, nutrients, water, and space, combined with having larger seed outputs and seedbanks, and fewer seed predators outside of the native distributions (D'Antonio and Vitousek, 1992; Ayres *et al.*, 2004; Guido *et al.*, 2019). Reductions in native plant species can subsequently result in biodiversity loss and changes in habitat structure and species composition (Godfree *et al.*, 2017; Damasceno *et al.*, 2018).

Flory and Clay (2010) reported that the invasive grass *Microstegium vimineum* (Trin.) A. Camus suppressed the regeneration of native trees in Indiana (USA) by reducing the recruitment and colonisation rates of small-seeded species, resulting in an overall decrease in native community biomass. Some of the possible mechanisms behind the suppression of natives included the shallow root system of *Microstegium*, which allows for it to capture large quantities of water and nutrients before the deeper-rooted natives can access it. The dense growth of the invader may also reduce the amount of light received by establishing native seedlings. Additionally, the large quantities of biomass cover produced by the invader may pose a physical barrier to germinating smaller seedlings. Dense stands of *Microstegium* can also provide shelter to small mammals (e.g. mice and voles) that feed on native trees and seeds.

Similarly, in a study of the impacts of the invasive African grasses *Melinis minutiflora* P. Beauv. and *Urochloa brizantha* Hochst. ex A. Rich. in the Brazilian Cerrado, Damasceno *et al.* (2018) found that the greater quantities of dead biomass produced by the invasive species hampered seedling establishment and development of native grasses. Changes in biomass can lead to altered nutrient cycling, decomposition rates, and overall community structure (D'Antonio and Vitousek, 1992; Vilà *et al.*, 2011). It has even been shown that some invasive grasses can change soil microbial communities, which can inhibit the growth of native species and contribute to a positive feedback loop in which the invasives further dominate an ecosystem (Batten *et al.*, 2008).

1.3.2 Fire cycles

The impacts of invasive grasses on fire cycles are some of the most concerning. A recent study by Fusco *et al.* (2019) in the United States found that fire occurrence and frequency increased by 230% and 150%, respectively, across different ecoregions and vegetation types. Similar findings have been reported in Australia and Central and South America (D'Antonio and Vitousek, 1992; Rossiter *et al.*, 2003; Setterfield *et al.*, 2010) (Fig. 1.2 A). Grass-fire cycles facilitate grass invasions via positive feedback loops by reducing native shrub and tree cover, where invasive grasses recover faster and dominate landscapes; thereby further increasing fuel loads (D'Antonio and Vitousek, 1992; Freifelder *et al.*, 1998). Fires can also change nutrient levels in the soil (e.g. carbon and nitrogen), which in turn can alter ecosystem functioning (D'Antonio and Vitousek, 1992).

Reed *et al.* (2005) reported how the invasion of *Andropogon bladhii* (Retz.) S.T.Blake in a prairie dominated by native *A. gerardii* Vitman in Kansas (USA) affected carbon and nitrogen cycling and overall species diversity following spring fires. The authors found that the invasive grass had significantly higher biomass, lower soil nitrogen and carbon cycling, higher C:N ratios in foliar and root tissues, and suppressed native species diversity. Frequent fire cycles are thus expected to facilitate further invasions, leading to decreased long-term soil fertility, erosion, and an overall decrease in native biodiversity.

Table 1.1: South African grasses that are confirmed invaders (as per the definition by Richardson *et al.* (2000)) around the world. Species marked with an asterisk (*) are endemic to South Africa, and those marked with a plus (+) are major ecosystem transformers in their invaded range. Adapted from Pyšek *et al.* (2017).

Species	**Common name**	**Invaded countries**
+ *Andropogon gayanus* Kunth	Gamba grass	Australia
+ *Cenchrus ciliaris (= Pennisetum ciliare)* L.	Buffel grass	Australia, North America, Pacific Islands
Cynodon dactylon L. (Pers.)	Kweek/couch grass	North America, Pacific and Atlantic Islands
Digitaria eriantha Steud.	Pangola grass	Central and South America, the Caribbean, Australia, Pacific Islands
Ehrharta calycina Sm.	Purple veldtgrass	Australia, Europe, South America
* *Ehrharta erecta* Lam.	Panic veldtgrass	Australia, New Zealand, Europe, North America, Pacific Islands
Eragrostis curvula (Schrad.) Nees.	African lovegrass	Australia, Europe, Asia
* *Eragrostis lehmanniana* Nees	Lehmann lovegrass	Australia, North America
Eragrostis plana Nees	South African lovegrass	South America
+ *Hyparrhenia hirta* L. (Stapf)	Thatching grass	Australia, Pacific Islands
+ *Hyparrhenia rufa* Nees (Stapf)	Giant thatching grass/jaragua	Australia, North, Central and South America, Asia, Pacific Islands
Imperata cylindrica L. P. Beauv.	Cogongrass	Numerous regions
Megathyrsus maximus (=Panicum maximum) Jacq.	Guinea grass	North America, Pacific Islands
+ *Melinis minutiflora* P.Beauv.	Molasses grass	North and South America, Pacific and Atlantic Islands
Melinis repens Willd. Zizka	Natal grass	Australia, North and South America, Asia
Panicum repens L.	Torpedograss	Australia, Central America, Asia
Pennisetum macrourum Trin.	African feathergrass/jaagbesem	Australia, New Zealand
Sporobolus natalensis (Steud.) Dur. & Schinz	Giant rat's tail	Australia
Sporobolus pyramidalis P. Beauv.	Giant rat's tail	Australia

Key references to the impacts of the weeds in Table 1.1:
Andropogon gayanus: Rossiter-Rachor *et al.* (2009); Setterfield *et al.* (2010); Rossiter-Rachor *et al.* (2017) ***Cenchrus ciliaris***: Daehler and Goergen (2005); Smyth *et al.* (2009); Franklin and Molina-Freaner (2010); Marshall *et al.* (2012) ***Cynodon dactylon***: Mau-Crimmins (2007); Barnes *et al.* (2013); Guido *et al.* (2020) ***Digitaria eriantha***: Vélez-Gavilán *et al.* (2018) ***Ehrharta calycina***: Fisher *et al.* (2006); Stanbury *et al.* (2018); Phillips *et al.* (2019) ***Ehrharta erecta***: Manea *et al.* (2016); Ray *et al.* (2018); Stinca and Mei (2019) ***Eragrostis curvula***: Muranaka and Washitani (2004); Firn *et al.* (2009); Han *et al.* (2012) ***Eragrostis plana***: Dresseno *et al.* (2018); Guido *et al.* (2019, 2020) ***Hyparrhenia hirta***: McArdle *et al.* (2004); Chejara *et al.* (2006) ***Hyparrhenia rufa***: D'Antonio and Vitousek (1992) ***Imperata cylindrica***: Brewer (2008); Daneshgar *et al.* (2008); Holly *et al.* (2009); Estrada and Flory (2015) ***Megathyrsus maximus***: Rojas-Sandoval and Meléndez-Ackerman (2012); Ellsworth *et al.* (2014) ***Melinis minutiflora***: Hoffmann and Haridasan (2008); Rossi *et al.* (2014); Zenni *et al.* (2019) ***Melinis repens***: Stevens and Fehmi (2009) ***Pennisetum macrourum***: ***Sporobolus natalensis*** and ***Sporobolus pyramidalis***: Witt and McConnachie (2004); Palmer *et al.* (2008)

Invasive buffelgrass (*Cenchrus ciliaris* L.) has fuelled wildfires in Australia (Butler and Fairfax, 2003), Mexico (Búrquez-Montijo *et al.*, 2002), and parts of the USA (Wilder *et al.*, 2021). Some authors have warned that parts of the lower Sonoran Desert in the USA could be transformed into an African grassland by frequent, high-intensity fires (Burgess *et al.*, 1991; Rutman *et al.*, 2002). The ecological effects of a single fire can last for decades, sometimes taking up to 40 years for some native plants to recover (Abella, 2009), while some native cacti can face local extinction (Thomas, 2006). The iconic saguaro cactus (*Carnegia gigantea* Engelm. Britton and Rose) of Arizona, for example, can suffer mortality rates of 68 - 85% following an intense fire (Rogers, 1985) (Fig. 1.2 B). McDonald and McPherson (2013) reported that buffelgrass fuel loads in the Sonoran Desert were higher than in other comparable ecosystems, and that the grass posed a consistent annual fire hazard.

1.3.3 Water dynamics

Invasive riparian grasses, such as *Arundo donax* L. in the southwestern regions of the USA, consumes about three times more water than native vegetation in order to support its rapid vegetative growth rate (Seawright *et al.*, 2009) (Fig. 1.2 C). Gowda *et al.* (2011) reported that the evapotranspiration rate of *A. donax* along the Rio Grande River Basin in 2009 was about 5.2 mm water per day, while Watts and Moore (2011) reported higher values of 8.8 ± 0.9 mm of water per day during the peak of the 2008 growing season.

Seawright *et al.* (2009) estimated that if the water consumption by *A. donax* was diverted to agricultural crops, the farming sector could save in the region of US$ 98 - 160 million annually (at 2010 prices). The weed was reported to result in water loss to the value of US$ 12 million a year on the Santa Ana River (USA) (Coffman, 2020). Jain *et al.* (2015) found that streamflow was significantly reduced in the Nueces River in central Texas after *A. donax* had completely displaced native stands of switchgrass (*Panicum virgatum* L.). The researchers also reported that the water uptake of *A. donax* was 7.2% greater than in the native species.

1.3.4 Agriculture

The economic losses caused by invasive grasses can be significant, particularly to the agricultural industry (Fig. 1.2 D). In Australia, for example, species in the *Sporobolus indicus* (L.) R. Br. complex can reduce pasture carrying capacity by 10 - 80% and cause annual losses of at least AU$ 60 million (at 2001 price estimates) to the livestock industry (Witt and McConnachie, 2004). Guinea grass (*Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs) has been reported to reduce global sugarcane yields by up to 40% in some cases (Rhodes *et al.*, 2021a).

Johnsongrass (*Sorghum halepense* (L.) Pers.), native to Eurasia, has been reported to cause substantial decreases in crop yields around the world (Klein and Smith, 2021). Keeley and Thullen (1989) reported reductions in cotton yields of up to 90% when herbicide treatments were delayed, with similar reports in soybean (88% yield reduction) (Williams and Hayes, 1984) and maize (80% yield reduction) (Mitskas *et al.*, 2003).

1.3.5 Hybridisation

Alien invasive species can sometimes hybridise with native species to form hybrid offspring that can outcompete their native parents (Ellstrand and Schierenbeck, 2000), or result in a hybrid population with lower fitness and the eventual possible extinction of native populations (Prentis *et al.*, 2007). Hybridisation between smooth cordgrass (*Spartina alterniflora* Loisel = *Sporobolus alterniflorus*) and native California cordgrass (*Spartina foliosa* Trin.), for example, was found to result in hybrids that produced more seeds than natives, and quickly dominated niche space (Ayres *et al.*, 2004)).

1.3.6 Vectors of disease and spread of pathogens

Some alien invasive plants carry microorganisms and/or inquilines, or serve as refugia for other pests that can become problematic in an invaded range. Stands of *Arundo donax* in the USA, for example, was found to provide a favourable habitat for the cattle ticks *Rhipicephalus microplus* Canestrini and *R. annulatus* Say (Acari: Ixodidae) (Racelis *et al.*, 2012), which vector the protozoa causing bovine babesiosis. In another example, alien invasive purple fountain grass (*Pennisetum setaceum* (Försskal) Chiov.) in the Mediterranean is accompanied by the leafhopper, *Balclutha brevis* Lindberg (Hemiptera: Cicadellidae) (D'Urso *et al.*, 2017). Although not conclusive yet for this particular species of leafhopper, there are other species in the genus that vector plant diseases, such as *B. punctata* Fabricius that transmits mulberry dwarf phytoplasma to mulberries (Han, 2012).

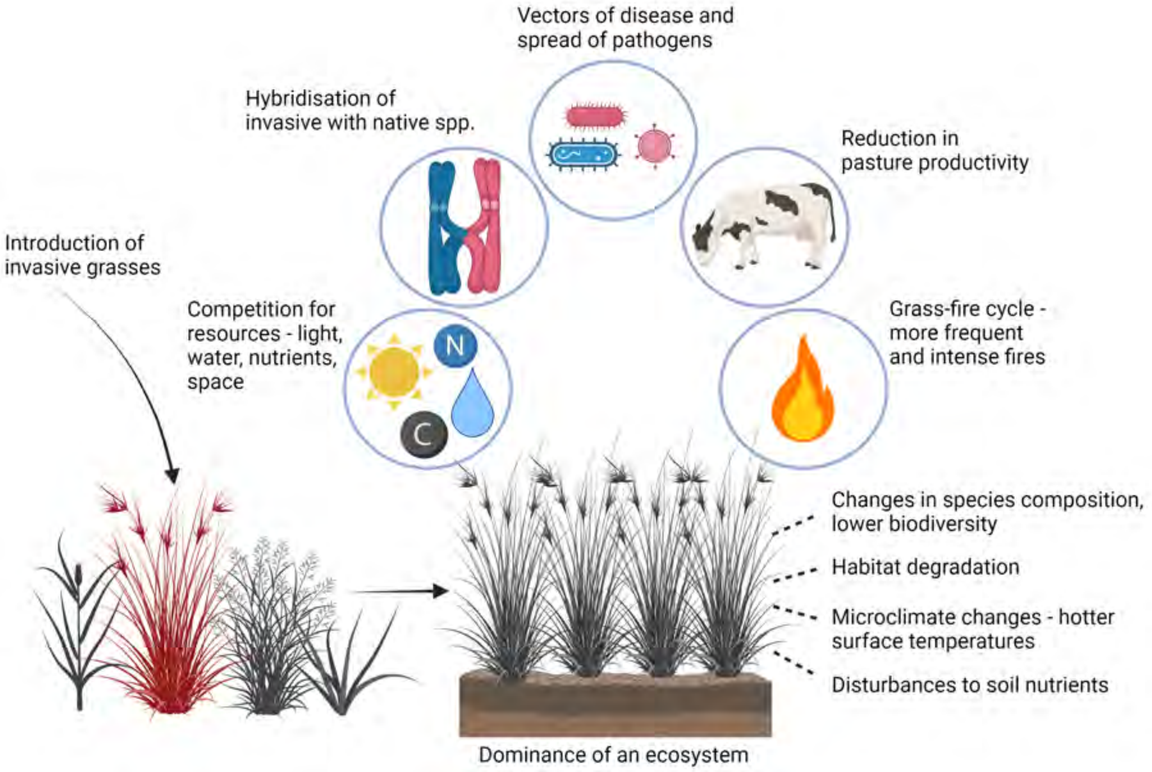


Figure 1.1: A summary of the main impacts of invasive grasses on an ecosystem. See the references in Section 1.3.

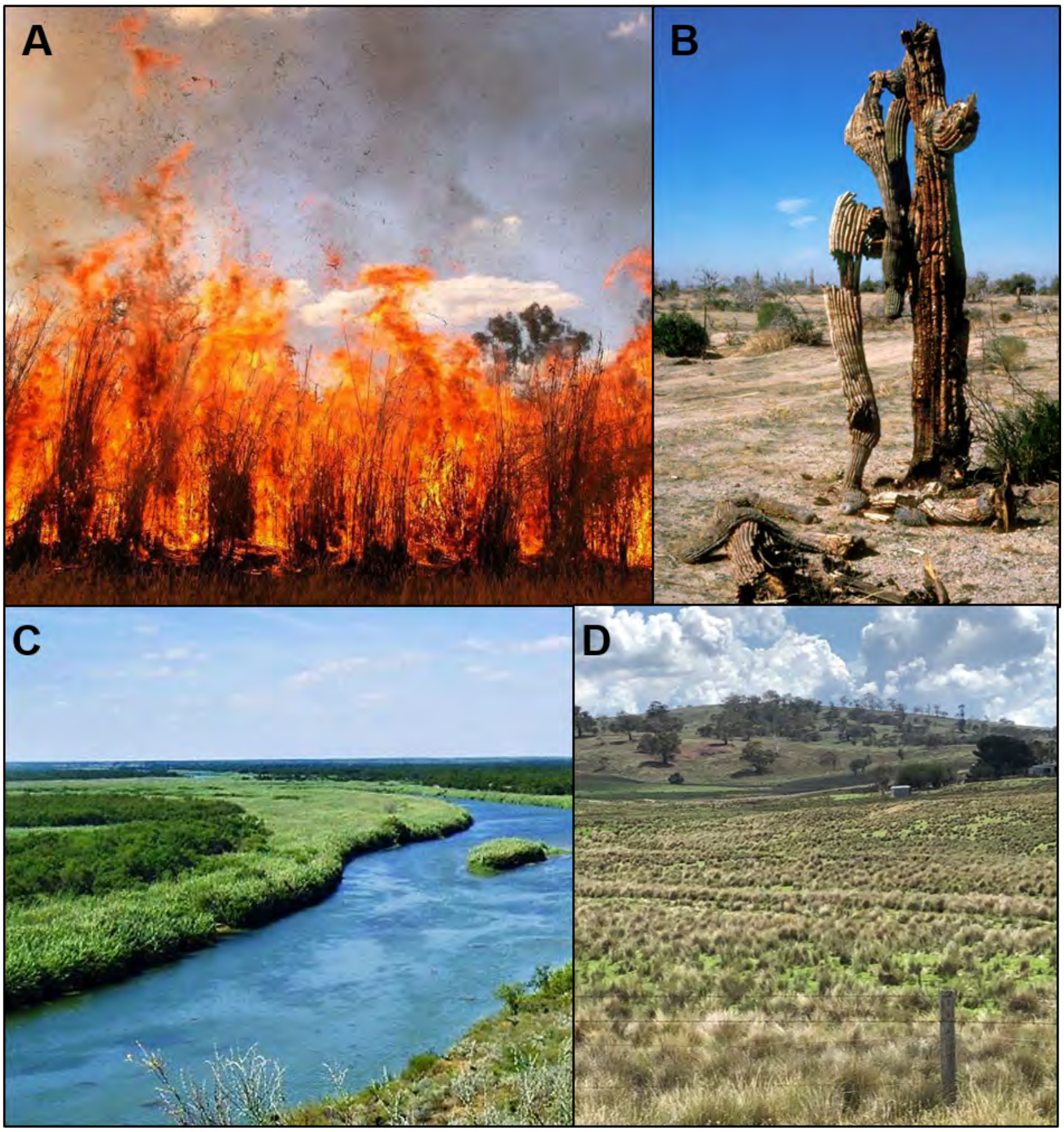


Figure 1.2: (A) burning gamba grass (*Andropogon gayanus*) in northern Australia (credit: CRC for Weed Management), (B) Saguaro cacti damaged by fire in the Saguaro National Park (credit: Perry Grissom), (C) invasive *Arundo donax* along the Rio Grande River, USA (credit: John Goolsby), and (D) African lovegrass (*Eragrostis curvula*) infestation in New South Wales, Australia (credit: Andrew McConnachie).

1.4 Graminivorous insects

Communities of grass-feeding insects (graminivores) are often inconspicuous, particularly endophages, and therefore represent a group with a large degree of undiscovered diversity (Tscharntke and Greiler, 1995). Ratios of endophages to ectophages are usually higher on hosts that are more defended, as endophages are adapted to avoiding chemical and physical defences (e.g. gallers or miners) and feeding selectively (Cornell, 1989; Tooker and Giron, 2020).

Herbivore assemblages on grasses were historically believed to be dominated by ectophages due to the perceived low levels of secondary metabolites produced by grasses (Tscharntke and Greiler, 1995), but the growing literature on grass metabolomics has begun to challenge this assumption (Li *et al.*, 2014; Cibils-Stewart *et al.*, 2022; Li *et al.*, 2022).

In general, graminivorous insects are more diverse on grass species that are abundant and structurally large, compared to those that are rare with simple architectures (Tscharntke and Greiler, 1995). For example, *Phragmites australis* (Cav.) Trin. ex Steud. has exceptionally long shoots, and was reported to be attacked by about 100 insect species, of which 93% were monophagous (Tscharntke, 1993). Additionally, perennial species appear to have larger herbivore assemblages compared to annuals; presumably due to the fast growth and short exposure time exhibited by annuals (Tscharntke and Greiler, 1995). Differences between C3 and C4 grasses may also affect herbivore assemblages due to a suite of differing physiological and chemical traits in the host plant; where C3 grasses are hypothesised to generally be more nutritious and easier to consume (Caswell *et al.*, 1973; Johnson *et al.*, 2014).

Many grasses display high levels of genetic variation, giving rise to multiple varieties or "biotypes" within a species (Ramakrishnan *et al.*, 2004; Cidade *et al.*, 2013; Ahrens *et al.*, 2020). These varying genotypes can affect herbivore assemblages due to differences in host-specificity and/or oviposition preferences (Hoffman *et al.*, 2019; Michereff *et al.*, 2022). Differences in the life-histories, genetic structure, and physical traits of invasive grasses are therefore all important factors in understanding invasion pathways and developing management strategies.

1.5 Invasive grass control methods

1.5.1 Manual

Manual and mechanical control methods of invasive weeds typically take the form of (1) cutting or mowing the plant down to its base toward the end of the growing season (e.g. *Phragmites australis* (Cav.) Trin. ex Steud. in Derr (2008)), (2) digging out or severing (referred to as "disking") the plant's rhizomes (Marks *et al.*, 1994), (3) carrying out prescribed burning

(Ditomaso *et al.*, 2006), (4) applying soil solarisation (also termed "tarping" or "mulching") (Marushia and Allen, 2011), or (5) targeted grazing (Rhodes *et al.*, 2021b). These methods are usually labour-intensive and costly, unless applied to small areas (e.g. control of *A. donax* along the Santa Margarita River in California, USA, cost approximately US$ 8100 per 0.4 hectares in 2005) (Lawson *et al.*, 2005).

Annual cutting before tassel development and seed set at the end of the growing season can be an effective means of control, as this removes the food reserves stored by the plant during the growing season, and decreases the plant's seedbank (Flory and Lewis, 2009; Flory and Clay, 2010). However, cutting at the wrong times of the year can have the opposite effect, and facilitate further invasion (e.g. through soil disturbance and re-sprouting from lower axillary stems) (Marks *et al.*, 1994). Some studies have suggested that a combination of well-timed mowing and prescribed burning can be effective, for example for controlling Japanese stiltgrass (*M. vimineum*) (Flory and Lewis, 2009).

Digging out or disking the rhizomes of invasive grasses can promote further invasion if the rhizomes are not disposed of correctly (since even small rhizome pieces can produce new plants) (Marks *et al.*, 1994). The practicality of the process is also dependent on the soil type present, where sandy soils make root removals relatively easier than mud or clay. The rhizomes of some grasses are deep (up to 5 m in the case of *A. donax* (Moore *et al.*, 2016)), and their removal can damage an ecosystem and even increase the risk of spread (e.g. downstream spread in the case of riparian species (Boland, 2008)).

Prescribed burning alone is often ineffective in controlling invasive grasses unless the rhizomes are burnt, but it can, in some cases, reduce accumulated leaf litter and allow for the germination of native seeds (Marks *et al.*, 1994). Repeated burning initiatives have been successful in the control of some annual and perennial grasses, such as medusahead (*Taeniatherum caput-medusae* L.) and Kentucky bluegrass (*Poa pratensis* L.), respectively, especially when combined with timely herbicidal applications (Ditomaso *et al.*, 2006). If not timed properly, however, fires can promote the regrowth of invasive grasses (Bell, 1997), and can further damage ecosystems (e.g. riparian habitats) (Mal and Narine, 2004).

Soil solarisation involves covering soil with clear or black polyethylene sheets or plastic mulch in order to raise the soil temperature to kill invasive weeds and pathogens (Katan, 2015). This method is ineffective, however, if the target species have heat-tolerant seeds (Melander and Jørgensen, 2005). This method has had some success with *P. australis*, where die-off began within 3 - 4 days in an experiment reported in Marks *et al.* (1994). Soil solarisation can become costly for controlling large infestations, where Holl *et al.* (2014) estimated that the method can cost an order of magnitude more than herbicidal applications.

Targeted grazing makes use of particular types of livestock during specific seasons, and at pre-planned intensities and durations to control invasive plants and other unwanted vegetation (Bailey *et al.*, 2019). This method is often preferred because it does not involve toxic herbicides, is applicable over large spatial areas, and can be removed at any time (Bailey *et al.*, 2019). Targeted grazing using cattle has been successful with the control of *M. maximus* and *C. ciliaris* in Texas, USA (Rhodes *et al.*, 2021b,a). General guidelines for the control of invasive grasses using this method are, however, lacking due to the uncertainties surrounding the effects of seasonality, precipitation, and effects on native plant communities (Frost and Launchbaugh, 2003).

1.5.2 Chemical

Invasive grasses are usually treated with amino acid synthesis inhibitors and photosynthetic inhibitors such as glyphosates (e.g. Rodeo®), imazapyr, or a mixture of the two combined with a surfactant to ensure adhesion (Minogue *et al.*, 2012; Rhodes *et al.*, 2021a). These are both non-selective, broad-spectrum herbicides (also referred to as graminicides), and can negatively impact non-target species in close proximity, especially if a spray-only approach is used (Florencia *et al.*, 2017; Isbister *et al.*, 2017).

Spray-only herbicides are applied to leaves and stems as a foliar spray, while a cut-and-spray approach first cuts the plant down to the base of the stem before applying the chemical formulation (Kaiser-Bunbury *et al.*, 2015). A cut-and-spray approach is more targeted, and poses relatively less risk to surrounding plants (Gettys and Sutton, 2004). Some more recent experiments have found that growth-regulator (GR) herbicides, such as dicamba and picloram, are effective against Japanese brome (*Bromus japonicus* Thunb.), where Rinella *et al.* (2010)

reported reduced seed production of nearly 100% when the GR herbicides were applied in the late growth stages, just before seed setting. Sebastian *et al.* (2017) reported that the cellulose-biosynthesis-inhibiting herbicide, indaziflam, has the potential to eliminate the seed banks of invasive winter annual grasses such as downy brome (*B. tectorum* L.).

Although the chemical control of some invasive weeds can be effective if applied correctly, especially when combined with other integrative methods, it can become exorbitantly expensive over large spatial and temporal scales (Van Wyk and Van Wilgen, 2002; Culliney, 2005; Maluleke *et al.*, 2021), induce resistance (DiTomaso, 2000; Délye *et al.*, 2013; Heap, 2014), and damage the environment (Relyea, 2005; Wagner and Nelson, 2014; Carpenter *et al.*, 2020).

1.5.3 Biological Control: a general overview

Biological control utilises the natural enemies of a pest or weed to suppress its growth and spread (McFadyen, 1998; van Driesche *et al.*, 2009). These natural enemies are termed "biological control agents". The practice should be cost-effective (with high cost-to-benefit ratios) (Maluleke *et al.*, 2021), sustainable and environmentally-friendly, target-specific, such that non-target organisms are not harmed, and often compatible with other management interventions in an integrated pest management programme (McFadyen, 1998; Culliney, 2005; van Wilgen and Lange, 2011; Zachariades *et al.*, 2017; Schwarzländer *et al.*, 2018). Successes from biological control can, however, be variable and difficult to predict, and can take a long time to implement (Waage and Greathead, 1988). For these reasons, chemical and mechanical methods are still the most widely-applied in weed control, particularly in the agricultural sector (Schwarzländer *et al.*, 2018).

The biological control of invasive alien plants has a long history and a high success rate (McFadyen, 1998; Zachariades *et al.*, 2017; Schwarzländer *et al.*, 2018). The top five countries implementing biological control are the USA, Australia, South Africa, Canada, and New Zealand (McFadyen, 1998). South Africa's first biological control programme took place in 1913, where the cochineal insect *Dactylopius ceylonicus* Green (Hemiptera: Dactylopiidae) was released to control invasive drooping prickly pear, *Opuntia monacantha* Haw. (Moran *et al.*, 2013). The insect was highly damaging, and permanently reduced cactus infestations (Paterson *et al.*, 2021b).

The negative effects of biological control are negligible when compared to the negative impacts of the weeds themselves and the chemical and mechanical methods applied to control them (Howarth, 1991). A total of 468 biological control agent species have been released onto 175 weed species from 48 plant families across the world (Schwarzländer *et al.*, 2018; Winston *et al.*, 2021). Approximately 71% of these agents have established successfully, 66% of the target weed species have been controlled to some degree, and only 7% of releases have been unsuccessful (i.e. no impact recorded) (Schwarzländer *et al.*, 2018). The safety track-record of biological control programmes is solid, with less than 1% of all releases yielding negative non-target effects (Suckling and Sforza, 2014; Hinz *et al.*, 2019; Paynter *et al.*, 2020).

A review by Hinz *et al.* (2019) stated that of the 457 species of biological control agents intentionally released between the years 1863 and 2008, 60 (13.1%) were associated with non-target attacks (NTAs). Of those 60 NTAs, 42 (9.2% of the total 457 agents released globally) involved attacks on native species. These NTAs, however, did include a large proportion of spillover effects and cases of minimal feeding damage. Spillover occurs when biological control agents feed and/or develop on non-target host species growing in close proximity to the target weed, but agent populations are not sustained in the target weed's absence. Additionally, Hinz *et al.* (2019) found that the proportion of agent releases associated with NTAs has decreased despite the increase in releases over the years. This is most likely due to a combination of more stringent host specificity testing measures and stricter regulations regarding the release of biocontrol agents. Hinz *et al.* (2019) emphasise that post-release monitoring should be as important as pre-release host specificity testing in order to accurately quantify agent impact and NTAs.

The four main approaches to biological control are (1) "classical", where natural enemies from the native range of a target pest (which share an evolutionary history) are used to achieve control (e.g. the biological control programmes against the invasive aquatics *Salvinia molesta* D.S. Mitch., *Pistia stratiotes* L., *Myriophyllum aquaticum* (Vell.) Verdc., and *Azolla filiculoides* Lam. (Coetzee *et al.*, 2011)), (2) "neo-classical", which makes use of agents that share no previous evolutionary history with the target weed, and are termed "new associations" (e.g. North American *Dactylopius opuntiae* and *D. confusus* Cockerell cochineals on South American *Opuntia megapotamica* Arechav. (Paterson *et al.*, 2021b)), (3) "augmentative" or "inundative", where the natural enemies of a target pest are mass-reared and released regularly to achieve control (e.g. the inundative releases of the *Megamelus scutellaris* Berg delphacid to control *P. crassipes* on the Hartbeespoort Dam in South Africa (Coetzee *et al.*, 2022), and *T. romana* for the control of *A. donax* in Texas, USA (Moran *et al.*, 2014)), and (4) "conservation", which focuses on maintaining the fitness and efficacy of natural enemy populations (e.g. parasitoids, pathogens, or predators, usually of arthropod pests (Shields *et al.*, 2019)) (McFadyen, 1998; Collier and Van Steenwyk, 2004; van Driesche *et al.*, 2009; Gullan and Cranston, 2014; Begg *et al.*, 2017).

Classical biological control is the most common and successful approach for the control of alien invasive weeds (Seastedt, 2015). A typical biological control programme begins with native range surveys, followed by the identification and selection of potential agents, host-specificity testing, mass rearing of the chosen agent/s, releases into target areas, and post-release evaluations (McFadyen, 1998).

1.5.4 Native range surveys

Native range surveys can be logistically difficult and expensive, but are pivotal in identifying which natural enemies are suitably host-specific and appropriate for consideration as biological control agents (Goolsby *et al.*, 2006b). Biological control practitioners need to first establish the exact origin/s of the target weed through literature and herbarium searches, and through the use of taxonomic and molecular tools (e.g. DNA sequencing and fragment analysis for genotype matching (Goolsby *et al.*, 2006a,b; Paterson *et al.*, 2009)). It is also important to determine whether the weed is a hybrid, as this can affect the performance of herbivores across different parental crosses (e.g. *Lantana camara* L. (Zalucki *et al.*, 2007), *Nymphaea mexicana* Zucc. (Reid *et al.*, 2021), and *Tamarix* L. spp. (Gaskin and Schaal, 2003)).

The biogeography of the target plant across its distribution is important to consider because geographically separated intraspecific populations can host differing suites of herbivores (Goolsby *et al.*, 2003). A plant's distribution can cover a wide spectrum of climate and habitat types, and so surveys should be as comprehensive as possible. Bell *et al.* (2013), for example, showed how the arthropod assemblages on *Parkinsonia aculeata* L. differed significantly across different biogeographical areas. Climatic matching between the native and introduced ranges of a weed can assist in prioritising sites to survey that are likely to be suitable for the establishment of prospective insect agents (Robertson *et al.*, 2008), but these projections should not be used as a predictor of agent performance (Goolsby *et al.*, 2006b).

1.5.5 Identification of target weeds and their agents

Correctly identifying target weeds and their hybrids is integral to finding their sources of origin and searching for and selecting appropriate potential biological control agents (Smith *et al.*, 2018). Misidentifications and taxonomic confusion have posed a number of challenges to biological control programmes in the past (Wardill *et al.*, 2005; Smith *et al.*, 2018), where even different conspecific genotypes of a weed have led to differences in agent damage (Gaskin *et al.*, 2013).

Equally as important to the safety and success of a biological control programme is the correct identification of potential agents, many of which may not yet be described (Goolsby *et al.*, 2006a; Andersen and Wagner, 2016; Smith *et al.*, 2018; Bin *et al.*, 2012; McCulloch *et al.*, 2022). Advances in genetic identification tools have facilitated the verification of taxonomic relationships and intraspecific population structures, and has revealed that a number of arthropod species previously thought to be polyphagous generalists are actually host-specific cryptic species. This has important implications in host-specificity testing, taxonomic classification, hybridisation studies, and government regulation (Smith *et al.*, 2018).

Cryptic species

Cryptic species are defined as two or more species that are reproductively isolated but are morphologically indistinguishable, and thus incorrectly classified as one species (Bickford *et al.*, 2007; Gaskin *et al.*, 2011). Cryptic species complexes can be positive for biological control, where a greater diversity of potential agents with restricted host ranges may be available (Smith *et al.*, 2018). It could also, however, result in the unintended release of cryptic species that practitioners are unaware of (Madeira *et al.*, 2006; Sha *et al.*, 2007; Paynter *et al.*, 2008; Paterson *et al.*, 2019). These species may have broader host ranges, resulting in non-target damage or unexpected ecological interactions. Alternatively, they may be unsuited to the target and deliver little or no control (Toševski *et al.*, 2011; Sheikh *et al.*, 2022). It is important to consider cryptic species at the level of both the biological control agent and invading plant, as different intraspecific plant lineages/haplotypes can present cryptic invasions that can be difficult to manage (Canavan *et al.*, 2020). A few examples from the literature are discussed in the following paragraphs, where genetic techniques have been used for cryptic species discovery.

Cydia succedana The gorse pod moth *Cydia succedana* Denis and Schiffermüller (Lepidoptera: Tortricidae), was released in New Zealand in 1992 as a host-specific biological control agent for gorse weed (*Ulex europaeus* L.). Post-release field surveys, however, revealed that the moth had a broader host range than previously predicted, where it infested a number of other exotic species in the Genisteae and Loteae tribes (Paynter *et al.*, 2008). It was found that the original *C. succedana* population, sourced from the United Kingdom (UK), also contained progeny from a Portuguese population. Although these populations were not genetically differentiated enough to be classified as cryptic species, the Portuguese population appeared to display a broader host range than the UK population that was used for host-specificity testing. The use of population genetic tools would have likely enabled the detection of these different taxonomic groups earlier on. The asynchrony between the flowering period of *U. europaeus* and the flight period of *C. succedana* was also found to potentially play a role in the differences in host utilisation. Although the non-target attacks occurred on other exotic species such that no negative environmental or economic impacts resulted, unexpected outcomes such as this need to be avoided at all costs to ensure the safety of biological control programmes.

Eccritotarsus catarinensis The discovery of two geographically isolated populations of *Eccritotarsus catarinensis* Carvalho (Hemiptera: Miridae), used for the control of the water weed *Pontederia crassipes*, was split into *E. catarinensis* and *E. eichhorniae* Henry following genetic and interbreeding experiments (Paterson *et al.*, 2016), and a morphological analysis (Henry, 2017) (Fig. 1.3 A - D). The initial genetic work by Taylor *et al.* (2011), using inter-simple sequence repeats (ISSRs) and COI sequences, revealed that the independent collections from Brazil and Peru were in fact two different mirid genetic groups. A difference in thermal tolerance was later found between the two species, where *E. catarinensis* performed better at lower, and *E. eichhorniae* at higher temperatures (Paterson *et al.*, 2019). This has very important implications for the efficacy of these agents at release sites with differing climatic conditions.

Mecinus janthinus The stem-mining weevil *Mecinus janthinus* Germar (Coleoptera: Curculionidae) was released in North America in 1991 for the control of invasive toadflax species (*Liniaria* Mill.), but was later found to comprise at least two cryptic species: *M. janthinus* Germar and *M. janthiniformis* Toševski & Caldara (Toševski *et al.*, 2011, 2013). The larvae were found to display different host preferences, where *M. janthinus* is associated with yellow toadflax (*L. vulgaris* Mill.) and *M. janthiniformis* with broomleaf toadflax (*L. genistifolia* (L.) Mill.) and Dalmatian toadflax (*L. dalmatica* (L.) Mill.). The discovery of these cryptic species can contribute to explaining the cause of the differential success of the biological control programme, and can assist in selecting the most appropriate agent for a particular target weed.

Psylliodes chalcomerus The stem-boring flea beetle, *Psylliodes chalcomerus* Illiger (Coleoptera: Chrysomelidae), is a potential biological control agent for yellow starthistle, *Centaurea solstitialis* L. (Antonini *et al.*, 2008). *Psylliodes chalcomerus* feeds on a variety of host plants in its indigenous range, but since the populations collected (> 10) were morphologically identical, they were considered a single species. Genetic analyses using mitochondrial markers later revealed that this group comprised at least three cryptic species (Antonini *et al.*, 2008; De Biase *et al.*, 2019). This has important implications for host-specificity testing and suitability, as each species (and possibly their hybrids) may display differing host breadths and levels of damage, and may possess varying physiological and ecological traits (e.g. thermal tolerance and competition dynamics (Paterson *et al.*, 2016, 2019)).

Trichosirocalus horridus The weevil *Trichosirocalus horridus* Panzer is used a biological control agent of invasive thistles in the *Carduus* L., *Cirsium* Mill., and *Onopordum* L. genera (Asteraceae, Carduoideae), and was initially recognised as one species (De Biase *et al.*, 2016). It was then split into three species in 2002 based on morphology (*T. horridus* Panzer, *T. briesei* Alonso-Zarazaga & Sánchez-Ruiz, and *T. mortadelo* Alonso-Zarazaga & Sánchez-Ruiz), each with different host ranges and geographic distributions (Alonso-Zarazaga and Sanchez-Ruiz, 2002). In 2016, based on a combination of genetic, morphological, and host range data, De Biase

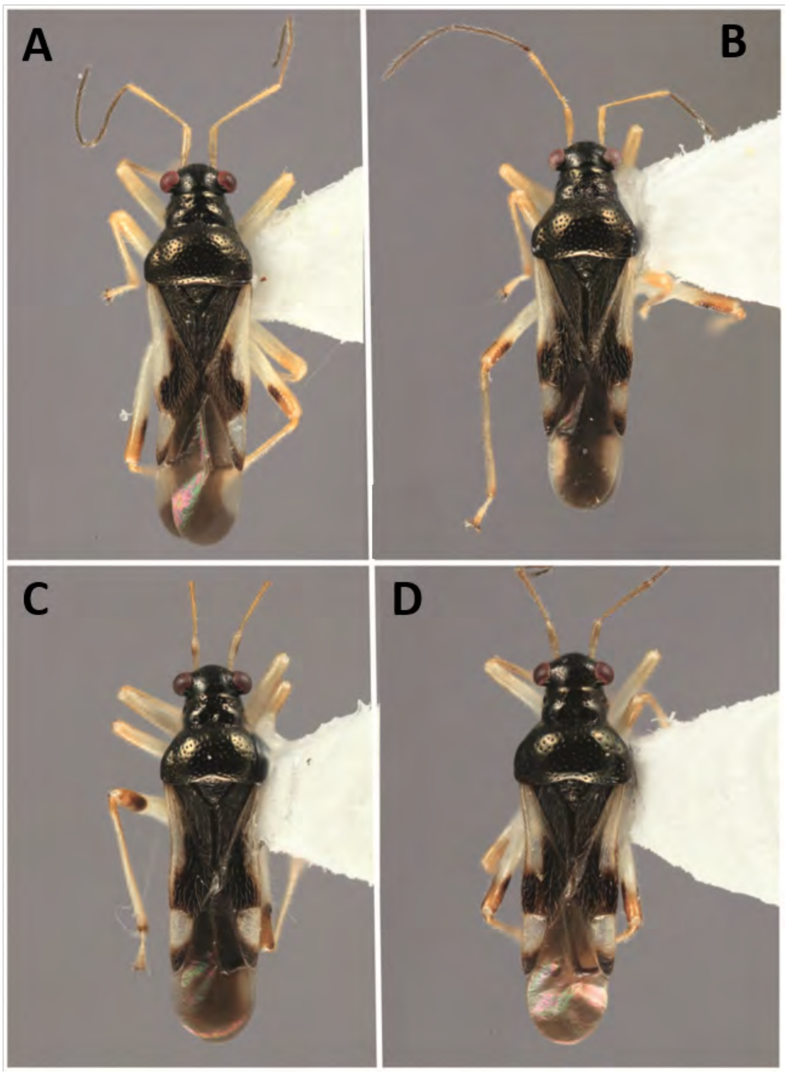


Figure 1.3: Examples of cryptic species, where groups are morphologically similar but genetically distinct. (A) and (B) *Eccritotarsus catarinensis* male and female, respectivley, (C) and (D) *E. eichhorniae* male and female, respectively (Henry, 2017).

et al. (2016) found that *T. horridus* and *T. mortadelo* were synonymous, and that there were only two distinct species. *Trichosirocalus horridus* has a broader host range, feeding on *Carduus, Cirsium, and Onopordum* species in the field, while *T. briesei* appears to be host-specific to *Onopordum* species. This means that while *T. horridus* and *T. mortadelo* were considered one species, releases of the former may have resulted in some degree of non-target damage, and inadvertently precluded the use of *T. mortadelo*, which would have otherwise been an effective agent.

1.5.6 Agent selection and prioritisation

There are usually numerous phytophagous species associated with a particular host plant (Frost, 1954; Southwood, 1961), which all inflict varying levels of damage. Since host specificity testing is time-and resource intensive (McFadyen, 1998), the prioritisation of agents is integral, such that only the most effective candidates are selected. A number of factors need to be considered in order to achieve this, which include the agent's degree of host-specificity and damage delivered, generation time (e.g. multivoltine) and overall fecundity, susceptibility to natural enemies, feeding behaviour (e.g. gregarious), compatibility with other agents, distribution, and previous biological control track record (Harris, 1973; Goeden, 1983).

As discussed earlier, ecological modelling and climatic and genotypic matching are frequently incorporated into agent-selection procedures, where factors such as disturbance regimes, competition, plant succession, different life stages of the host plant, climatic variation and compatibility, and the genetic variation in both the host plant and agent are considered (Cullen, 1995; Iline and Phillips, 2004; Kriticos, 2003; Dhileepan *et al.*, 2006; Goolsby *et al.*, 2006a; Van Klinken and Raghu, 2006; Robertson *et al.*, 2008; Paterson *et al.*, 2009; van Driesche *et al.*, 2009; Paterson *et al.*, 2014; Fischbein *et al.*, 2019; Jourdan *et al.*, 2019; Sutton, 2019).

1.5.7 Host specificity testing

The safety of a biological control programme is vital, and requires a thorough risk assessment of the potential non-target effects that could arise following the release of an agent into a target area (McEvoy, 1996; Secord and Kareiva, 1996; McFadyen, 1998; van Klinken, 1999; Schaffner, 2001; Heard, 2002; van Driesche *et al.*, 2009). This involves host specificity testing, which assesses the host range breadth of candidate agents and their relative acceptability of different host plant species (van Klinken, 1999). There are three central steps in the host specificity testing process, as outlined by van Klinken (1999): (1) identifying which stages in an agent's life history need to be host-specific, (2) estimating the fundamental host range through the use of the centrifugal-phylogenetic method proposed by Wapshere (1974), and (3) determining the realised host range through field-based experiments. Host-specificity testing and impact assessments usually take an average of five years in South Africa, and typically costs about US$ 247 828 per novel agent (at 2020 values) (Ehlers *et al.*, 2020).

The centrifugal-phylogenetic method tests an array of plant species in the agent's host range, beginning with those most closely related to the target weed, working outwards to more distantly related species (see for example Hinz *et al.* (2008); Briese and Walker (2008); Wheeler and Madeira (2017)). Phylogenies are important in host-range testing because it allows researchers to prioritise test species that are most at risk of non-target damage. Plants occurring in the same habitat, that share similar chemical and/or morphological traits, or serve as hosts to congeneric insects to that of the target weed are also included in the testing procedure. Economically important crops and rare and endangered species in the same family as the target weed are prioritised. The testing procedure encompasses choice and no-choice tests (Schaffner, 2001), continuation, oogenesis, and open-field tests (van Driesche *et al.*, 2009; Schaffner *et al.*, 2018).

A risk:benefit assessment is required in cases where non-target species may be at risk in order to determine whether the relative value of the non-target species is higher than the damage caused by the target weed (McFadyen and Marohasy, 1990; Cristofaro *et al.*, 1998; McFadyen, 1998; Haye *et al.*, 2006; van Driesche *et al.*, 2009). Olckers *et al.* (1995), for example, found that the two defoliating beetles *Leptinotarsa texana* Schaeffer and *L. defecta* Stål (Coleoptera: Chrysomelidae), considered for the control of alien invasive *Solanum elaeagnifolium* Cavanilles in South Africa, could develop on cultivated egg plant (*Solanum melongena* L.) and five native South African *Solanum* species. Futher experimentation revealed that the threat to these non-target plants was negligible, and that the minimal NTAs would be a fair trade-off for controlling the aggressive invader. Both agents were released in 1992, and *L. texana* in particular has had significant benefits, with no non-target impacts being reported (Hoffmann *et al.*, 1998; Winston *et al.*, 2021).

1.5.8 Mass-rearing, release, and post-release evaluations

The mass-rearing of biological control agents aims to produce as many disease-free insects as possible, while minimising labour and resource costs, in order to release sufficiently large numbers to control target pests (Fig. 1.4 A, B, C) (Hill *et al.*, 2021). Maintaining healthy insect cultures depends on a number of factors, including (1) the provision of high quality food, (2) exposure to optimal climatic conditions, (3) minimising inbreeding depression, and (4) protection from natural enemies (Racelis *et al.*, 2010; Moran *et al.*, 2014).

The mass-rearing and inundative releases of *Tetramesa romana* for the control of *Arundo donax* in Texas, for example, has resulted in population densities of 39-fold greater than in the insect's native range, causing considerable damage to the grass (Marshall *et al.*, 2018). The wasps are released through an aerial-release system, where cardboard boxes are opened and dropped over *A. donax* stands from single-engined Cessna aircraft (Fig. 1.4 D) (Racelis *et al.*, 2010).

Post-release evaluations are often neglected and underfunded, but they are important to monitor the long-term impacts of a biological control programme (Schaffner, 2001; Morin *et al.*, 2009). It is particularly important to assess the changes in native species and community structure in response to a reduction in weed populations (e.g. it might be that the ecosystem is restored, or it might lead to a secondary invasion (Pearson *et al.*, 2016; Coetzee *et al.*, 2021)), potential non-target effects, and reasons for the success or failure of the programme (Carson *et al.*, 2008).

1.6 Control of Invasive Grasses

Mechanical and chemical methods of controlling invasive grasses have been largely ineffective and impractical due to the large spatial scales involved (Quirion *et al.*, 2018), the development of resistance to herbicides (Peterson *et al.*, 2018), non-target effects on the environment and native species (Reeg *et al.*, 2017), and extreme costs due to the necessity of repeated applications (Grice *et al.*, 2012). Grasses are therefore an ideal target group for the use of biological control.

Very few invasive grasses have been targeted using biological control programmes in the past, mainly due to (1) potential non-target attacks on economically-valuable crops (e.g. wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), sugarcane (*Saccharum officinarum* L.), sorghum (*Sorghum bicolor* (L.) Moench), and corn (*Zea mays* L.)), (2) lack of host-specificity, and (3) tolerance to herbivory (Sutton *et al.*, 2021a). The low levels of secondary metabolites in the grass family (McNaughton *et al.*, 1985; Huitu *et al.*, 2014) raised concerns surrounding the

Figure 1.4: The Centre for Biological Control's (CBC) (A) Waainek water weeds and (B) Uitenhage cactus mass rearing facilities for biological control agents. (C) The release of *Dactylopius opuntiae* 'stricta' lineage on *Opuntia stricta* in the Eastern Cape, South Africa. (D) The release of boxes of *Tetramesa romana* over the Rio Grande River, USA, from a Cessna aircraft. Images A, B, and C: credit to the CBC; image D taken from Racelis *et al.* (2010).

lack of host-specific natural enemies (Witt and McConnachie, 2004). This is because secondary chemicals, such as terpenoids, alkaloids, tannins, and cyanogenic glycosides, are considered key drivers of herbivore specialisation and resulting monophagy (Ehrlich and Raven, 1964; Moore and Johnson, 2017; Kariñho-Betancourt, 2020) (but see Bernays and Graham (1988)), and as such, grasses were expected to host a limited suite of specialist herbivores. This would make them inappropriate targets for biological control since the practice relies on the host-specificity of herbivores. Some recent studies have, however, found evidence of the production of specialised metabolites in grasses (e.g. switchgrass, *Panicum virgatum* L.) (Li *et al.*, 2022). Additionally, symbiotic fungal endophytes are known to be able to produce distinct alkaloid classes which may influence herbivory resistance (Schardl *et al.*, 2004; Saikkonen *et al.*, 2013). This suggests that the metabolomic profiles of grasses may not be as depauperate as previously reported.

Since grasses have evolved under strong grazing pressures from herbivorous insects and mammals (Tscharntke and Greiler, 1995), it was believed that releasing biological control agents would have a negligible effect (Sutton *et al.*, 2021a). These perceptions have resulted in grasses not being targeted for biological control in the past despite the large number of invasive species.

The belief that grasses were poor candidates for biological control has changed in recent years due to increased reports of putative host-specific natural enemies that are damaging to their grass hosts (Witt and McConnachie, 2004; Anderson *et al.*, 2017; Blossey *et al.*, 2018; Casagrande *et al.*, 2018; Blossey *et al.*, 2020). This may be due to other factors selecting for narrow host ranges, such as plant structural defences (Bernays and Graham, 1988; War *et al.*, 2018) and predator avoidance (driving what is termed 'enemy-free space') (Bernays, 1988). At the time of the first review of grass biological control by Sutton *et al.* (2021a), 23 invasive grass species were currently, or had already been, investigated as targets for biological control using herbivorous arthropods and fungal pathogens. To date, the catalogue maintained by Winston *et al.* (2021) listed 14 releases of seven biological control agent species onto five invasive grass species between 1997 and 2019 (Table 1.2). These include the release of *Tetramesa romana* Walker (Hymenoptera: Eurytomidae) on *Arundo donax* in Mexico, the United States of America (USA), and New Zealand, *Rhizaspidiotus donacis* Leonardi (Hemiptera: Diaspididae) on *A. donax* in Mexico and the USA, and *Prokelisia marginata* van Duzee (Hemiptera: Delphacidae) on *Spartina alterniflora* and *S. anglica* in the USA. Two *Archanara* species, *A. geminipuncta* Haw. and *A. neurica* Hübner (Lepidoptera: Noctuidae), were recently petitioned for release on *Phragmites australis* in the USA (Blossey *et al.*, 2020), and the rust fungus *Uromyces pencanus* Arth. & Holw. was approved for release in New Zealand for the control of *Nassella neesiana* Barkworth (Anderson *et al.*, 2017).

There are a number of other potential grass biological control agents currently under consideration (e.g. Eriophyid mites (Acari: Eriophyoidea) (Reinert *et al.*, 2007; Smith *et al.*, 2010; Brown *et al.*, 2021), shoot-galling flies (Diptera: Chloropidae) (Nartshuk, 2014), and fungal pathogens (e.g. Pikatek *et al.* (2015))), but the stem-galling members of the *Tetramesa* Walker (Hymenoptera: Eurytomidae) genus are particularly promising due to their reported host-specificity and the previous success of *T. romana* on invasive *Arundo donax* in the USA (Moran and Goolsby, 2009; Sutton *et al.*, 2019).

Of the 23 invasive grass species that have been under consideration for biological control around the world, eight are under evaluation in Australia; namely *Andropogon gayanus* Knuth. (gamba grass), *Eragrostis curvula* (Schrad.) Nees (African lovegrass), *Sporobolus natalensis* (Steud.) Dur. & Schinz, *Sporobolus pyramidalis* P. Beauv. (giant rat's tail grass), *Avena fatua* L. (wild oats), *Hymenachne amplexicaulis* (Rudge) Nees (West Indian marsh grass), *Nassella neesiana* (Trin. & Rupr.) Barkworth (Chilean needlegrass), and *Nassella trichotoma* (Nees) Hack. ex Arechav. (serrated tussock) (Sutton *et al.*, 2019). Additionally, *M. maximus* is being targeted for biological control in the USA (Vacek *et al.*, 2021; Gaskin *et al.*, 2022).

Sutton *et al.* (2021a) prioritised five target grasses that are feasible biological control targets in South Africa based on the Biological Control Target Selection system developed by Paterson *et al.* (2021a); namely the category 1b-listed (1) *A. donax* L., (2) *Cortaderia jubata* Stapf., (3) *Cortaderia selloana* Asch. & Graebn., (4) *Nassella trichotoma* Hack., and (5) *Glyceria maxima* Holmb. Biological control programmes for the first four of these species have already been initiated elsewhere in the world, making transfer or repeat programmes in South Africa more cost-effective options. There is sufficient evidence to suggest that the biological control of grasses is equally as feasible and likely to be as effective as other plants, and that the *Tetramesa* wasps are promising potential agents that may be prioritised in many cases.

Table 1.2: A list of all the agents released on invasive Poaceae from 1997 - 2019, extracted from Winston *et al.* (2021).

Weed	**Agent**	**Country, year**	**Established?**	**Impact**
Arundo donax	*Rhizaspidiotus donacis* Leonardi (Hemiptera, Diaspididae)	Mexico, 2011	Established	Medium
Arundo donax	*Tetramesa romana* Walker (Hymenoptera, Eurytomidae)	Mexico, 2009	Established	Medium
Arundo donax	*Rhizaspidiotus donacis* Leonardi (Hemiptera, Diaspididae)	USA, 2010	Established	Medium
Arundo donax	*Tetramesa romana* Walker (Hymenoptera, Eurytomidae)	USA, 2009	Established	Medium
Arundo donax	*Tetramesa romana* Walker (Hymenoptera, Eurytomidae)	USA	Established	Medium
Arundo donax	*Lasioptera donacis* Coutin (Diptera, Cecidomyiidae)	USA, 2017	Not established	N/A
Arundo donax	*Tetramesa romana* Walker (Hymenoptera, Eurytomidae)	New Zealand, 2017	Unknown	Unknown
Arundo donax	*Tetramesa romana* Walker (Hymenoptera, Eurytomidae)	South Africa	Unknown*	Slight
Arundo donax	*Lasioptera donacis* Coutin (Diptera, Cecidomyiidae)	Mexico, 2018	Not established	N/A
Phragmites australis subsp. *australis*	*Lenisa geminipuncta* Haworth (Lepidoptera, Noctuidae)	Canada, 2019	Unknown	Unknown
Phragmites australis subsp. *australis*	*Archanara neurica* Hübner (Lepidoptera, Noctuidae)	Canada, 2019	Unknown	Unknown
Poa annua	*Xanthomonas campestris pv. poae* (Gammaproteobacteria, Xanthomonadales)	Japan, 1997	N/A	Heavy
Spartina alterniflora	*Prokelisia marginata* Van Duzee (Hemiptera, Delphacidae)	USA, 2000	Established	Variable
Spartina anglica	*Prokelisia marginata* Van Duzee (Hemiptera, Delphacidae)	USA, 2003	Established	Variable

* This is an adventive population of *Tetramesa romana* in South Africa, and not an intentionally released (Canavan *et al.*, 2019a).

Note: *Ischnodemus variegatus* (Signoret) (Hemiptera: Blissidae) for the control of *Hymenachne amplexicaulis* (Rudge) Nees is pending acceptance in Australia (Diaz *et al.*, 2009), and the rust fungus *Uromyces pencanus* Arth. & Holw. (Uredinales) for the control of *Nassella neesiana* (Trin. & Rupr.) Barkworth has been approved in New Zealand and is pending export permits (Anderson *et al.*, 2017).

1.7 The *Tetramesa* wasps

1.7.1 Overview

There are approximately 88 genera and 1424 described hymenopteran species in the Eurytomidae family (Chalcidoidea: Eurytomidae: Eurytominae) (Noyes, 2012). A number of eurytomids have been released, or considered, as biological control agents of invasive weeds in the past (such as *Tetramesa romana* on *Arundo donax* and *Eurytoma bryophylli* on *Bryophyllum delagoense* (Neser, 2008)). Only three eurytomid genera feed exclusively on grasses; namely *Cathilaria* Burks, *Eurytomocharis* Ashmead, and *Tetramesa* Walker (DiGaulio, 1998).

1.7.2 Taxonomy

The taxonomic history of the *Tetramesa* genus is convoluted, with 12 generic synonyms in use since the first description made by Francis Walker in 1832, in which he named the group "*Isosoma*" (Walker, 1832) (Fig. 1.5). Following a revision of the group in 1919 (Phillips and Emery, 1919), Gahan (1924) suggested that the name *Isosoma* be replaced with the name *Harmolita* Motschulsky, as it was discovered that there was already a genus of Coleoptera named *Isosoma*. The German entomologist, R. Hedicke, refused this suggestion, calling the genus *Isthmosoma* instead (Gahan, 1924). By 1958, Michael Claridge suggested that the name *Tetramesa* should be treated as a synonym for *Isosoma*, and replace *Harmolita* (Claridge, 1961), which it retains at present.

As far back as 1922, Gahan (1922) wrote that the Eurytomidae family was in need of a major revision, and that the *Harmolita* genus would have to serve as a container for new species descriptions even though some of the species were "*perhaps slightly extralimital in some characters*". More than 80 years later, in a molecular phylogenetic study of the Eurytomidae, Chen *et al.* (2004) reported that the Eurytominae subfamily has been treated as a "trash can" for taxa that do not fit into the other subfamilies (currently comprising Buresiinae, Rileyinae, and Heimbrinae), and that the Rileyinae subfamily was more closely related to the Torymidae than to Eurytomidae. Similarly, Gates (2008) wrote that "*although various families of the*

Eurytomidae have been recognised, roughly the same taxa are merged or separated depending on the author's perspective". Since morphological and molecular evidence suggest that the Eurytomidae family is not monophyletic (Campbell *et al.*, 2000; Chen *et al.*, 2004; Lotfalizadeh *et al.*, 2007b; Munro *et al.*, 2011), an increased effort is required to apply an integrative taxonomic approach to start assembling the missing puzzle pieces in this complex insect group.

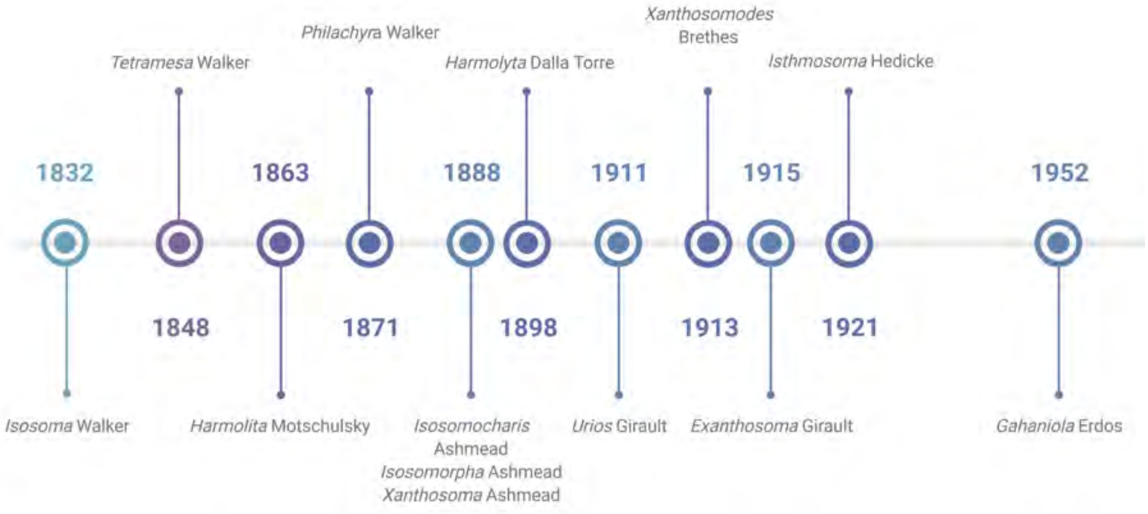


Figure 1.5: Synonyms used for the *Tetramesa* Walker genus since its first description in 1832. Data were sourced from the Natural History Museum Universal Chalcidoidea Database (`https://www.nhm.ac.uk/our-science/data/chalcidoids/database`).

The *Tetramesa* genus currently comprises at least 200 species (202 according to the Natural History Museum (2021), plus the newly-described *T. amica* Lotfalizadeh, sp. nov. (Lotfalizadeh *et al.*, 2020), and 205 according to Al-Barrak (2006)). *Tetramesa* wasps are highly host-specific; typically specialising on only one species or genus, or on closely-related genera in the Poaceae (Phillips, 1936; Claridge, 1961). Adults feed on nectar (or do not feed at all), and lay their eggs in the stems of their host plant (Al-Barrak, 2006). The larvae are endophagous herbivores, and are either stem borers or gallers (Claridge, 1961). Several *Tetramesa* species are serious agricultural pests to grains and forage, including *T. tritici* Fitch (wheat jointworm), *T. grandis* Riley (wheat strawworm), *T. hordei* Harris (barley jointworm), and *T. secale* (Fitch) (rye jointworm) (Goolsby and Moran, 2009). Jones *et al.* (1952) wrote that *T. tritici* '*ranks next in destructiveness to the hessian fly [on wheat]*'. Spears and Barr (1985), for example, reported that *Tetramesa* infestations reduced seed weight, percentage germination, and germination rate in four grass species native to North America (*Aristida longiseta* Steud. *Sitanion hystrix*

Nutt. J.G. Smith, *Sporobolus cryptandrus* Torr. A. Gray, and *Stipa comata* Trin. & Rupr); with a 99% reduction in the seed germination of *A. longiseta* Steud. The highly host-specific behaviour of the *Tetramesa*, and the damage that they can cause to their host plants, makes this group an ideal candidate species complex for biological control agents of invasive grasses.

1.7.3 *Tetramesa* as biological control agents

Only one *Tetramesa* species, *T. romana* (the arundo gall wasp), has been used as a grass biological control agent to date. The host-specificity of *T. romana*, collected from a population in its native range in Spain, was sufficient for it to be released as a control agent for *Arundo donax* L. (giant reed or carrizo cane) along the Rio Grande in Texas (USA), and Mexico, for the first time in 2009 (Table 1.2 and Figure 1.6) (Goolsby and Moran, 2009; Moran and Goolsby, 2009; Racelis *et al.*, 2010). The wasp was also later released in Arizona and California (Moran *et al.*, 2017). *Arundo donax* is an invasive perennial reed native from Mediterranean Europe to India (Hardion *et al.*, 2014). It has become invasive across the world, particularly in the arid riparian regions of the southern USA, California, and Mexico (Moran *et al.*, 2017). Dense infestations occur along the creeks and rivers in the Rio Grande basin in south and southwest Texas, where the reed covers over 30 000 hectares (ha) (Everitt *et al.*, 2005; Moran *et al.*, 2017). Post-release evaluations indicate that *T. romana* has spread over 800 km along the river, and is having a considerable impact on *A. donax* (Goolsby *et al.*, 2016). A 32% reduction in biomass was recorded at selected sites between 2014 and 2016, as well as the recovery of native plant communities, and the reduction in agricultural water loss (see Racelis *et al.* (2021)) estimated at $\sim$ US$ 4.4 million annually (Moran *et al.*, 2017).

A number of other *Tetramesa* species are currently being considered as potential grass biocontrol agents. Witt and McConnachie (2004) reported the presence of an unidentified *Tetramesa* sp. on *S. africanus*, *S. natalensis*, and *S. pyramidalis* in southern Africa (*Tetramesa sp. 1*). About 33% of the *S. pyramidalis* surveyed were infested with the wasp, resulting in malformed inflorescences in 60% of those sampled. It was also reported that the wasps significantly reduced culm lengths, but this requires further experimentation to eliminate the possibility that the wasps selected for shorter culms. A second unidentified *Tetramesa* species

(*Tetramesa sp. 2*) was found on *S. natalensis* and *S. pyramidalis* by Sutton *et al.* (2019, 2021b) during native range surveys, where the wasp was found only on these two target weeds. Sutton *et al.* (2021b) found that both *Tetramesa* species significantly decreased tiller survival and had a deleterious impact on tiller reproduction, where *Tetramesa sp. 1* was reported to be the most damaging of the two wasps. *Tetramesa sp. 1* and *Tetramesa sp. 2* are currently under evaluation as potential agents for *S. natalensis* and *S. pyramidalis* in Australia (Witt and McConnachie, 2004; Sutton *et al.*, 2019), and were imported into quarantine in Australia for the first time in September 2022 to undergo host-specificity testing.

The newly-described *T. amica* Lotfalizadeh, sp. nov. on *Taeniatherum caput-medusae* L. Nevski (Medusahead) is also being evaluated as a potential biological control agent (Lotfalizadeh *et al.*, 2020). *Taeniatherum caput-medusae* is an annual grass native to the Mediterranean region, and has become an invasive weed in the USA, the north and northwestern parts of Europe, Chile, and Australia (Peters, 2013).

1.7.4 Extent of species discovery to date

Almost all of the sampling effort in collecting and describing *Tetramesa* species has taken place in the Northern Hemisphere (Al-Barrak, 2006) (Fig. 1.7). Only four African species have so far been described; namely *T. aristidae* Risbec from Senegal, *T. decaryi* Risbec and *T. tananarivense* Risbec from Madagascar, and *T. macalusoi* De Stefani from Somalia (van Noort, 2020). To date, no *Tetramesa* species have been described from South Africa (van Noort, 2020), although the existence of novel taxa appears certain (see above). It is very likely that there is a high diversity of native southern African species that can be utilised as potential biological control agents of African grasses that are invasive outside their native range. This is particularly important to investigate because Africa is such a large donor of invasive grasses (Visser *et al.*, 2016), and so surveying for natural enemies will be greatly beneficial to biological control programmes.

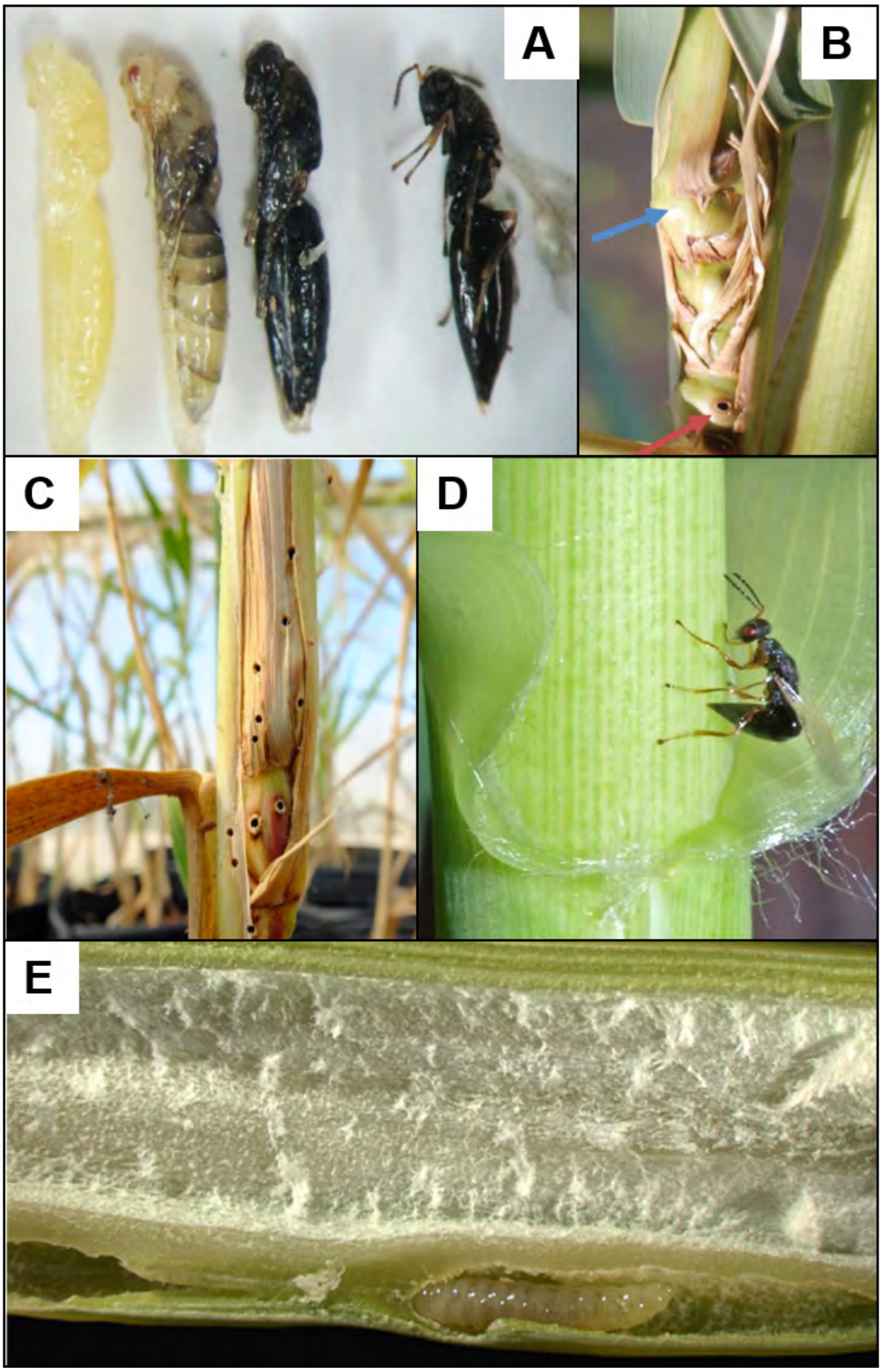


Figure 1.6: (A) *Tetramesa romana* pupal stages and mature adult (adult females measure ∼ 5.4 mm in length (Moran and Goolsby, 2009)), (B) gall formation (blue arrow) and adult wasp exit hole (red arrow) in *Arundo donax*, (C) multiple exit holes in *A. donax*, (D) an adult *T. romana* female ovipositing in the stem of *A. donax*, and (E) the endophagous feeding of a *Tetramesa* larva. Figures A and B taken from Jiménez *et al.* (2017), Figure C credit: Quentin Paynter, Figures E and E credit: John Goolsby.

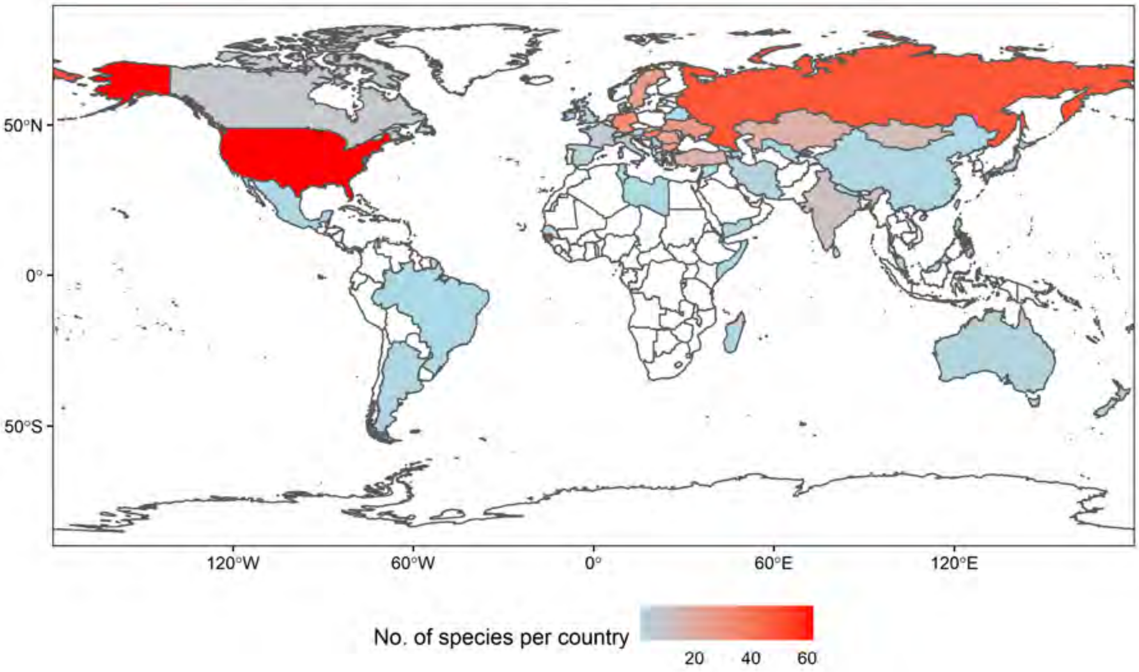


Figure 1.7: The global distribution of recorded and described *Tetramesa* species, as sourced from the literature (see the file in the 'maps' folder in `https://github.com/clarkevansteenderen/PhD_files`, compiled by Guy Sutton).

1.7.5 Molecular tools and taxonomy

The morphological uniformity of the adult and larval stages makes the *Tetramesa* extremely challenging to identify (Dawah, 1987; Ghajarieh *et al.*, 2006). Genus-level identifications delineating between the *Tetramesa* and *Eurytoma* have proven difficult (Henneicke *et al.*, 1992), and even reliable synapomorphies at the family-level are still lacking (Lotfalizadeh *et al.*, 2007b; Gates, 2008). In many cases, this is likely due to morphological convergent evolution within the Chalcidoidea (e.g. due to the utilisation of similar ecological niches and hosts) (Chen *et al.*, 2004; Peters *et al.*, 2018). For example, head shape in female Sycoecinae and Agaoninae fig wasps (Hymenoptera: Chalcidoidea: Agaonidae), has been under the same strong selection pressure due to the morphological constraints of their host fig trees (*Ficus* L.) (Noort and Compton, 1996). This has led to similar morphological features that have arisen independently in these two lineages.

Genetic barcoding has become an integral tool in species delimitation projects since it gained popularity in the early 2000s (Hebert *et al.*, 2003a,b). The Barcode of Life Database (BOLD) currently contains over 12 million genetic barcodes representing 246 000 animal, 72 000 plant, and 24 000 fungal species (BOLD, 2023). The mitochondrial cytochrome oxidase I (COI) marker is known as the "barcoding gene", and is usually preferred for metazoans due to its faster mutation rate relative to nuclear regions, and its high abundance in cells (Ballard and Whitlock, 2004; Piganeau *et al.*, 2004; Waugh, 2007; Haag-Liautard *et al.*, 2008). "Barcoding" regions are, however, not only limited to COI (for example the use of the ITS region in fungi, and *matK* and *rbcL* in plants (Casiraghi *et al.*, 2010)). In order to differentiate between putative species, the interspecific genetic distance between groups needs to be larger than their intraspecific distance, referred to as the "barcode gap" (Hebert *et al.*, 2003a). Using the COI marker, a genetic sequence divergence of 3% and above is suggested to indicate species delineation in insects, and 2% and above for vertebrates (Hebert *et al.*, 2003a). These are, however, merely ballpark figures and should be assessed on a case-by-case basis. Since the fundamental genetic composition remains the same throughout an organism's lifecycle, genetic barcoding offers the advantage of identifying putative species from material taken from any sex or developmental stage; which might be otherwise impossible to identify morphologically. Barcoding tools are by no means silver bullets, and their limitations have been reviewed by numerous authors (Moritz and Cicero, 2004; Will and Rubinoff, 2004; Rubinoff and Holland, 2005; Rubinoff *et al.*, 2006; Zamani *et al.*, 2020, 2022). These criticisms are predominantly aimed at the use of DNA barcoding alone to delineate and describe new species through what is termed a "minimalist" approach (Meierotto *et al.*, 2019). Despite the shortcomings of DNA barcoding, if used appropriately and in conjunction with other independent lines of evidence (e.g. morphological, ecological, biogeographical, behavioural characteristics, and multilocus genetic markers), the methods can be highly informative.

There have been a number of studies that have used COI barcoding tools to conduct species delimitation analyses in hymenopteran families, including the Eurytomidae (Zhang *et al.*, 2022), Braconidae (Fagan-Jeffries *et al.*, 2018; Sharkey *et al.*, 2021a,b), Formicidae (Oberprieler *et al.*, 2018; Williams *et al.*, 2022), Apidae (Milam *et al.*, 2020), Pompilidae (Waichert *et al.*, 2020), Ichneumonidae (Schwarzfeld and Sperling, 2015), and Gasteruptiidae (Parslow *et al.*, 2021). Genetic analyses of the *Tetramesa* are limited, however, with the only published studies carried out by Madjdzadeh (2004) for Iranian species using COI, COII, and cytochrome *b* markers, Al-Barrak *et al.* (2004) using random amplified polymorphic DNA (RAPD) markers for United Kingdom (UK) species, and Ghajarieh *et al.* (2006) for species in the UK using COI markers. Although not the focus of the study, Chen *et al.* (2004) sequenced a number of eurytomid wasps, including *T. bambusae* Phillips, using mitochondrial COI and 16S, and nuclear 18S and 28S markers. These, and a number of other genetic studies involving chalcidoid wasps, have been successful in delineating and uncovering cryptic species (Li *et al.*, 2010; Zhang *et al.*, 2014; Powell *et al.*, 2019; Zhang *et al.*, 2019b, 2022).

Molecular tools, particularly DNA barcoding, have become indispensable in the field of biological control (Gaskin *et al.*, 2011). Apart from enabling the identification of target weeds and insect agents, molecular tools can also aid in (1) determining the origin/s of weeds (Goolsby *et al.*, 2006a; Paterson *et al.*, 2009), (2) investigating population structure and gene flow (Brede *et al.*, 2008), (3) testing whether the agents or weeds can hybridise (Szűcs *et al.*, 2011; Havill *et al.*, 2012; Williams *et al.*, 2014), (4) the identification of immature life stages (Rector *et al.*, 2010; Rauth *et al.*, 2011), (5) establishing test plant lists based on phylogenetic relatedness (Wapshere, 1974; Kelch *et al.*, 2004; Briese and Walker, 2008), (6) determining host associations (e.g. through gut analysis (Rougerie *et al.*, 2011; Unruh *et al.*, 2016)), and (7) identifying endosymbionts associated with insect agents (e.g. *Wolbachia* that may contribute to reduced mitochondrial genetic diversity in their hosts (Roehrdanz *et al.*, 2006)).

1.8 Research Objectives

The aim of this thesis was to investigate the diversity and host plant affiliations of *Tetramesa* in South Africa. This was done to gain a better understanding of the diversity and evolution of the taxon, to investigate the radiation of cryptic species of insect herbivores, and to determine the utility of these species for the biological control of invasive African grasses in other parts of the world. More specifically, the aims of each chapter are listed below.

1. To determine the species diversity of *Tetramesa* and their host ranges in South Africa, including a dating analysis to estimate divergence times (Chapter 2).
2. To investigate the cophylogenetic relationships between the *Tetramesa* and their host plants to gain a better understanding of host specificity, and whether it is stable (Chapter 3).
3. Because it is often difficult to distinguish between speciation events and intraspecific

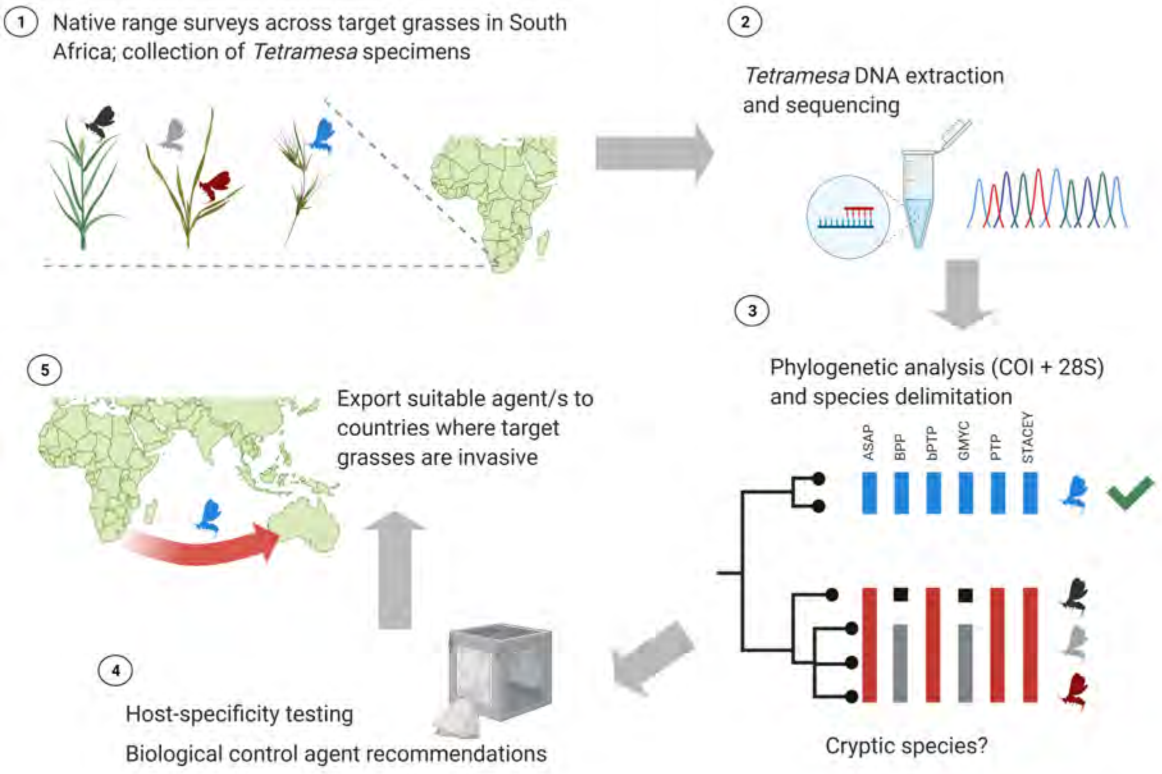
population divergences, a novel software programme, “SPEDE-sampler” was developed to investigate how computational choices and sample size can affect the results obtained from the Generalised Mixed Yule Coalescent (GMYC) species delimitation model (Chapter 4).

4. To provide recommendations on how *Tetramesa* could be used for biological control of African grasses that are invasive globally (Chapter 5).

Chapter 2

Tetramesa Phylogenetics

Graphical Overview



2.1 Introduction

2.1.1 Native-Range Surveys

Native-range field surveys are integral to biological control programmes to enable the screening of candidate agents such that only those that are host-specific to the target weed are selected

(Goolsby *et al.*, 2006b; Cabrera Walsh *et al.*, 2013; Minuti *et al.*, 2021; Sutton *et al.*, 2021a; Rafter *et al.*, 2022). DNA barcoding tools have become indispensable in cases where candidate agents are difficult to identify (e.g. immature stages, cryptic species), and can assist in better understanding their host preferences (Smith *et al.*, 2006; Li *et al.*, 2010; Zhang *et al.*, 2011; Egli *et al.*, 2020; Nawaz *et al.*, 2021).

In a similar scenario to the present study, Egli *et al.* (2020) conducted native range surveys for candidate biological control agents on *Senecio madagascariensis* Poiret (Asteraceae), which is native to southern Africa but an invasive weed in Australia. The authors used COI barcoding to differentiate between candidate agents and to determine their host-use. As there is a high diversity of native *Senecio* species in Australia, it is vital that the candidate agents are host-specific to *S. madagascariensis*. Phylogenetic trees revealed that the stem-boring curculionids and tortricids, and the capitulum-feeding pterophorids and tephritids collected were suitably host-specific, while the stem-boring agromyzids were not. Similarly, native range surveys on *Acacia auriculiformis* A. Cunn. ex Benth. (Fabaceae) and the subsequent DNA barcoding of potential agents in a study by Nawaz *et al.* (2021) revealed 143 MOTUs (Molecular Operational Taxonomic Units); 85% of which were associated with a single *Acacia* species. Finding highly specialised candidate agents during surveys is not surprising given the high percentage of insect herbivores that are specialists.

Less than 10% of phytophagous insects feed on host plants in more than three different families (Bernays and Graham, 1988). A number of possible reasons for the selection of host specialisation have been presented, and include fitness tradeoffs (e.g. an adaptation to one host plant may reduce fitness on other hosts), reduced competition for resources, reduced time spent searching for a suitable host, increased likelihood of finding a mate, predator and parasite avoidance (termed "enemy-free space"), specialised nutritional needs, and coevolved microbial symbionts (Bernays and Graham, 1988; Bernays, 1988; Futuyma and Moreno, 1988; Jaenike, 1990).

As discussed in Chapter 1 Section 1.5.5, a number of biological control agents were believed to comprise only one species, but molecular and other integrative methods found that they were in fact cryptic species complexes with different host ranges and damage potential. Cryptic species and host-use have important implications for biological control, as practitioners need to be confident that they are releasing an agent that has been identified correctly, is suitably host-specific, and that will deliver optimal control. Some cryptic species may be more effective than others under different environmental conditions due to, for example, thermal tolerance (e.g. *Eccritotarsus catarinensis* vs *E. eichhorniae* in Paterson *et al.* (2019)), and may deliver varying levels of control at different release sites. The application of genetic techniques during the stage of field surveying for new agents can help in avoiding identification problems, and separating otherwise morphologically identical groups.

2.1.2 Taxonomy and Molecular Species Delimitation

Molecular species delimitation methods use genetic data to estimate species boundaries and the number of distinct categories in a particular study system, which are often referred to as "Molecular Operational Taxonomic Units" (MOTUs), "genospecies", "phylospecies", "phylotypes", or "Recognisable Taxonomic Units" (RTUs) (Wiens, 2007; Fontaneto *et al.*, 2015; Luo *et al.*, 2018). These methods rely either on the construction of phylogenetic trees, or on genetic distance matrices (Fontaneto *et al.*, 2007). DNA-based tools have become valuable, sometimes indispensable, complements to traditional morphological taxonomy; especially for taxa that (1) comprise groups that are morphologically indistinguishable and may contain multiple cryptic species (Anguita-Salinas *et al.*, 2017), (2) are morphologically plastic in response to their environment, leading to either morphological convergence or divergence (Guo *et al.*, 2018), (3) are microscopic in size (Mills *et al.*, 2017), or (4) undergo larval stages that are challenging or impossible to identify (Collin *et al.*, 2020).

Defining what constitutes a "species", and how living organisms can be delineated in a consistent, biologically-meaningful, and objective manner, is, however, a long-debated topic in biology that remains unresolved (De Queiroz, 2005; Hey, 2006; Zachos, 2018; Wang *et al.*, 2020c). There are over 30 different species concepts (Zachos, 2016), with new additions appearing regularly in the literature (Hill, 2017; Shanker *et al.*, 2017; Hong, 2020; Seifert, 2020). One of the major challenges to species delimitation is distinguishing between species- and population-level divergences (Derkarabetian *et al.*, 2019). In other words, determining when genetically distinct groups are different species, and when are they intraspecific populations. Species numbers may regularly be overestimated due to the sensitivity of delimitation algorithms to population genetic structure caused by, for example, geographic sub-structuring (Sukumaran and Knowles, 2017).

Species delimitation methods should be approached with the understanding that they are imperfect representations of real-world biological systems (Carstens *et al.*, 2013). Asexual organisms (Dudgeon *et al.*, 2017), naturally fragmented populations, and hybridisation and introgression (Dowling and Secor, 1997; Winter *et al.*, 2018), for example, can create grey areas in species boundaries (Wiens, 2007; Galtier, 2019). An integrated approach is recommended, using a variety of methods which include genetic, biogeographical, behavioural, life-history, and morphological information (referred to as "integrative taxonomy") (see for example Hey *et al.* (2003); Hey (2006); Padial *et al.* (2010); Schlick-Steiner *et al.* (2010); Yeates *et al.* (2011); Carstens *et al.* (2013); Stanton *et al.* (2019), and also Zamani *et al.* (2020)). This thesis uses the term "host-specific taxonomic unit" (HSTU) when referring to phylogenetic clades that utilise a single host. This term is not proposing a new species concept, but may be a more useful term in a biological control context when referring to distinct host-specific genetic groups.

Species discovery is not only important for biological control, but also has important implications for the better understanding of species diversity on our planet, and to facilitate conservation and management initiatives (Bickford *et al.*, 2007; Trontelj and Fišer, 2009). This is particularly relevant to the protection of threatened and endangered species (Cook *et al.*, 2008; Delić *et al.*, 2017; Yan *et al.*, 2018; Rubinoff *et al.*, 2020); many of which are not even yet known to science (Tedesco *et al.*, 2014; Lees and Pimm, 2015).

The Centre for Biological Control (CBC) at Rhodes University, South Africa, has been collaborating with Australian biological control practitioners to prospect for biological control agents targeting invasive African *Eragrostis curvula* (African lovegrass), *Andropogon gayanus* (Gamba grass), *Sporobolus natalensis*, and *Sporobolus pyramidalis* (giant rat's tail grass) grasses in Australia (Fig. 2.2). The CBC is also currently surveying for prospective agents for *M. maximus* in collaboration with researchers from the USA. Native range surveys on African grasses in South Africa since 2017 have yielded a number of unidentified *Tetramesa* specimens, but the lack of described *Tetramesa* species in Africa (largely due to their uniform adult morphology as well as a Northern Hemisphere sampling bias) has made field-based host-specificity testing difficult to conduct (Sutton *et al.*, 2019, 2021b). The present study is not a taxonomic revision of the *Tetramesa* genus, but is the first of its kind to distinguish between African *Tetramesa* species, determine their host-specificity, and to gain a better understanding of the diversification processes among taxa feeding on native South African grasses that are invasive in Australia and the United States of America.

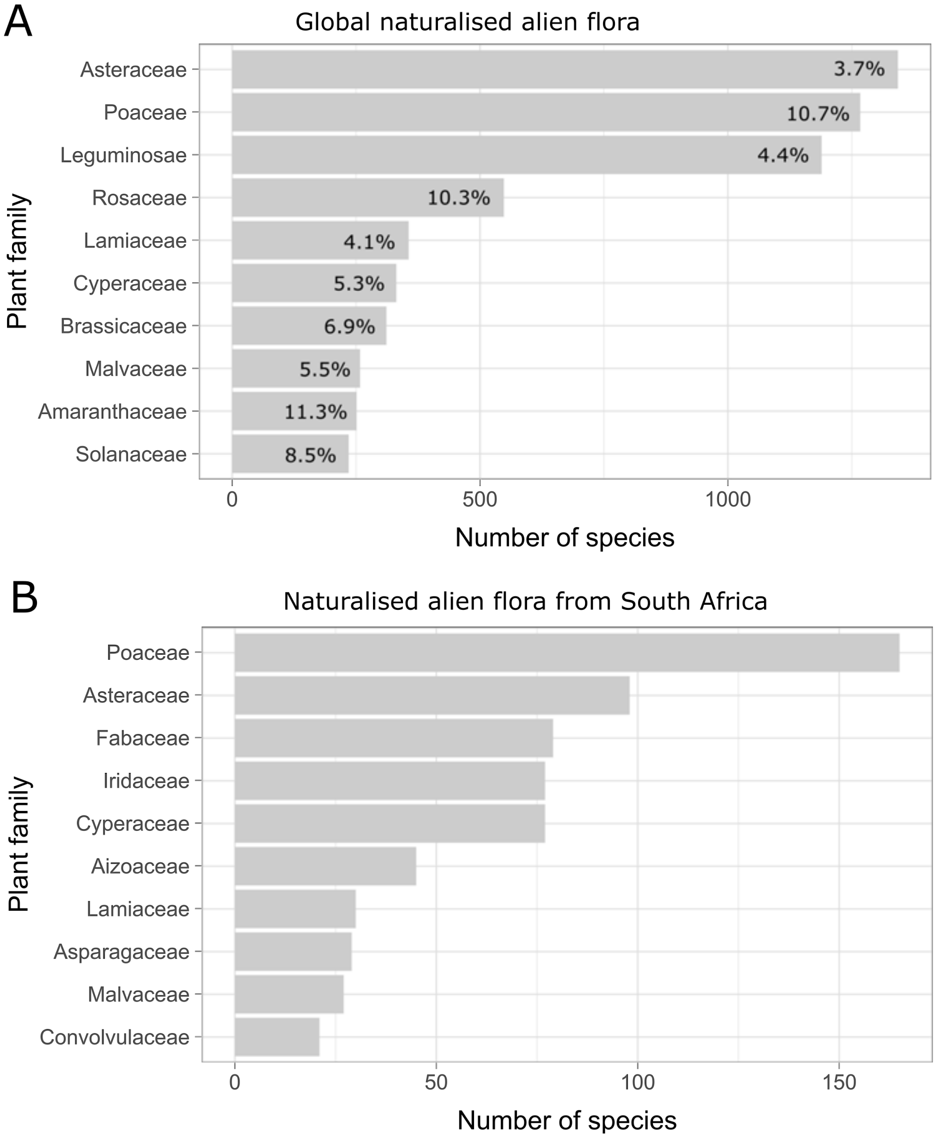


Figure 2.1: The top ten invasive plant families (A) most represented on a global scale (adapted from (Pyšek *et al.*, 2017)), and (B) originating from South Africa (adapted from Pyšek *et al.* (2020)). The percentages in plot (A) are the proportions of invasive species relative to the total number of described species in the family.

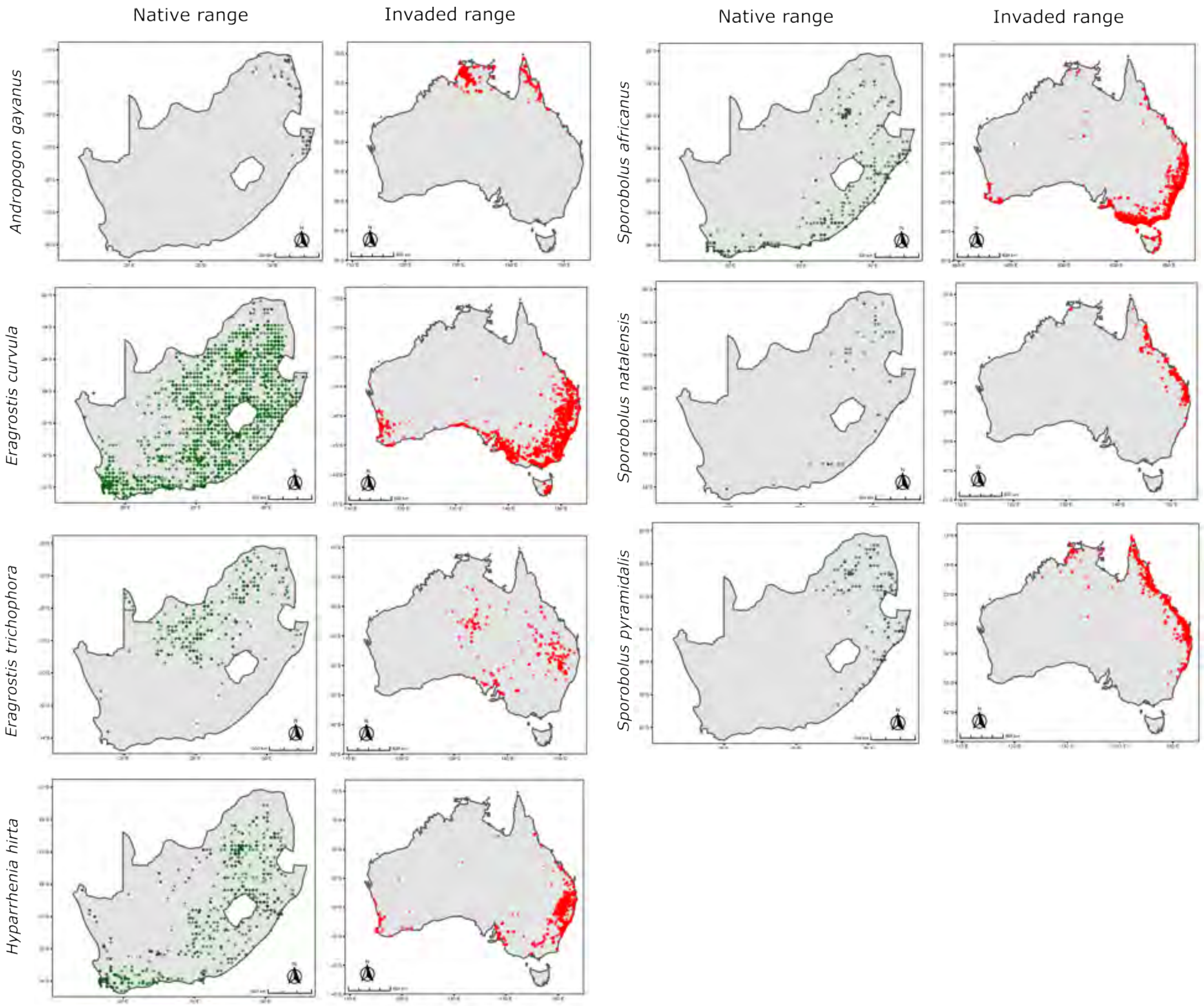


Figure 2.2: Distributions of some problematic African grasses in their native ranges, and invaded ranges in Australia. Data were sourced from the GBIF (Global Biodiversity Information Facility) database using the R spocc (SPecies OCCurrence) package (`https://github.com/ropensci/spocc`).

2.1.3 Aims

This chapter aimed to address the taxonomic gap in the *Tetramesa* genus by generating a genetic database and estimating species boundaries between taxonomic groups; particularly investigating interspecific differentiation and intraspecific substructuring. It then investigated the host-specificity of these wasp groups in order to make recommendations for their potential use for the biological control of grasses.

2.2 Materials and Methods

2.2.1 Sample Collection

Surveys and collections of herbivore communities on grasses across South Africa have been performed since 2017 across 129 sites and 49 grass species (Sutton *et al.*, 2021b). These included bi-monthly surveys at 22 sites in KwaZulu-Natal for a one-year period to assess changes in insect assemblages over time. As opposed to collecting external adults, the surveys were performed through either stem dissections or emergence chambers to ensure that the host-plant record was a true interaction and not due to chance. Voucher specimens were identified to the lowest taxonomic level by experts at the South African National Insect Collection in Pretoria (ARC-PPRI). These taxonomic classifications were used as a baseline in the present work.

For this study, wasp samples for DNA analysis (COI = 193, 28S = 199) were collected from six South African provinces and 19 grass host plants (Fig. 2.4, Fig. 2.5, and Table S1). Host grasses were selected based on their invasive status and prioritisation in control programmes in Australia and the USA. The main targets were *Andropogon gayanus* (Fig. 2.3 A), *Eragrostis curvula* (Fig. 2.3 B), *Megathyrsus maximus (= Panicum maximum)* (Fig. 2.3 C), *Sporobolus natalensis*, and *S. pyramidalis* (Fig. Fig. 2.3 D). *Hyparrhenia hirta* and *Eustachys paspaloides* were also included because they are also problem species in Australia and might be considered for future biological control programmes.

Figure 2.3: A) *Andropogon gayanus*, B) *Eragrostis curvula*, C) *Megathyrsus maximus*, and D) *Sporobolus pyramidalis*. Images taken from Plants of the World Online (POWO) (`http://www.plantsoftheworldonline.org/`). Facilitated by the Royal Botanic Gardens, Kew.

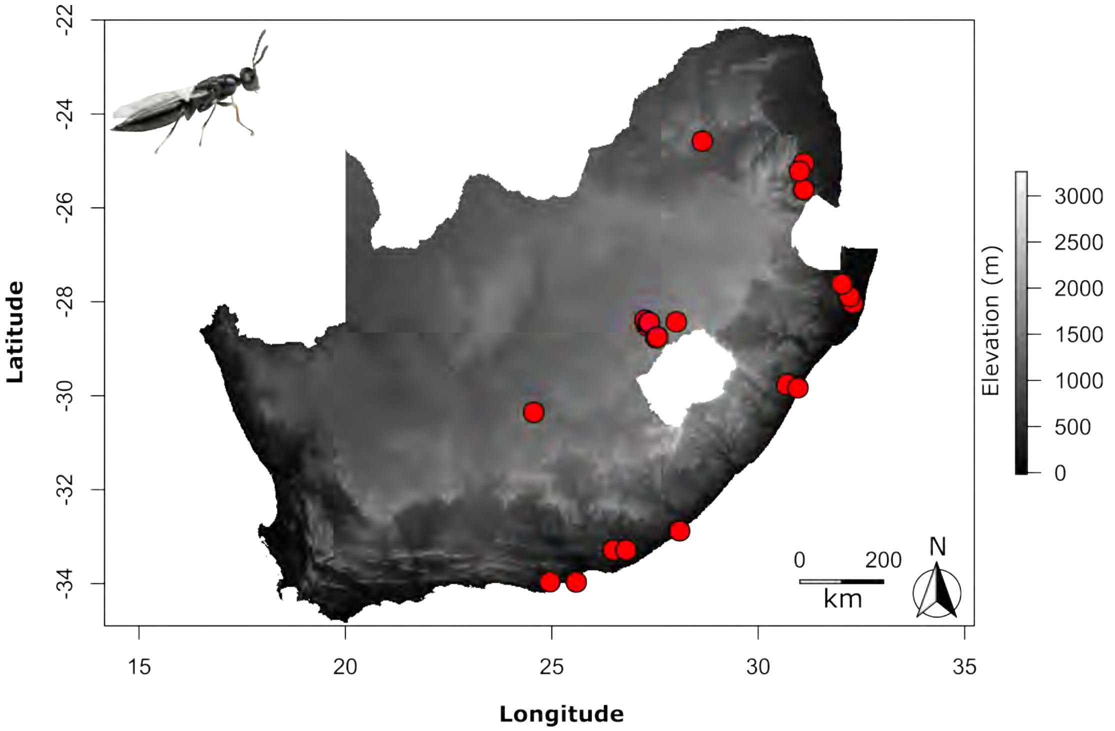


Figure 2.4: An elevation map of South Africa showing the collection sites of *Tetramesa* specimens used in the present study.

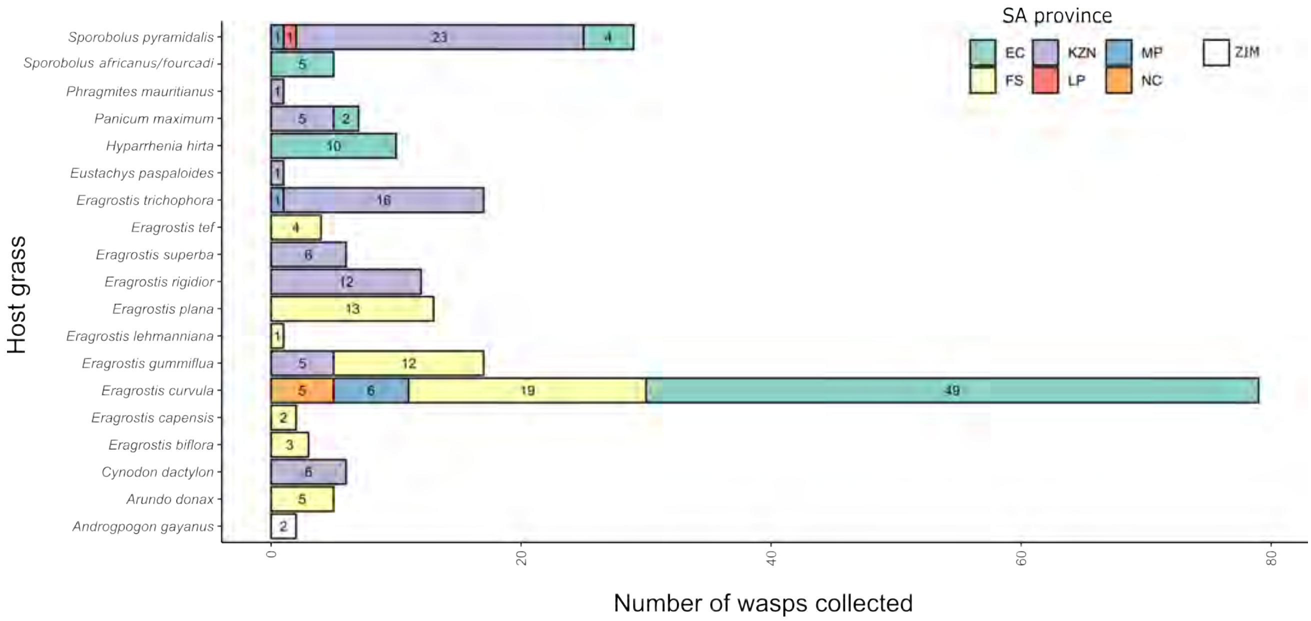


Figure 2.5: Counts of the wasp specimens collected across 19 host grasses. The bars represent the distribution of specimens collected, where *Eragrostis curvula* had the greatest sampling effort due to its prioritisation as a target for biological control. EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, LP = Limpopo, MP = Mpumalanga, NC = Northern Cape, ZIM = Zimbabwe. All species, except *Arundo donax*, are native South African grasses.

Sampling sites were selected based on the known distributions of the target host grasses, and where *Tetramesa* wasps had been found during previous surveys. The eurytomids collected were divided into morphospecies based on their host plants and on the presence or absence of a pronotal spot (Claridge, 1961) (Fig. 2.6 A, B). This feature is abbreviated "PNS" henceforth. Wasps without the feature are abbreviated "NPNS" (non-pronotal spot). It is important to note that the PNS (*Tetramesa* sp. 1) wasp recorded on *S. natalensis* and *S. pyramidalis* in Sutton *et al.* (2021b) is the same wasp species; the same is true for the NPNS wasp on these two grasses.

The Wapshere Centrifugal Phylogenetic Model (WCPM) (Wapshere, 1974) was used as a primary criterion to select other species within close phylogenetic proximity to the target weeds, focusing particularly on native congeners. Successively less sampling effort was applied to distantly related grasses, although non-target grasses that (1) occurred in sympatry in both the native and invaded range (Briese, 2003), (2) had a high relative abundance (Moffat *et al.*, 2013), (3) were structural analogues (Futuyma, 2000), and (4) were known hosts of other *Tetramesa* species were also taken into account (see Tables S1 and S2 in Sutton *et al.* (2021b)). The grass phylogenetic work by Shrestha *et al.* (2003), Shrestha *et al.* (2005), Peterson *et al.* (2010), Peterson *et al.* (2014), and Barrett *et al.* (2020) were used to guide the WCPM approach.

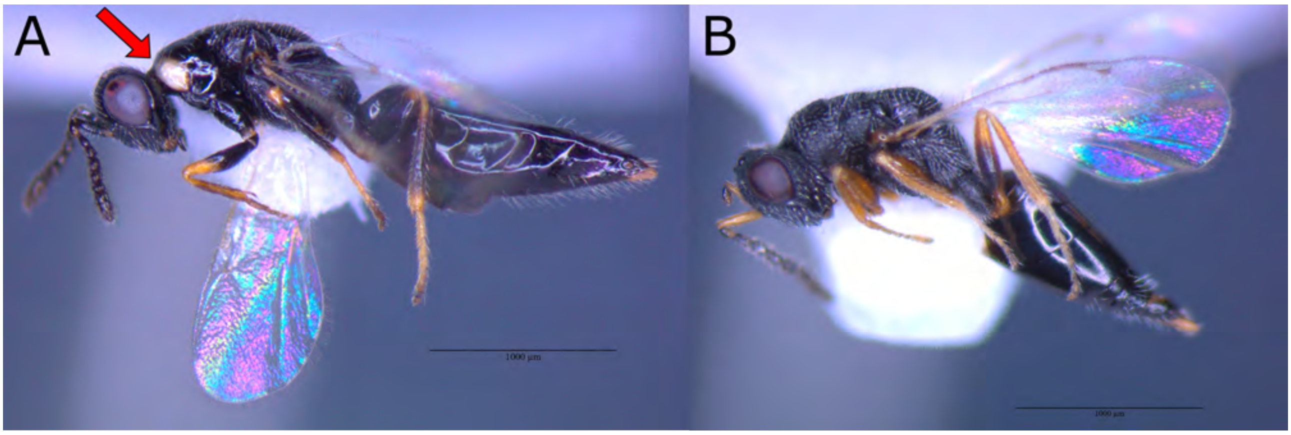


Figure 2.6: A) PNS *Tetramesa* (KCP 2021 00194) and B) NPNS eurytomid specimen (KCP 2021 00245). The prominent pronotal spot in (A) is shown with a red arrow. Scale bars represent 1 mm.

2.2.2 Sample preservation

All insect specimens were stored in 95% ethanol at -20°C. Reference specimens (Fig. S1 A - Q) were photographed under a ZEISS Stemi 508 stereo microscope using ZEN imaging software (ZEN Digital Imaging for Light Microscopy (RRID:SCR_013672)), and CombineZP software was used for z-stacking to create the final images. A full database of photographs is housed on the GitHub project repository (`https://github.com/clarkevansteenderen/PhD_files/tree/main/Microscope%20Images`).

2.2.3 Wasp genetics

DNA extraction

Three legs from each specimen were removed under a dissecting microscope so that the remaining whole specimens could be used for direct comparisons after sequencing. Genomic DNA extractions were performed using a PureLink^{TM} Genomic DNA Mini Kit (ThermoFisher Scientific, catalogue number K182002) according to the manufacturer's protocols. DNA extracts were eluted in a total volume of 35 μL Genomic Elution Buffer to account for lower DNA yields, and stored at -20°C. The concentration and purity of DNA extracts were recorded using a ThermoScientific NanoDrop 2000^{TM} spectrophotometer.

Polymerase Chain Reaction (PCR)

Mitochondrial cytochrome *c* oxidase I (COI) sequences were obtained using the forward FWPTF1 (Li *et al.*, 2010) and reverse LepR1 (Hebert *et al.*, 2004) primers (Table 2.1). These primers were designed by Li *et al.* (2010) for determining the host-specificity of *Sycophila* wasps (Hymenoptera: Eurytomidae), and worked well for species delimitation.

Each PCR reaction was carried out in a 25 μL volume, comprising 12.5 μL PCR master mix (2x iTaq^{TM} Universal SYBR® Green Supermix, Bio-Rad, catalogue number 1725121), 2 μL forward and reverse primer (10 μM concentration), and template DNA ($\sim$ 5 - 10 ng/μL). Thermocycling conditions were as follows: 5 min denaturation at 94°C, followed by 38 - 40 cycles of 40 s at 94°C / 35 s at 53°C / 45 s at 72°C / and a final extension of 10 min at 72°C.

Nuclear internal transcribed spacer II (ITS2) primers from White *et al.* (1990) (ITS2F and ITS2R) and Lotfalizadeh *et al.* (2007a) (ITS2F_lotf and ITS2R_lotf) (Table 2.1) were tested on *Tetramesa* specimens, where PCR and thermocycling conditions followed the protocols as per the respective references. Despite running gradient PCRs to find the optimal annealing temperature, sequence reads contained consistent multiple overlapping peaks and substantial background noise.

It was therefore decided to exclude this region from the study, and test 18S (White *et al.*, 1990; Carmean *et al.*, 1992) and 28S rRNA (Campbell *et al.*, 1994) markers as alternatives (Chen *et al.*, 2004). Both the 18S and 28S markers successfully amplified target fragments and produced clean sequence reads. Test sequences (n = 5) using these markers were assessed for average genetic diversity, which yielded 0% and 3%, respectively. It was therefore decided to continue using 28S to complement the COI data. The 28S region was also been found to be more informative relative to 18S in the barcoding of mites in Zhao *et al.* (2020), and was suggested as a more suitable alternative to ITS2. This was due to the variability of the ITS2 region in terms of both sequence length and composition, which resulted in alignment issues. This might explain the difficulty in amplification in the present work. Thermocycling conditions for the 18S region were as follows: 5 min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C / 30 s at 52°C / 30 s at 72°C / and a final extension of 10 min at 72°C (modified from Murphy *et al.* (2007)). Conditions for the 28S region were: 5 min denaturation at 94°C, followed by 35 cycles of 1 min at 94°C / 1 min at 55°C / 1.5 min at 72°C / and a final extension of 10 min at 72°C (modified from Schmidt *et al.* (2006)).

Table 2.1: List of primers used for *Tetramesa* samples in this study.

Region	**Name**	**Sequence (5'→ 3')**	**Reference**
COI	FWPTF1	CCTGGTTCTTTRATTGGTAATGATC	Li *et al.* (2010)
	LepR1	TAAACTTCTGGATGTCCAAAAA	Hebert *et al.* (2004)
ITS2	ITS2F	ATTCCCGGACCACGCCTGGCTGA	White *et al.* (1990)
	ITS2R	TCCTCCGCTTATTGATATGC	
ITS2	ITS2F_lotf	TGTGAACTGCAGACATG	Lotfalizadeh *et al.* (2007a)
	ITS2R_lotf	AAATGCTTAAATTTAGGGGGTA	
18S	18S.up1	TGGTTGATCCTGCCAGTAG	White *et al.* (1990);
	18SV4.lo1	CRTHYTYGGCAAATGCTTTCGC	Carmean *et al.* (1992)
28S	D2F	AGTCGTGTTGCTTGATAGTGCAG	Campbell *et al.* (1994)
	D2R	TTGGTCCGTGTTTCAAGACGGG	

2.2.4 Nucleotide Sequencing

Gels were prepared using a 1% agarose solution (TBE buffer (1X), Seakem$^{\text{TM}}$ agarose, and ethidium bromide) and run at 90 V for 40 min using a Bio-Rad Powerpac$^{\text{TM}}$. Bands were visualised

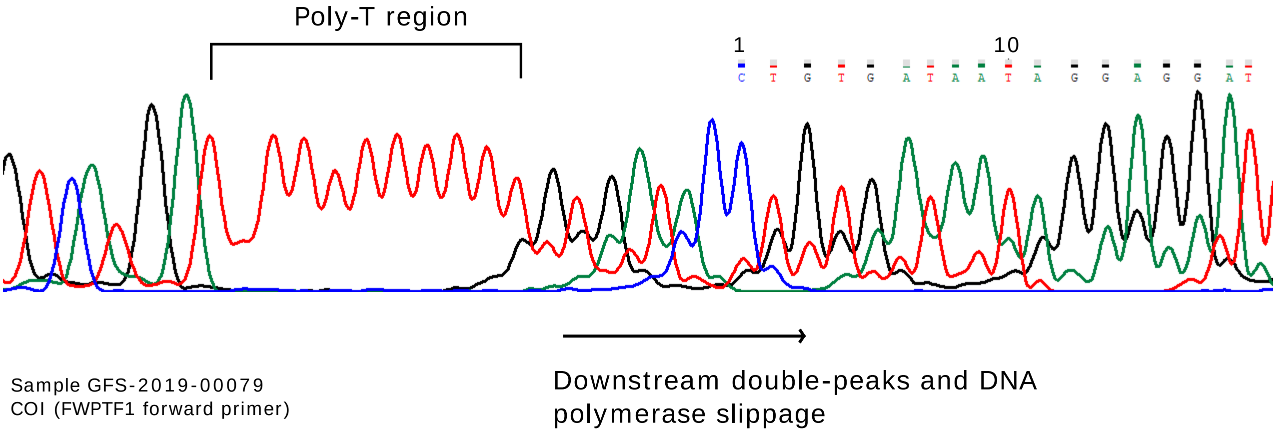


Figure 2.7: An example of a poly-T region at the start of a COI read, sequenced in the forward direction using primer FWPTF1 for *Tetramesa* sample GFS-2019-00079. The presence of this homopolymer often disrupts the sequencing process, causing the slippage pattern and double peaks visible here.

on a Bio-Rad Geldoc^{TM} molecular imaging system, and photographed using Image Lab^{TM} software. Successfully amplified PCR products were sent to Macrogen Inc. in the Netherlands (https://dna.macrogen-europe.com/eng/) for post-PCR purification and sequencing. The quality of each DNA chromatogram was checked manually using Chromas v2.6.4 (Technelysium Pty Ltd.), and trimmed such that the "noisy" beginning and end regions were removed.

For *Tetramesa* specimens, despite using the FWPTF1 forward COI primer designed by Li *et al.* (2010) to avoid a poly-T region at the 5' end of the barcode region, this homopolymer was observed in most COI sequences. Homopolymers are known to cause DNA polymerase slippage patterns which disturb the sequencing process (Ivády *et al.*, 2018) (Fig. 2.7). The resulting overlapping peaks downstream from the poly-T region was avoided by sequencing in the reverse direction (LepR1 primer), and then reverse-complementing the reads. PCRs were repeated for samples with sequences that had above-normal background noise and/or ambiguous base calls throughout, and were re-sequenced. Sequences were stored on the Centre for Biological Control (CBC) database, and will be submitted to GenBank once morphological identifications have been finalised (Table S1).

2.2.5 Sequence Alignment

Chromatograms were opened in Chromas v2.6.4 (Technelysium Pty Ltd.), where sequences were trimmed to remove messy beginning and end sections ($\sim$ 50 - 70 bp). These were saved as FASTA files and opened in AliView (Larsson, 2014), where their base-calls were verified against their corresponding chromatograms. Poor-quality sequences were excluded from the alignments. Gaps at the beginning and end of the sequence alignments were padded with Ns. Alignments were performed online using the default parameters in MAFFT v7 (`https://mafft.cbrc.jp/alignment/server/`) (Katoh *et al.*, 2019), and improved manually where appropriate. To check that the amplified COI gene region did not include any numts (nuclear mitochondrial DNA) that could result in barcoding "COI-like" sequences from the nuclear genome rather than the targeted protein-coding mitochondrial region (Lopez *et al.*, 1994), the COI alignment was translated into amino acids in its longest open reading frame (ORF) to check for the presence of stop codons or indels (Buhay, 2009).

2.2.6 Phylogenetic Analyses

Additional 28S and COI sequences were obtained from the GenBank (`https://www.ncbi.nlm.nih.gov/genbank/`) and BOLD (`https://www.boldsystems.org/index.php/`) databases, and are listed in Table S1. Until further clarification is attained, *Tetramesa romana* and *T. bambusae* have been used as reference specimens for the *Tetramesa* genus. Barring *Tetramesa bambusae*, all the historically archived *Tetramesa* sequences were only identified to genus. *Tetramesa bambusae* has been recorded to form galls in *Phyllostachis* Siebold & Zucc. stems (Asian bamboo) (Phillips, 1936), which are predominantly native to East Asia and the Himalayas (Isagi and Torii, 1997). All the BOLD COI specimens from Germany were assigned to genus by Dr. Stefan Schmidt at the Bavarian State Collection of Zoology in Munich, Germany, and the Canadian specimens by Dr. Angela Telfer at the Centre for Biodiversity Genomics in Guelph, Ontario, Canada. These were named based on COI sequence Barcode Index Numbers (BINs) rather than on morphological identifications. All of these specimens were collected in Malaise traps, and therefore did not have any associated host plant information. Additionally, the host range for these specimens is also unknown (Stefan Schmidt and Angela Telfer, pers. comm.). Outgroups were chosen based on the work by Chen *et al.* (2004), and comprised three *Sycophila* (Eurytomidae) species for the COI analysis (GenBank codes AY317230 - AY317232), and the genera *Neorileya* Ashmead (Eurytomidae), *Megastigmus* Dalman (Megastigmidae), *Monodon-*

tomerus Westwood (Torymidae), and *Brachymeria* Westwood (Chalcididae) for the 28S analysis.

At the time of the writing of this thesis, the specimens used for DNA analysis in the data set were sent to Iran for morphological identification by the world expert in *Tetramesa* taxonomy (Hossein Lotfalizadeh, Iranian Research Institute of Plant Protection). Selected wasps collected from *Sporobolus pyramidalis* were confirmed to genus by expert taxonomists Gerhard Prinsloo (Department of Agriculture and Animal Health, University of South Africa) and Werner Strümpher (Department of Zoology and Entomology, University of Pretoria).

Mitochondrial COI and nuclear 28S gene regions were analysed separately due to the presence of unresolved clades within both phylogenies, and differing topologies. Chen *et al.* (2004) reported a similar topological incongruence between their nuclear (28S + 18S) and mitochondrial data sets (COI + 16S), and suggested a number of possible causes that include horizontal gene transfer, ancient polymorphism, or differing inheritance pathways or mechanisms.

Optimal substitution models and maximum likelihood phylogenies were estimated using the IQ-TREE online server (`http://iqtree.cibiv.univie.ac.at/`) (Nguyen *et al.*, 2015; Chernomor *et al.*, 2016; Trifinopoulos *et al.*, 2016), where the best models were found by MODELFINDER (Kalyaanamoorthy *et al.*, 2017). Default parameters were applied in IQ-TREE, with 1000 ultrafast bootstrap repetitions.

The COI data set was partitioned by codon position, where the optimal models were TIM3 + F + Γ (position 1), and TIM + F + Γ (positions 2 and 3) according to the Bayesian Information Criterion (BIC). The optimal model for the 28S data set was GTR + F + I + Γ (BIC). Bootstrap values between 90 - 100% were considered strong, 70 - 89% as medium, and 50 - 69% as weak support.

Bayesian phylogenies were created in MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001), using random starting trees, four chains (three hot and one cold), two runs, 25 million generations, and the model parameters obtained from IQ-TREE. Trees were sampled every 1000 generations with a 25% burn-in. Tracer v1.7 (Rambaut *et al.*, 2018a) was used to check for MCMC convergence (ESS scores > 200). Posterior probability values between 0.9 - 1.0 were considered strongly supported. All analyses were carried out on the CIPRES Science

Gateway v3.3 online server (http://www.phylo.org/) (Miller *et al.*, 2010). Output tree files were prepared using the "ggtree" (Yu *et al.*, 2017) and "treeio" (Wang *et al.*, 2020b) R packages in R v4.0.2 (R Core Team, 2013). Haplotype networks were generated for the 28S PNS (n = 91) and NPNS (n = 59) clades using the TCS networks method (Clement *et al.*, 2002) in PopART (Leigh and Bryant, 2015), where haplotypes were coloured by host plant.

The clade naming convention used in this thesis followed the form of:

MORPHOTYPE.HOST PLANT.NUMBER

where morphotype refers to the morphotype of pronotal spot (PNS), non-pronotal spot (NPNS), or unknown (U) form. The host plant was abbreviated to the first letter of the genus, followed by a recognisable few characters denoting the species name. For example, PNS.ECUR.1 means the first clade (1) representing a pronotal spot morphotype (PNS), collected on *Eragrostis curvula* (ECUR). In some cases, the host plant is unknown (U), or the clade might comprise multiple host plants (MIX). See the list of abbreviations at the start of this thesis (page xv). Ordered clade numbers are also provided for quick navigation.

2.2.7 Species delimitation

Since species delimitation methods can sometimes produce varying outputs (Blair and Bryson Jr, 2017; Hofmann *et al.*, 2019; Ranasinghe *et al.*, 2022), six independent methods were explored in this work to estimate species boundaries within the *Tetramesa* COI and 28S genetic dataset. These were the (1) Automatic Barcode Gap Discovery (ABGD), (2) Assemble Species by Automatic Partitioning (ASAP), (3) Generalised Mixed Yule Coalescent model (GMYC), (4) Haplowebs, (5) Poisson Tree Processes (PTP), and (6) Bayesian Tree Processes (bPTP) methods. Outgroups were excluded from all analyses. Intra- and interspecific divergence values for selected clades were calculated in MEGA-11 (Kumar *et al.*, 2018) using uncorrected distances. Threshold ranges were explored using a novel R script (https://github.com/clarkevansteenderen/PhD_files, Fig. S7 and Fig. S8), where the final values chosen ranged between 0.3 and 3% for 28S, and 2 - 5% for COI. The COI barcode gap was not clear-cut, and so the lower and upper limits of the threshold range were additionally guided by the ABGD results (4.3% barcode gap).

Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP)

The ABGD (Puillandre *et al.*, 2012) and ASAP (Puillandre *et al.*, 2021) methods apply a hierarchical clustering algorithm to genetic pairwise distances in order to estimate a relevant barcode gap and species boundaries.

The ASAP and ABGD analyses were run on their respective online servers. ABGD and ASAP were run using the default settings and the K2P substitution model, but the X-value for the COI data set was reduced to 1. The ABGD analysis was run using the K80 substitution model, with a minimum barcode gap width of 0.013 and 0.043 for 28S and COI, respectively. These parameters were manually selected based on the cross-referencing of clade groupings seen in the associated phylogenies.

Generalised Mixed Yule Coalescent model (GMYC)

The GMYC model is a tree-based tool for species delimitation that implements maximum likelihood statistics to single-locus genetic data (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Fujisawa and Barraclough, 2013). The model assesses when branching rates in an ultrametric phylogeny transition from the species (inter-specific) to the population (intra-specific processes) level, where genetic cluster groups are separated by longer internal branch lengths (Fujisawa and Barraclough, 2013).

A Bayesian ultrametric phylogeny was generated in BEAST v2.6.3 (Suchard *et al.*, 2018), where the GTR site model, strict clock (clock rate = 1), and Yule tree prior with a uniform birth rate distribution were set, with 15 million MCMC generations. The resulting phylogenies were combined in TreeAnnotator, and were subsequently used to run the single-threshold Generalised Mixed Yule Coalescent (GMYC) model (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Fujisawa and Barraclough, 2013) in R using the “splits” package (Ezard *et al.*, 2009).

Haplowebs

Haplowebs (Flot *et al.*, 2010) uses a sequence alignment to determine the number of haplotypes in the data, and a subsequent minimum spanning network and species partition matrix.

The Haplowebs analysis was not included for COI data, as the method was found to substantially oversplit groups (estimated MOTUs = 139, which was in the region of double that of the other methods). Dellicour and Flot (2015) reported a similar outcome, and reported that Haplowebs performed better with less variable markers (suggesting that nuclear markers may be better indicators than mitochondrial for this method), smaller population sizes, and lower speciation rates.

Possion Tree Processes (PTP) and Bayesian Poisson Tree Processes (bPTP)

The bPTP tool uses the mean number of substitutions per site to differentiate between intra and inter-specific processes, which are represented by differences in branch lengths (Zhang *et al.*, 2013). The Poisson tree processes (bPTP) (Zhang *et al.*, 2013) analysis was run on the bPTP web server using the default settings, and 200 000 MCMC generations with a 0.25 burnin value. The option of removing distantly-related outgroups was selected to improve delimitation results.

2.2.8 Genetic divergence across geographic space

A Mantel test assesses the relationship between two matrices, and was first applied in a population genetics context by Sokal (1979). The test is now frequently used in phylogenetic studies to investigate how spatial processes can influence population genetic structure by testing the association between genetic and geographic distance matrices (Diniz-Filho *et al.*, 2013).

The Mantel test was run on a subset of the COI data such that only the PNS *E. curvula Tetramesa* specimens were included. These specimens were collected from four different provinces; namely Mpumalanga, Limpopo, and the Northern- and Eastern Cape (Fig. 2.8). The PNS *E. curvula* group was selected because the COI phylogeny showed support for four separate clades utilising this host from two different provinces. Since *E. curvula* is a target for biological control, investigating whether these are different intraspecific populations or interspecific taxa is of importance. The test was run using the `mantel()` function in the vegan R package (Oksanen *et al.*, 2022), using the p-distance matrix for the COI sequence subset (uncorrected distances obtained in MEGA-11), and a matrix of the associated GPS coordinates for each sample. The number of permutations was set to 9999.

An AMOVA (analysis of molecular variance), analagous to an ANOVA (analysis of variance), tests genetic structuring and the proportion of variation within and among populations of a particular species using various hierarchical levels (e.g. geographic location, biome, host) (Excoffier *et al.*, 1992). Two AMOVAs were run in Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) (K2P, permutations = 1000) on the PNS *E. curvula Tetramesa* across (1) provinces, and (2) populations within the Eastern Cape. A TCS haplotype network was generated for the Eastern Cape populations in PopArt (Leigh and Bryant, 2015).

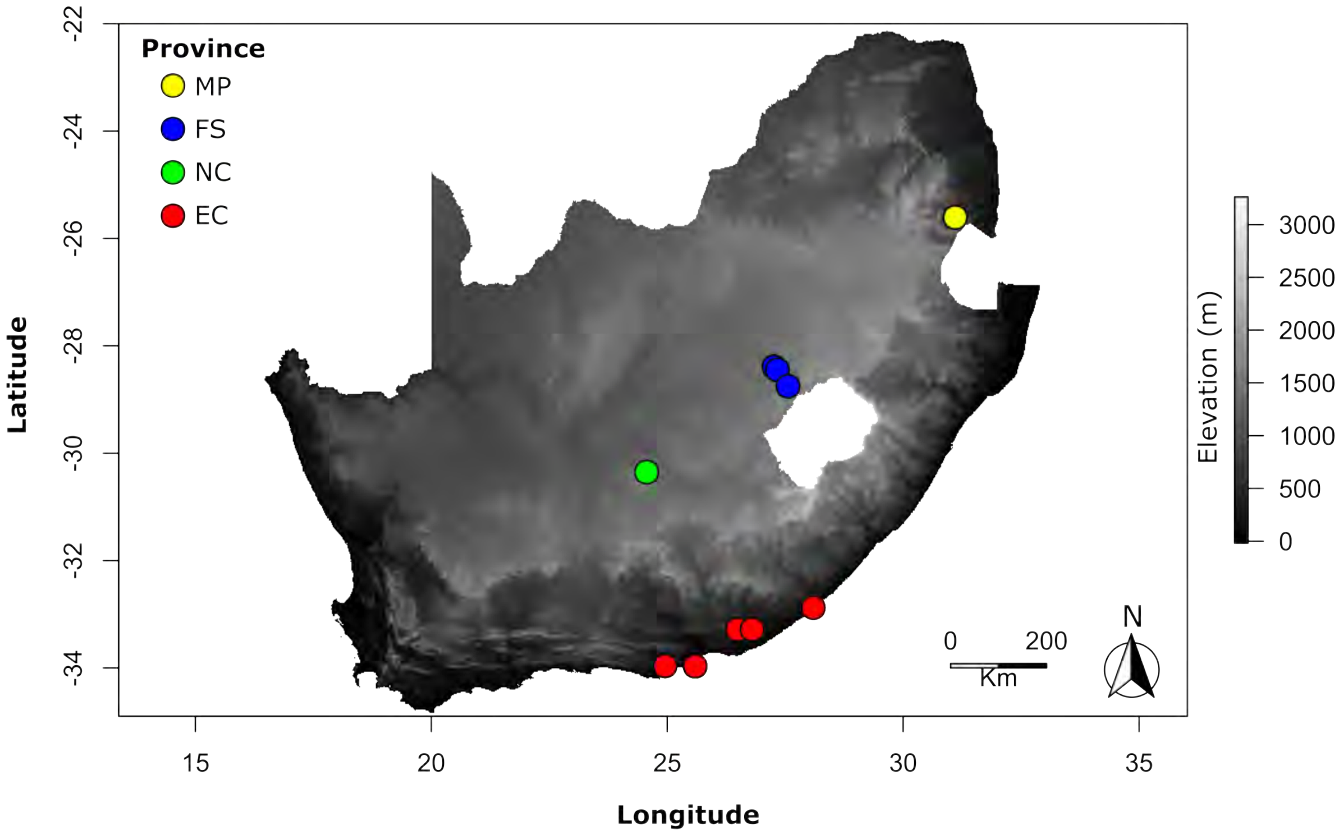


Figure 2.8: An elevation map of South Africa showing the localities of PNS *Tetramesa* specimens collected on *E. curvula* host plants. EC = Eastern Cape, FS = Free State, MP = Mpumalanga, NC = Northern Cape.

2.3 Molecular clock dating

Numerous studies have relied solely on molecular clocks to generate dated phylogenies for various taxonomic groups (Buckley *et al.*, 2010; Marshall *et al.*, 2016; Vuataz *et al.*, 2016; Winkler *et al.*, 2018), and it is generally accepted that molecular clocks can be good approximations of reality when (1) applied to closely related species and/or intraspecific populations, (2) appropriate nucleotide substitution models, molecular clock models, and tree priors are applied, and (3) molecular dates are considered in terms of their associated confidence intervals to account for imprecision (Dos Reis *et al.*, 2016). Acknowledging that molecular dating estimates are imprecise does not mean that they have no use in evolutionary biology. The phylogenies presented in this thesis should thus be treated as relative timescales and not absolute divergence times until such a time that they can be calibrated with fossil or other appropriate geological evidence.

Some of the oldest chalcidoid fossils (Eulophidae and Trichogrammatidae) were found preserved in amber in Ethiopia, and were dated to the mid-Cretaceous between $\sim$ 93 - 95 mya (Schmidt *et al.*, 2010). The only recorded eurytomid fossils have been described as *Eurytoma sepulta* Brues (Miocene), *E. sequax* Brues (Miocene), and *Decatoma antiqua* Scudder (Oligocene) (Yoshimoto, 1975). These dates have not been used as calibration points in this study because the *Eurytoma* are not a monophyletic group (Chen *et al.*, 2004).

Due to the absence of reliable fossil or other geological calibration points relating to the Eurytomidae, a nuclear clock rate of 0.06 $\pm$ 0.03% per My (Papadopoulou *et al.*, 2010) was applied to the 28S rRNA region to estimate divergence times. The nuclear rather than the COI mitochondrial region was selected for the dating analysis due to the conservative properties of nuclear RNA and its known reliability in inferring deep phylogenetic relationships (Hillis and Dixon, 1991; Hwang and Kim, 1999). Additionally, Chen *et al.* (2004) recommended the use of nuclear above mitochondrial regions for further investigations of the Eurytomidae due in part to the unknown inheritance dynamics and evolutionary processes of mitochondrial DNA in this hymenopteran group.

Two independent Bayesian analyses were run in BEAST v2.6.3 (Suchard *et al.*, 2018), applying the relaxed (uncorrelated lognormal) molecular clock (the automatic set clock rate mode option was deselected in BEAST). The same evolutionary model was applied as in section 2.2.6 (GTR + F + I + Γ). The tree prior was set to the Yule model, and the clock rate prior was set to a lognormal distribution centred on 0.0003, with a standard deviation of 0.00015. Default options were applied for the remaining prior and operator settings. Each analysis was run for 50 million generations, with trees sampled every 1000. The maximum clade credibility tree using median heights was built with TreeAnnotator v. 1.8.3 (Suchard *et al.*, 2018), where tree burnin was set to 75%. Log files were viewed in Tracer v1.7 (Rambaut *et al.*, 2018b) to check for convergence and effective sample size (ESS) scores > 200.

BEAST tree files were read into R via `phyloch::read.beast()` (Heibl, 2019), and the dated phylogeny plotted using `strap::geoscalePhylo()` (Bell and Lloyd, 2015). Lineage through time (ltt) plots were created using `ape::ltt.plot()` (Paradis *et al.*, 2004).

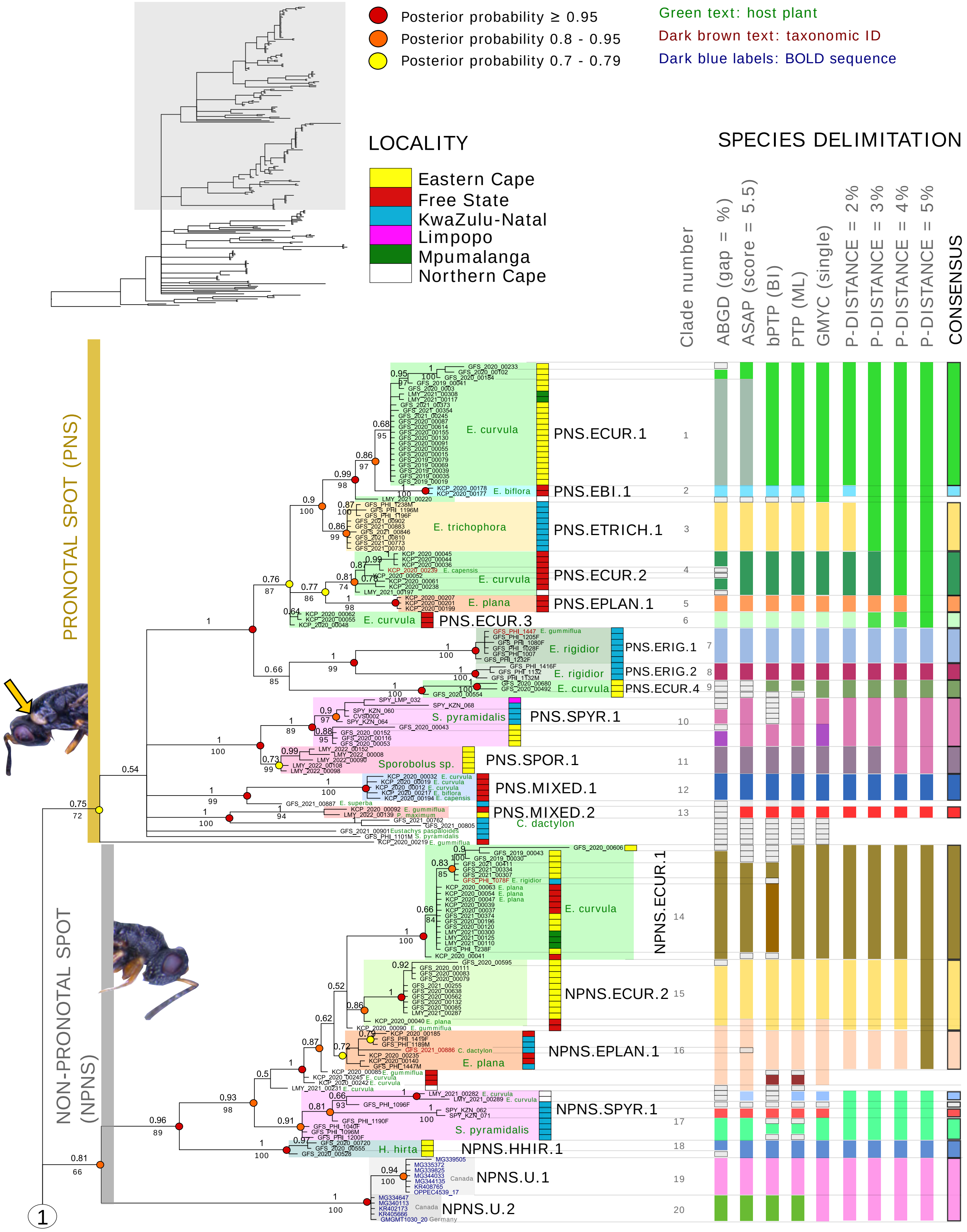
2.4 Results

Both the COI (545 bp) (Log-likelihood of the ML consensus tree = -10200.798, BI run 1 = -10641.18, BI run 2 = -10661.60) and 28S (605 bp) (Log-likelihood of the ML consensus tree = -3840.301, BI run 1 = -4097.81, BI run 2 = -4115.02) phylogenies showed a distinct separation between pronotal spot (PNS) and non-pronotal spot (NPNS) eurytomids, but the NPNS groups were unresolved. In the COI phylogeny, *T. romana* formed a polytomy (PNS.ADON.2; clade 25) with the *H. hirta* and *A. gayanus* wasps (NPNS.HHIR.2, NPNS.AGAYA.1, and NPNS.HHIR.3; clades 22 - 24) which fell outside the PNS clade (Fig. 2.9 and Fig. 2.10 A - H)), while in the 28S phylogeny, *T. romana* (PNS.ADON.1, clade 3) grouped within the PNS clade (clades 1-3), which together formed a sister group to the *H. hirta* (NPNS.HHIR.1, clade 4) and *A. gayanus* (NPSN.AGAYA.1, clade 5) wasps (Fig. 2.11). Both phylogenies supported (1) the *T. bambusae* specimen from Chen *et al.* (2004) as being most closely related to *T. romana*, and (2) the *Andropogon gayanus* wasps from Zimbabwe (NPSN.AGAYA.1) forming a sister group to those collected on South African *Hyparrhenia hirta* (NPNS.HHIR.1 (clade 4) in 28S, and NPNS.HHIR.2 (clade 22) and NPNS.HHIR.3 (clade 24) in COI).

The sequence divergences between *T. romana* and *T. bambusae* relative to the PNS groups revealed quite different patterns in the nuclear versus the mitochondrial data sets (Fig. 2.10 A - H), where COI divergences were surprisingly high (17.2 - 22.8%) for specimens expected to be congeneric (Fig. S5). This compared to a range of 2 - 2.7% in the 28S data (Fig S6).

The PNS wasps collected on an unidentified *Sporobolus* sp. (PNS.SPOR.1, COI clade 11) in East London (Eastern Cape, South Africa) formed a sister group to *Sporobolus pyramidalis* (PNS.SPYR.1, COI clade 10) (Fig. 2.9). The grass was barcoded using the nuclear ITS and chloroplast trnL and trnK regions (see chapter 3 for the associated methods). The ITS result suggested that it was closely related to *S. sessilis* B.K.Simon and *S. myrianthus* Benth. (Fig. S2). The chloroplast phylogeny showed the same pattern, but included *S. africanus* (Poir.) Robyns & Tournay as an additional close relative (Fig. S3). Since the native range of *S. sessilis* is eastern and north-eastern Australia, and *S. myrianthus* occurs across Nigeria and Kenya, and other tropical regions in southern Africa, it appears more likely that this is *S. africanus*. At the time of this writing, these *Sporobolus* specimens are being sent to an expert taxonomist for identification.

The *Tetramesa* collected on *Phragmites mauritianus* (KCP 2021 00247, between clades 16 and 17 in the 28S phylogeny) was found to be more closely related to *Sycophila*, *Eurytoma*, and *Bruchophagus* than to *Tetramesa*. This was also confirmed by a morphological inspection of the specimen (Fig. S6).


Posterior probability ≥ 0.95
Posterior probability 0.8 - 0.95
Posterior probability 0.7 - 0.79
Green text: host plant
Dark brown text: taxonomic ID
Dark blue labels: BOLD sequence
LOCALITY
Eastern Cape
Free State
KwaZulu-Natal
Limpopo
Mpumalanga
Northern Cape
SPECIES DELIMITATION
Clade number
ABGD (gap = %)
ASAP (score = 5.5)
bPTP (BI)
PTP (ML)
GMYC (single)
P-DISTANCE = 2%
P-DISTANCE = 3%
P-DISTANCE = 4%
P-DISTANCE = 5%
CONSENSUS
PRONOTAL SPOT (PNS)
NON-PRONOTAL SPOT (NPNS)
E. curvula
PNS.ECUR.1
1
E. biflora
PNS.EBI.1
2
E. trichophora
PNS.ETRICH.1
3
E. curvula
PNS.ECUR.2
4
E. plana
PNS.EPLAN.1
5
E. curvula
PNS.ECUR.3
6
E. rigidior
PNS.ERIG.1
7
E. rigidior
PNS.ERIG.2
8
E. curvula
PNS.ECUR.4
9
S. pyramidalis
PNS.SPYR.1
10
Sporobolus sp.
PNS.SPOR.1
11
PNS.MIXED.1
12
PNS.MIXED.2
C. dactylon
13
E. curvula
NPNS.ECUR.1
14
NPNS.ECUR.2
15
E. plana
NPNS.EPLAN.1
16
S. pyramidalis
NPNS.SPYR.1
17
H. hirta
NPNS.HHIR.1
18
Canada
NPNS.U.1
19
Canada
Germany
NPNS.U.2
20
1

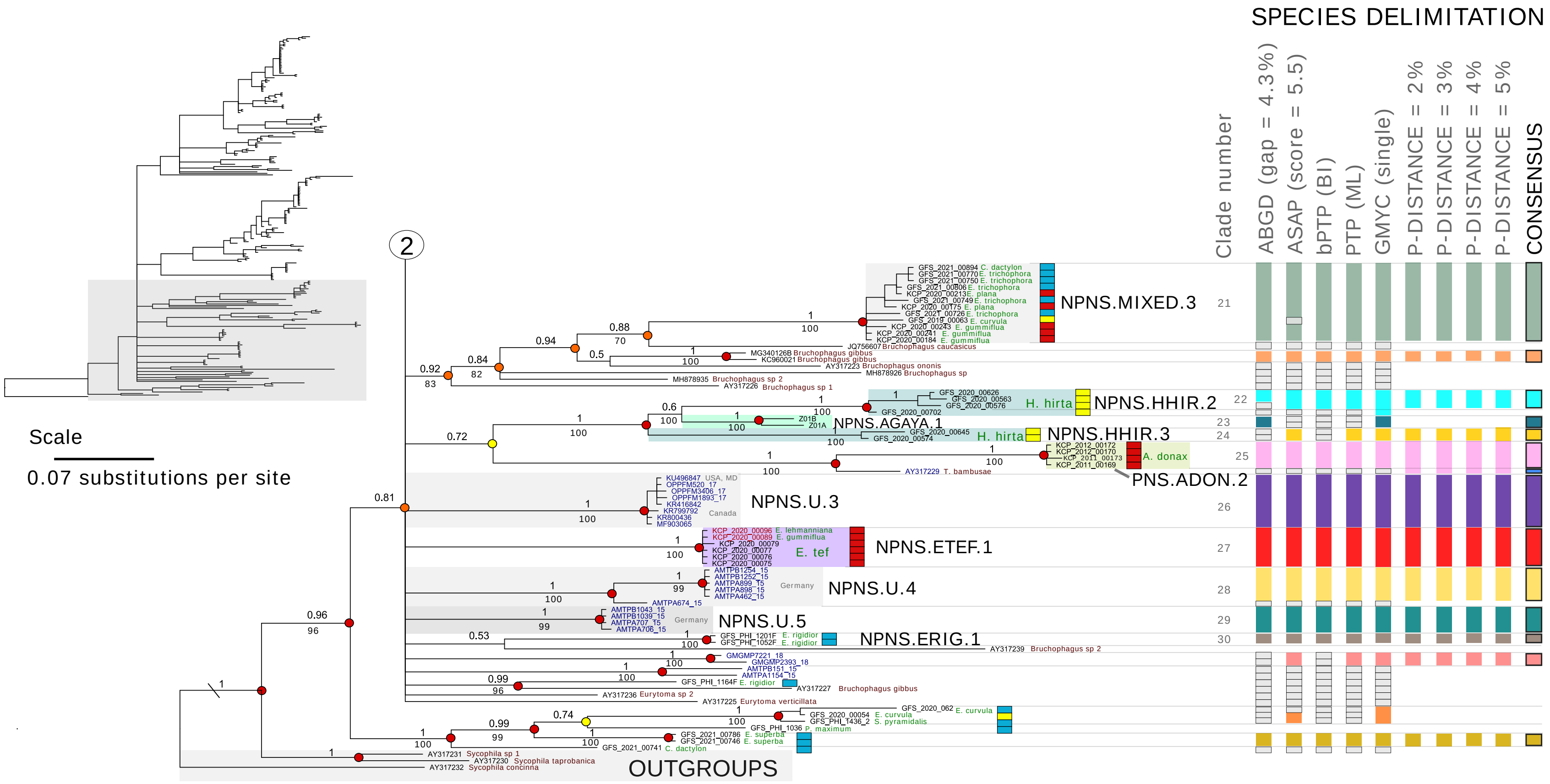
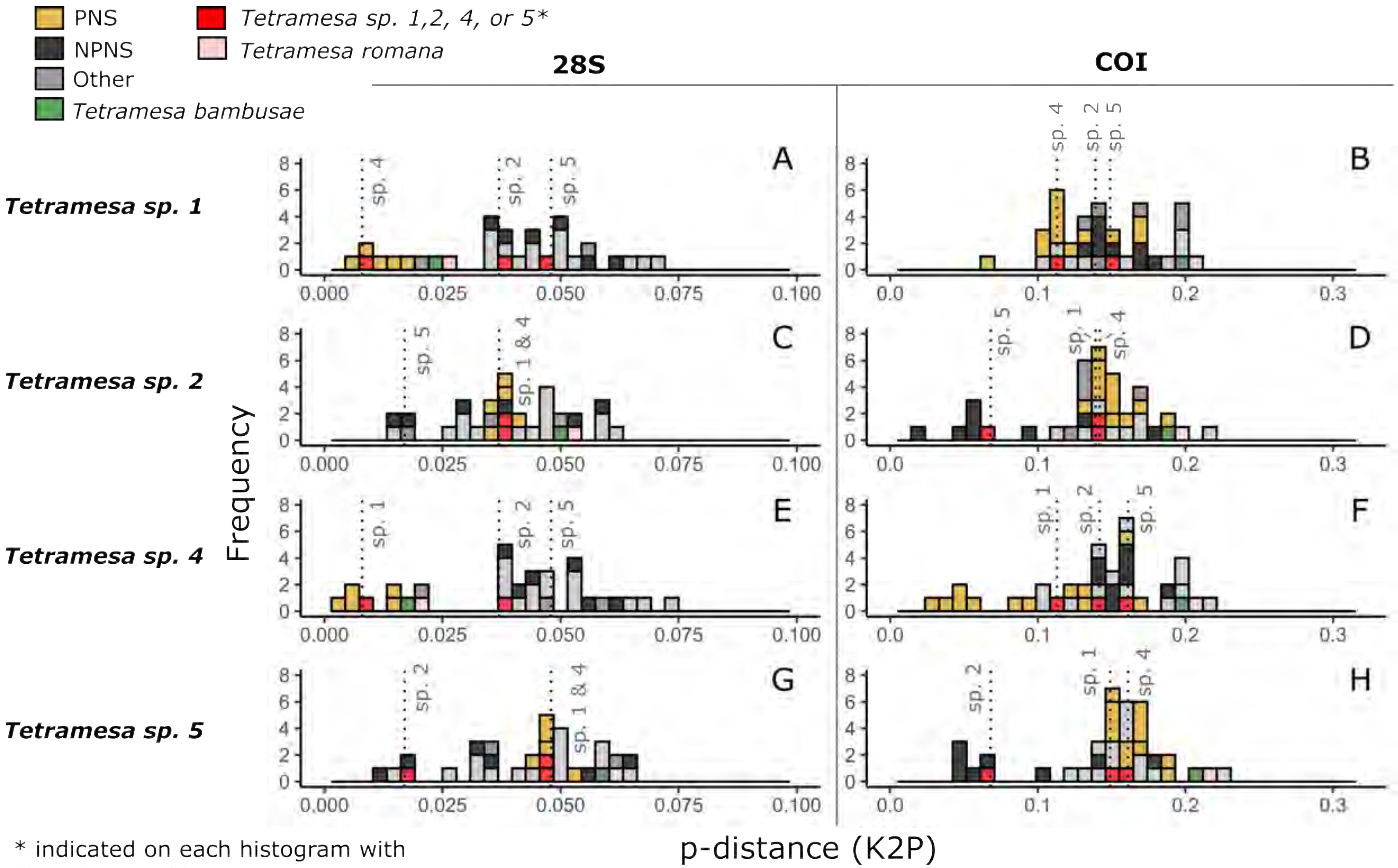
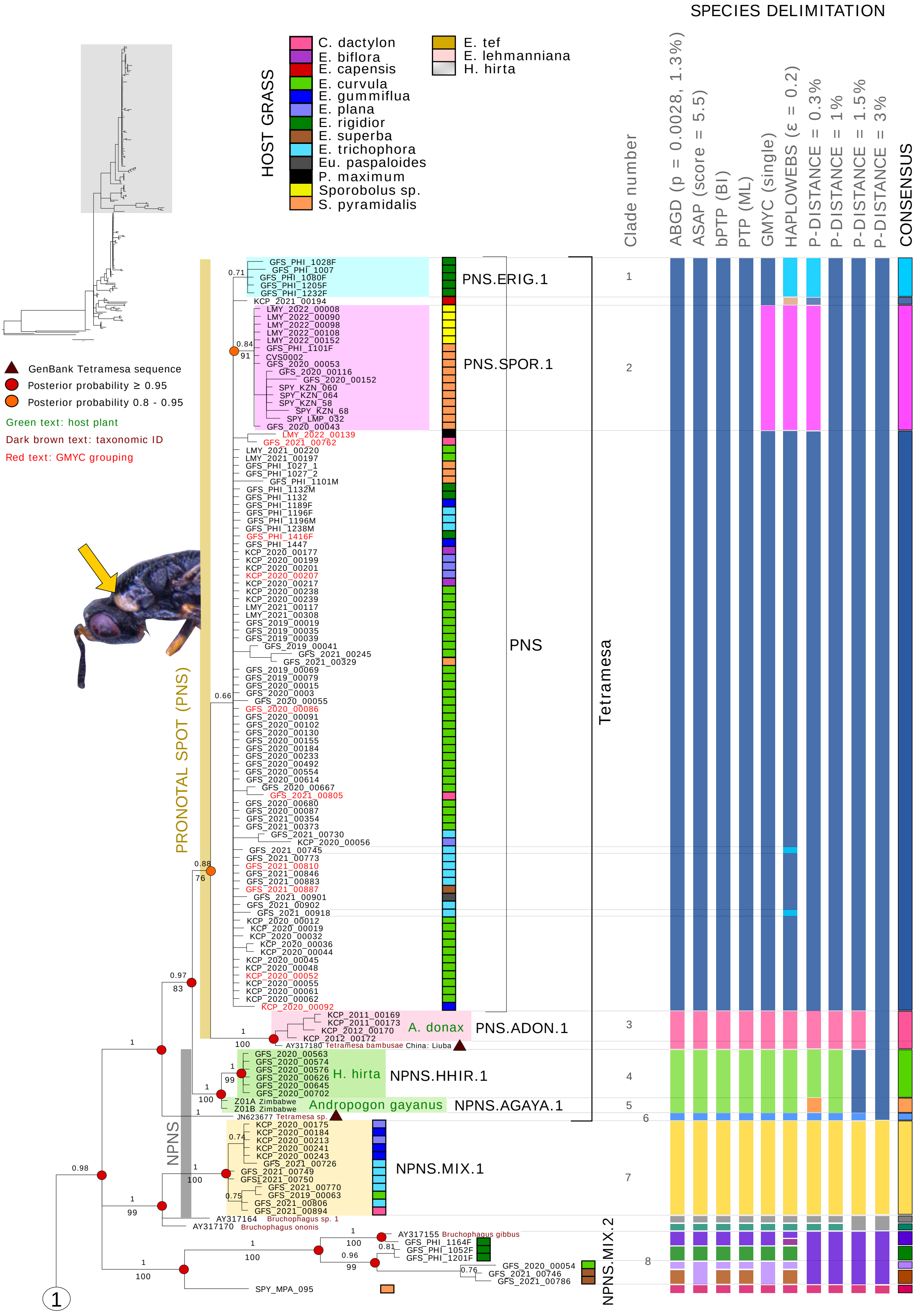


Figure 2.9: Bayesian inference phylogeny for the COI mitochondrial gene region. Coloured boxes next to sequence names indicate collection localities, as per the legend. Solid rectangular bars to the far right are the results of a suite of species delimitation tests. Samples that share the same coloured bar within each column belong to the same MOTU. (1) Blue text *Tetramesa* are sequences obtained from the BOLD database, (2) green text next to sample names indicate the host plant from which the sample was collected, (3) dark brown text indicates taxonomic identifications, (4) red and orange filled circles denote posterior probability values greater than or equal to 0.9, and between 0.8 and 0.95, respectively. Numbers above and below branches are BI posterior probability and ML bootstrap support values, respectively. Species delimitation results for the non-target polytomy groups are deliberately not displayed. See the list of phylogenetic clade abbreviations on page xv.



* indicated on each histogram with dotted vertical lines

Figure 2.10: Histograms A - H are interspecific 28S and COI p-distance plots (K2P, 1000 bootstrap repeats) for *Tetramesa sp. 1* (PNS, *Sporobolus pyramidalis*), *Tetramesa sp. 2* (NPNS, *Sporobolus pyramidalis*), *Tetramesa sp. 4* (PNS, *Eragrostis curvula*), and *Tetramesa sp. 5* (NPNS, *Eragrostis curvula*) relative to the other major groups in the associated phylogenies. PNS = pronotal spot, NPNS = non-pronotal spot.


SPECIES DELIMITATION
HOST GRASS
🟥 C. dactylon
🟪 E. biflora
🟥 E. capensis
🟩 E. curvula
🟦 E. gummiflua
🟦 E. plana
🟩 E. rigidior
🟫 E. superba
🟦 E. trichophora
⬛ Eu. paspaloides
⬛ P. maximum
🟨 Sporobolus sp.
🟧 S. pyramidalis
🟨 E. tef
⬜ E. lehmanniana
⬜ H. hirta
Clade number
ABGD (p = 0.0028, 1.3%)
ASAP (score = 5.5)
bPTP (BI)
PTP (ML)
GMYC (single)
HAPLOWEBS (ε = 0.2)
P-DISTANCE = 0.3%
P-DISTANCE = 1%
P-DISTANCE = 1.5%
P-DISTANCE = 3%
CONSENSUS
▲ GenBank Tetramesa sequence
● Posterior probability ≥ 0.95
● Posterior probability 0.8 - 0.95
Green text: host plant
Dark brown text: taxonomic ID
Red text: GMYC grouping
PRONOTAL SPOT (PNS)
NPNS
Tetramesa
PNS.ERIG.1
PNS.SPOR.1
PNS
PNS.ADON.1
NPNS.HHIR.1
NPNS.AGAYA.1
NPNS.MIX.1
NPNS.MIX.2
1
2
3
4
5
6
7
8
0.71
GFS_PHI_1028F
GFS_PHI_1007
GFS_PHI_1080F
GFS_PHI_1205F
GFS_PHI_1232F
KCP_2021_00194
0.84
91
LMY_2022_00008
LMY_2022_00090
LMY_2022_00098
LMY_2022_00108
LMY_2022_00152
GFS_PHI_1101F
CVS0002
GFS_2020_00053
GFS_2020_00116
GFS_2020_00152
SPY_KZN_060
SPY_KZN_064
SPY_KZN_58
SPY_KZN_68
SPY_LMP_032
GFS_2020_00043
LMY_2022_00139
GFS_2021_00762
LMY_2021_00220
LMY_2021_00197
GFS_PHI_1027_1
GFS_PHI_1027_2
GFS_PHI_1101M
GFS_PHI_1132M
GFS_PHI_1132
GFS_PHI_1189F
GFS_PHI_1196F
GFS_PHI_1196M
GFS_PHI_1238M
GFS_PHI_1416F
GFS_PHI_1447
KCP_2020_00177
KCP_2020_00199
KCP_2020_00201
KCP_2020_00207
KCP_2020_00217
KCP_2020_00238
KCP_2020_00239
LMY_2021_00117
LMY_2021_00308
GFS_2019_00019
GFS_2019_00035
GFS_2019_00039
GFS_2019_00041
GFS_2021_00245
GFS_2021_00329
GFS_2019_00069
GFS_2019_00079
GFS_2020_00015
GFS_2020_0003
0.66
GFS_2020_00055
GFS_2020_00086
GFS_2020_00091
GFS_2020_00102
GFS_2020_00130
GFS_2020_00155
GFS_2020_00184
GFS_2020_00233
GFS_2020_00492
GFS_2020_00554
GFS_2020_00614
GFS_2020_00667
GFS_2021_00805
GFS_2020_00680
GFS_2020_00087
GFS_2021_00354
GFS_2021_00373
GFS_2021_00730
KCP_2020_00056
GFS_2021_00745
GFS_2021_00773
0.88
76
GFS_2021_00810
GFS_2021_00846
GFS_2021_00883
GFS_2021_00887
GFS_2021_00901
GFS_2021_00902
GFS_2021_00918
KCP_2020_00012
KCP_2020_00019
KCP_2020_00032
KCP_2020_00036
KCP_2020_00044
KCP_2020_00045
KCP_2020_00048
0.97
83
KCP_2020_00052
KCP_2020_00055
KCP_2020_00061
KCP_2020_00062
KCP_2020_00092
KCP_2011_00169
KCP_2011_00173
KCP_2012_00170
KCP_2012_00172
A. donax
1
100
AY317180 Tetramesa bambusae China: Liuba
1
GFS_2020_00563
GFS_2020_00574
GFS_2020_00576
GFS_2020_00626
GFS_2020_00645
GFS_2020_00702
H. hirta
1
99
1
100
Z01A Zimbabwe
Z01B Zimbabwe
Andropogon gayanus
1
JN623677 Tetramesa sp.
KCP_2020_00175
KCP_2020_00184
0.74
KCP_2020_00213
KCP_2020_00241
KCP_2020_00243
GFS_2021_00726
1
100
GFS_2021_00749
GFS1_2021_00750
GFS_2021_00770
GFS_2019_00063
0.75
GFS_2021_00806
GFS_2021_00894
0.98
1
99
AY317164 Bruchophagus sp. 1
AY317170 Bruchophagus ononis
1
100
AY317155 Bruchophagus gibbus
GFS_PHI_1164F
0.81
GFS_PHI_1052F
GFS_PHI_1201F
1
100
0.96
99
GFS_2020_00054
0.76
GFS_2021_00746
GFS_2021_00786
1
100
SPY_MPA_095
1

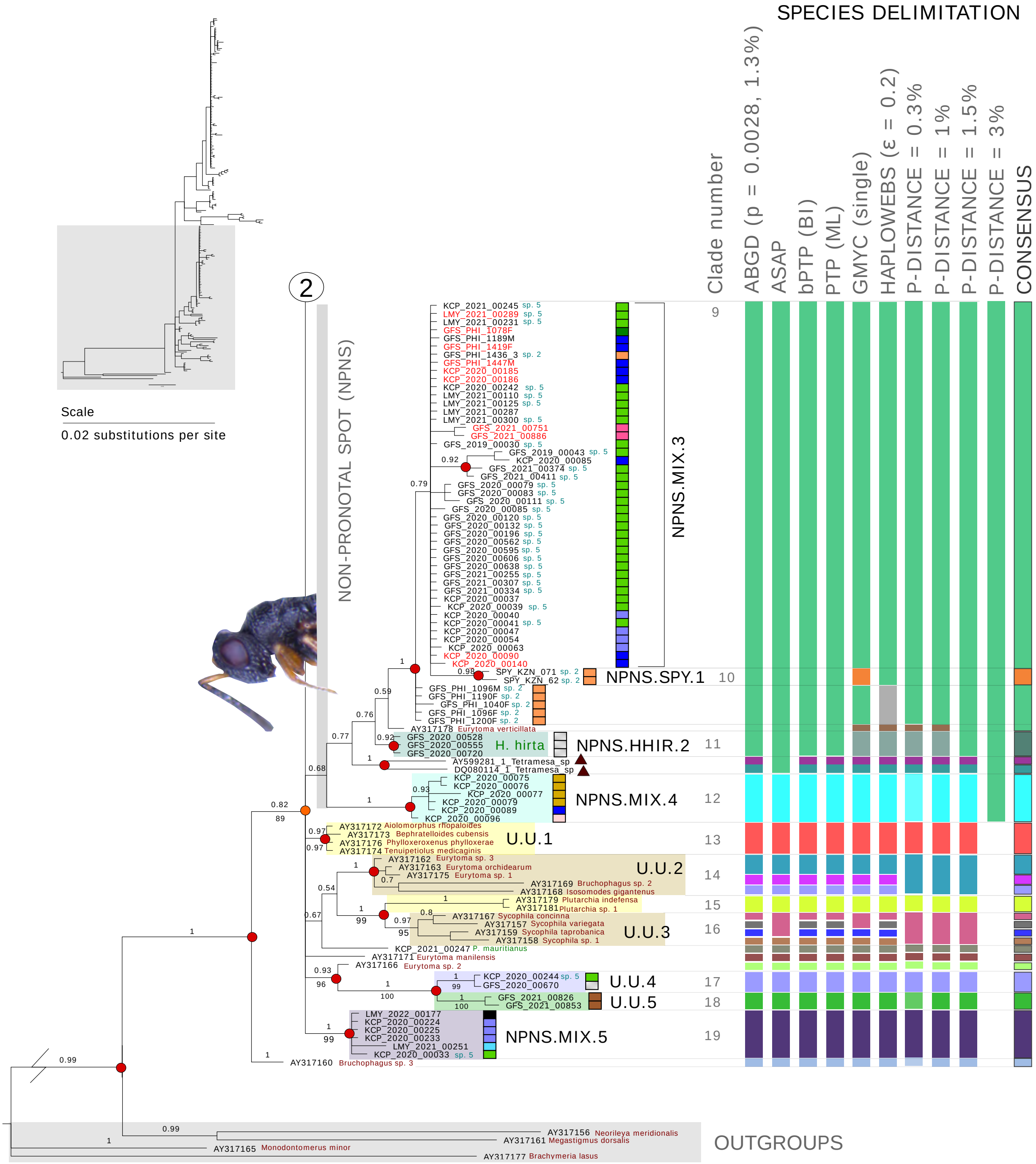


Figure 2.11: Bayesian inference phylogeny for the 28S nuclear gene region. Individually-coloured boxes next to sequence names represent host grass species as per the legend. Solid rectangular bars to the far right are the results of a suite of species delimitation tests. Samples that share the same coloured bar within each column belong to the same MOTU. (1) Brown triangles denote *Tetramesa* sequences obtained from GenBank, (2) green text next to sample names indicate the host plant from which the sample was collected, (3) dark brown text indicates taxonomic identifications, (4) red text marks samples that the GMYC analysis grouped as a separate MOTU, (5) red and orange filled circles denote posterior probability values greater than or equal to 0.9, and between 0.8 and 0.95, respectively. Numbers above and below branches are BI posterior probability and ML bootstrap support values, respectively. Species delimitation results for the non-target polytomy groups are deliberately not displayed. Genus names are shortened as: *C* = *Cynodon*, *E* = *Eragrostis*, *Eu* = *Eustachys*, *P* = *Panicum*, *S* = *Sporobolus*. See the list of phylogenetic clade abbreviations on page xv.

2.4.1 COI data

Overall inter-group and intra-group p-distances (excluding the outgroup) ranged from 1.6 - 26.3% and 0 - 3.2% respectively. Chen *et al.* (2004) reported interspecific distances ranging from 0.2 - 25.8%. Most of the clades in the COI phylogeny were unresolved, rendering taxonomic inferences based on this marker very challenging (Fig. 2.9).

The estimated number of MOTUs ranged from 52 - 87 across delimitation methods, where ABGD = 52, ASAP = 87, bPTP = 87, PTP = 73, and GMYC = 71 (clusters = 42, entities = 71, CI clusters = 36 - 43, CI entities = 62 - 77, lnL of null model = 1026.2, ML of GMYC model = 1082.666, $p < 0.001$, singletons = 29) (Fig. 2.9).

The PNS *E. curvula* clades (PNS.ECUR.1 - PNS.ECUR.4; clades 1, 4, and 6) displayed overall strong host-specificity (only one specimen was recorded on *E. capensis*; KCP 2020 00239), while those in the NPNS clades NPNS.ECUR.1 (clade 14) and NPNS.ECUR.2 (clade 15) contained specimens collected on three different *Eragrosis* species; namely *E. rigidior*, *E. plana*, and *E. gummiflua* (Fig. 2.9). The *E. trichophora* wasps (PNS.ETRICH.1, clade 3) formed a sister clade to the PNS *E. curvula* and *E. biflora* group (PNS.ECUR.1 + PNS.EBI.1; clades 1 and 2), and had a 2.9% sequence divergence from PNS.ECUR.1 (Fig. S5). The divergence between the PNS *E. biflora* (PNS.EBI.1, clade 2) and *E. curvula* (PNS.ECUR.1, clade 1) clades was 2.5% (Fig S5).

Unlike in the 28S phylogeny, the NPNS *H. hirta* (NPNS.HHIR.2 and NPNS.HHIR.3; clades 22 and 24) and *A. gayanus* (NPNS.AGAYA.1, clade 23) clades, along with *T. romana* (NPNS.ADON.1, clade 25) did not cluster with or form a sister group to the PNS clade, but rather formed a polytomy that had unexpectedly high genetic divergence values ranging between 15.5 - 19.4% Fig. S5). The sequence divergence between *T. romana* and *T. bambusae* (native to Europe and Asia, respectively) was 10.8%, and ranged between 17.2 - 22.8% when these two species were compared to all the southern African PNS clades, NPNS.HHIR.1 (clade 18), and NPNS.AGAYA.1 (clade 23) (Fig. 2.9 and Fig. S5). The PNS *S. pyramidalis* wasps (clades 10 and 11), which have been identified to genus by expert taxonomists, showed a divergence of 19.5% relative to *T. romana*. Clades NPNS.U.1 - NPNS.U.5 (clades 19, 20, 26, 28, and 29) comprised unidentified Northern Hemisphere (Germany and North America) *Tetramesa* specimens taken from BOLD (Table S1), and had a sequence divergence in a similar range of 17 - 22.8% and 17.5 - 19.6% relative to *T. romana* and *T. bambusae*, respectively (Fig. S5).

Sequence divergences within the southern African PNS groups ranged from 2.4 - 13.1% (Fig. S5). A sequence divergence of 2.8% was found between the PNS.ECUR.1 (clade 1) (PNS wasps on *E. curvula*) clade and its sister group PNS.ETRICH.1 (clade 3) (PNS wasps on *E. trichophora*), where the latter forms unique galls on *E. trichophora* that have not been observed on other grasses (Fig. 2.12).

Figure 2.12: The unique gall formed on *Eragrostis trichophora*. Image: Guy Sutton.

There were four separate clades associated with *E. curvula* within the PNS group (PNS.ECUR.1 - PNS.ECUR.4; clades 1, 4, 6, and 9), although only PNS.ECUR.1 (clade 1) (pp = 0.68, bt = 95) and PNS.ECUR.4 (clade 9) (pp = 1, bt = 100) were strongly supported (Fig. 2.9). The sequence divergence between PNS.ECUR.1 (clade 1) (Eastern Cape) and PNS.ECUR.2 (clade 4) (Free State) was 4.5% (Fig. 2.9 and Fig. S5).

The PNS.ECUR.4 (clade 9) clade formed a sister group to the *E. rigidior* clades PNS.ERIG.1 (clade 7) and PNS.ERIG.2 (clade 8) rather than grouping within PNS.ECUR.1 (clade 1) (sequence divergences ranged from 7.7 - 9.9% between PNS.ECUR.4 (clade 9) and PNS.ECUR.1 - PNS.ECUR.3; clades 1, 4, and 6) (Fig. S5). The wasps collected on *Sporobolus pyramidalis* and the unidentified *Sporobolus* sp. (but likely *S. africanus*) formed two distinct sister clades, with a sequence divergence of 3.9% (PNS.SPYR.1 (clade 10); pp = 1, bt = 89 and PNS.SPOR.1 (clade 11); pp = 0.73, bt = 99). Additionally, the *S. pyramidalis* wasps showed some evidence of geographic substructuring between the KwaZulu-Natal (pp = 0.9, bt = 97) and Eastern Cape (pp = 0.88, bt = 95) specimens. The two *E. rigidior* clades, PNS.ERIG.1 (clade 7) and PNS.ERIG.2 (clade 8), had a sequence divergence of 7.1% (Fig. S5).

Species delimitation

Delimitation results across the six methods were generally congruent, but the presence of singletons appeared to result in some disagreements in the ABGD, ASAP, bPTP and PTP output (Fig. 2.9). There were 13 consensus MOTUs in the PNS section of the phylogeny (clades 1 - 13), and six in the NPNS polytomy below it (clades 14 - 20; Fig. 2.9).

The GMYC was the only method to group the PNS *E. biflora* (PNS.EBI.1, clade 2) wasps with the PNS *E. curvula* (PNS.ECUR.1, clade 1) group (Fig. 2.9). Due to a sequence divergence of 2.5%, and the utilisation of different host species, these were treated as separate MOTUs. The PNS.SPYR.1 clade (clade 10) was supported as being one MOTU by the ASAP and PTP methods, but as two separate groups by the GMYC. This separation is more likely a population-level difference due to geographic separation between KwaZulu-Natal and Eastern Cape specimens, rather than a species-level difference. The wasps collected on the unidentified *Sporobolus* grass (PNS.SPOR.1, clade 11) were unanimously supported as a separate MOTU, as was the case for clades PNS.MIXED.1 and PNS.MIXED.2 (barring ABGD for PNS.MIXED.2, which split the two samples into singletons).

Clade NPNS.SPYR.1 (clade 17) was not supported as a single MOTU by any of the delimitation methods, and so the consensus bar shows four potential MOTUS within this group. It is, however, surprising that two specimens collected from *E. curvula* (LMY 2021

00282 and LMY 2021 00289) fell within the NPNS *S. pyramidalis* clade. They were, however, supported as separate MOTUs by the ASAP, PTP, and GMYC delimitation methods.

Only the GMYC supported clades NPNS.U.1 and NPNS.U.2 (clades 19 and 20) forming one MOTU. Due to the low sequence divergence between NPNS.U.1 and NPNS.U.2 (1.8%), they have been tentatively treated as one group. Only one sequence from Bavaria, Germany, grouped with these Canadian samples. All the other German specimens fell in clades NPNS.U.4 (clade 28) and NPNS.U.5 (clade 29).

The samples in NPNS.MIXED.3(clade 21) were well supported as a MOTU, and since seven *Bruchophagus* samples formed sister groups to it, these likely also belong to the *Bruchophagus* genus.

Although only the AGBD and GMYC methods supported NPNS.AGAYA.1 (clade 23) as a single MOTU, it was treated as such due to the unique host grass (*A. gayanus*) that the specimens were collected from in Zimbabwe. More sequences are, however, required to make further inferences.

Although clades HHIR.2 (clade 22), NPNS.HHIR.3 (clade 24), and PNS.ADON.1 (clade 25), NPNS.U.3 (clade 26), NPNS.ETEF.1 (clade 27), NPNS.U.4 (clade 28), and NPNS.U.5 (clade 29) were unresolved in the phylogeny, they were well supported by all the delimitation methods. Only NPNS.HHIR.3 showed some disagreement in the ABGD and bPTP results (Fig. 2.11). None of the delimitation methods included *T. bambusae* with the *T. romana* clade (PNS.ADON.1, clade 25).

2.4.2 28S data

Overall inter-and intraspecific p-distances (excluding the outgroup) ranged from 3 - 9.7% and 0 - 2.7%, respectively. Comparatively, Chen *et al.* (2004) reported interspecific distances of 1.69 - 13.5% for their eurytomid data set.

The estimated number of MOTUs ranged from 28 - 43 across delimitation methods, where ABGD = 32 spp, ASAP = 27, bPTP = 32, PTP = 32, GMYC = 36 (clusters = 23, entities = 36, CI clusters = 15 - 28, CI entities = 20 - 43, lnL of null model = 957.235, ML of GMYC model = 963.6845, $p < 0.01$, singletons = 13), and Haplowebs = 39 (Fig. 2.11).

The only three well-defined clades within the PNS group were the wasps collected on *Eragrostis rigidior* (PNS.ERIG.1; clade 1, posterior probability (pp) = 0.71), *Sporobolus* spp. (PNS.SPOR.1; clade 2, pp = 0.84, bootstrap support (bt) = 91), and *Arundo donax* (PNS.ADON.1, clade 3, pp = 1, bt = 100) (Fig. 2.11 and Fig. 2.13). The wasps from *H. hirta* formed two separate clades (NPNS.HHIR.1; clade 4 and NPNS.HHIR.2; clade 11), where NPNS.HHIR.1 formed a sister group to the *A. gayanus* wasps (NPNS.AGAYA.1; clade 5) and to the PNS group, while NPNS.HHIR.2 formed a sister group to the major NPNS clade (Fig. 2.11). *Tetramesa bambusae* (AY317229, (Chen *et al.*, 2004)) and *T. romana* (KCP 2011 00169, KCP 2011 00173, KCP 2012 00170, and KCP 2012 00172) had a sequence divergence range of 0.3 (to each other) - 8.1% (compared to all the other major clades) (Fig. S6). This range was 2 - 2.6% relative to the PNS clades only (PNS.ERIG.1 (clade 1), PNS.SPOR.1 (clade 2), PNS.ADON.1 (clade 3), NPNS.HHIR.1 (clade 4), and NPNS.AGAYA.1 (clade 5)). Divergence values between the other unidentified *Tetramesa* specimens (JN623677, DQ080114, AY599281) ranged from 3.2 - 7.1% relative to *T. bambusae* and *T. romana* (Fig. S6). *Tetramesa* samples AY599281 and DQ080114 clustered within the major NPNS clade, and not with or near JN623677 (*Tetramesa* sp.), *T. romana*, or *T. bambusae* (AY317180) (Fig. 2.11). *Bruchophagus* and *Eurytoma* specimens did not form monophyletic clades, and were scattered throughout the tree (Fig. 2.11).

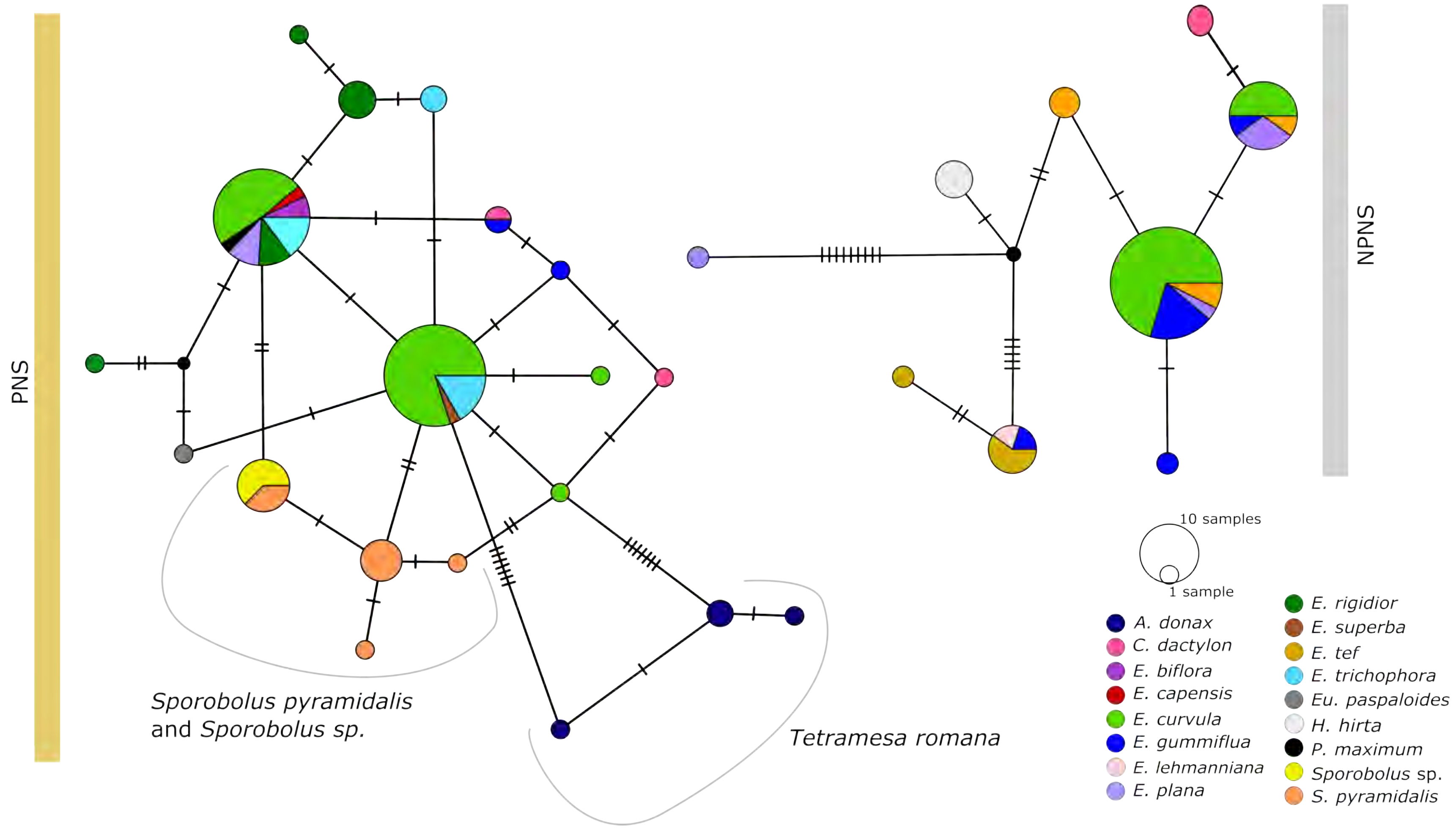


Figure 2.13: TCS Haplotype networks (28S gene region) for the pronotal spot (PNS, n = 91) and non-pronotal spot (NPNS, n = 59) clades marked with the vertical gold and grey bars in Figures 2.11 and 2.11. Hash marks denote the number of nucleotide differences between haplotypes, and the different colours represent the host grass species shown in the figure legend.

The nuclear marker shows that both the Eurytominae subfamily and the *Tetramesa* form well-supported monophyletic groups (Fig. 2.11, pp = 1). This work suggests that a sequence divergence of $\sim$ 3 - 3.5% and above likely does not belong to the *Tetramesa* genus (Table S2), and that major groups within the PNS clade can be delineated at a threshold of below 0.4%. The only NPNS wasps that fell within the *Tetramesa* group were those collected on *H. hirta* (NPNS.HHIR.1; clade 4) and *A. gayanus* (NPNS.AGAYA.1; clade 5) Fig. 2.11).

Species delimitation

The six delimitation methods were congruent, except for PNS.ERIG.1 (clade 1), PNS.SPOR.1 (clade 2), NPNS.SPY.1 (clade 10), and NPNS.HHIR.2 (clade 11) that were delimited by only the GMYC and/or Haplowebs methods (Fig. 2.11). These were retained as putative MOTUs due to their utilisation of different grass genera as hosts (namely *Eragrostis*, *Sporobolus*, and *Hyparrhenia hirta*).

There were six resolved consensus MOTUs within the group deemed to belong to the *Tetramesa* genus (Fig. 2.11). These were the wasps collected on *E. rigidior* (PNS.ERIG.1; clade 1), *Sporobolus pyramidalis* + *Sporobolus* sp. (PNS.SPOR.1; clade 2), *H. hirta* (NPNS.HHIR.1; clade 4), *A. gayanus* (NPNS.AGAYA.1; clade 5), and *T. romana* + *T. bambusae* (PNS.ADON.1; clade 3), and the unidentified *Tetramesa* specimen JN623677 collected in the USA, Maryland. Although unresolved, the two NPNS.AGAYA.1 (clade 5) specimens are likely a single sister group to NPNS.HHIR.1 (clade 4), and since they were collected on a different host, *Andropogon gayanus*, they have been treated as a putative MOTU. The NPNS.MIX.1 (clade 7) polytomy formed a sister group to the two *Bruchophagus* specimens AY317164 and AY317170 (*B. ononis*), but these were not supported as one shared MOTU by any of the delimitation methods.

NPNS.MIX.5 (clade 19), NPNS.MIX.4 (clade 12), U.U.4 (clade 17), and U.U.5 (clade 19) were supported as separate MOTUs across all methods (Fig. 2.11). Although a singleton, *Eurytoma* sp. 2 (AY317166) was sister to U.U.4 and U.U.5. The unidentified *Tetramesa* specimens AY599281 and DQ080114 formed two separate MOTUs that were supported by all the delimitation methods except ASAP (Fig. 2.11). Also shown by Chen *et al.* (2004) as a single clade, U.U.1 (clade 13) was unanimously supported as a single MOTU, despite containing four different genera (*Aiolomorphus rhopaloides* Walker, *Bephratelloides cubensis* Ashmead, *Phylloxeroxenus phylloxerae* Ashmead, and *Tenuipetiolus medicaginis* Gahan).

2.4.3 Pronotal spot *Eragrostis curvula Tetramesa* wasps: genetic divergence across geographic space

The Mantel test indicated that there was a significant correlation between genetic divergence and geographic distance for the PNS *Eragrostis curvula* wasps ($r = 0.45$, $p < 0.001$) (Fig. 2.14). This is important to investigate because it provides an indication of intraspecific population structure across spatially separated groups. The results of the AMOVA tests showed that there was a significant level of genetic substructuring in both the provincial and Eastern Cape-only populations, but that most of the variation across provinces was found within populations (69.8%), while the variation across the Eastern Cape showed the most variation among populations (84.4%) (Table 2.2). Pairwise FST comparisons across provinces ranged between 0.17 - 0.38, and showed a significant difference in genetic structure between the Eastern Cape and Free State populations ($FST = 0.38$, $p < 0.001$). Pairwise FST comparisons within Eastern Cape populations ranged between 0 - 0.91, where no genetic structuring was found between the Grahamstown and Port Elizabeth populations (Table 2.3). There was significant support for a high degree of genetic structuring between both the Grahamstown and Port Elizabeth populations compared to Mondplaas and East London (Fig. 2.15 and Table 2.3).

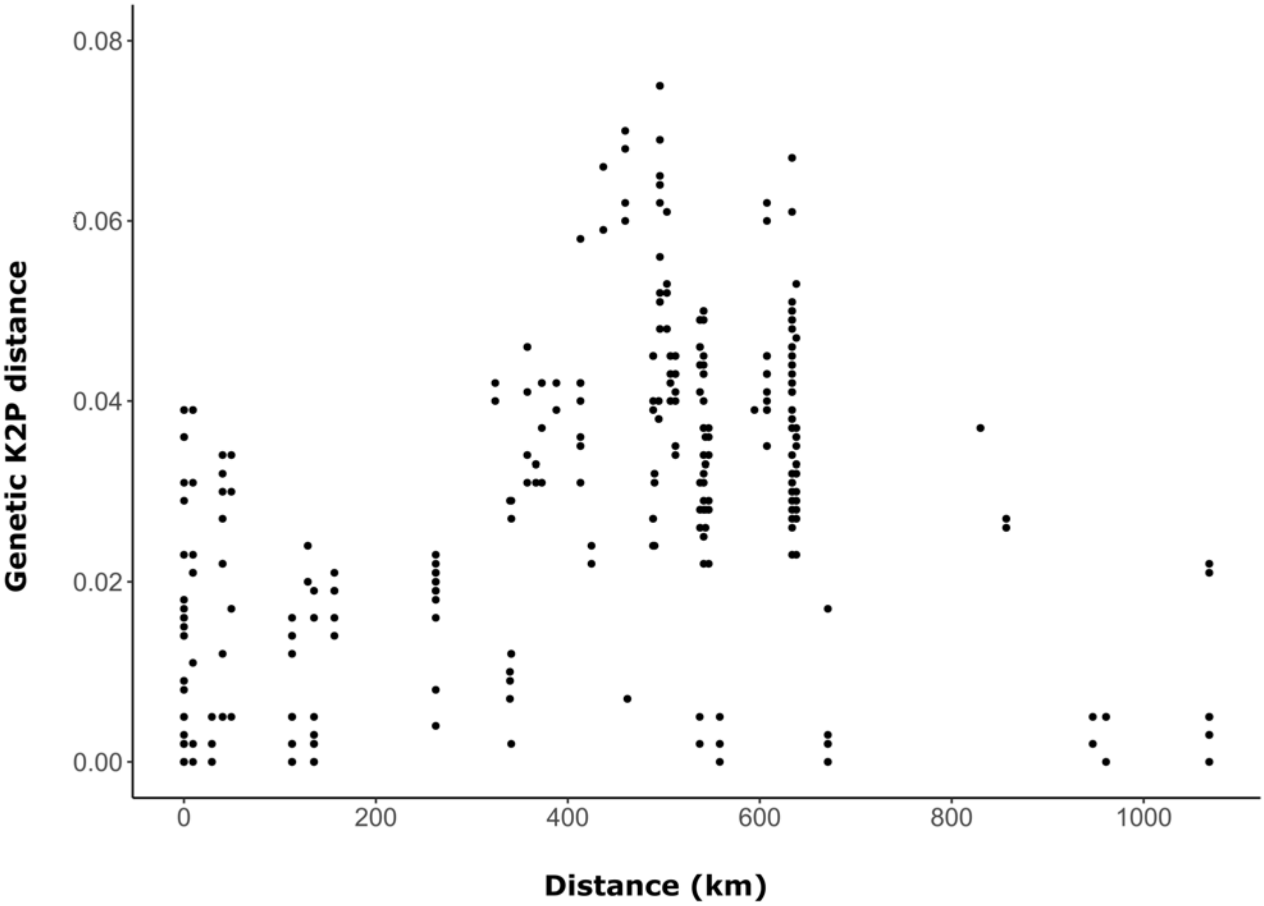


Figure 2.14: Genetic K2P distances plotted against geographic distance (km) as pairwise matrices, for *Tetramesa sp. 4* collected on *Eragrostis curvula* host plants.

Table 2.2: Analysis of molecular variance (AMOVA) results of COI data from the *Tetramesa* collected on *Eragrostis curvula*. The AMOVA for provincial comparisons included *Tetramesa* from the Eastern Cape (n = 21), Free State (n = 12), Mpumalanga (n = 2), and the Northern Cape (n = 2), and the AMOVA for populations within the Eastern Cape were for *Tetramesa* collected from Grahamstown (n = 6), Port Elizabeth (n = 10), East London (n = 2), and Mondplaas (n = 3). Df = degrees of freedom.

Source of variation	df	Sum of squares	Variance components	% of variation
Provincial comparisons				
Among populations	3	77.76	2.79	30.23
Within populations	33	212.29	6.43	69.77
Total	36	290.05	9.22	100
Fixation index (FST)	0.30*** ($p < 0.001$)			
Eastern Cape comparisons				
Among populations	3	90.79	6.28	84.4
Within populations	17	19.73	1.16	15.6
Total	20	110.52	7.44	100
Fixation index (FST)	0.84*** ($p < 0.001$)			

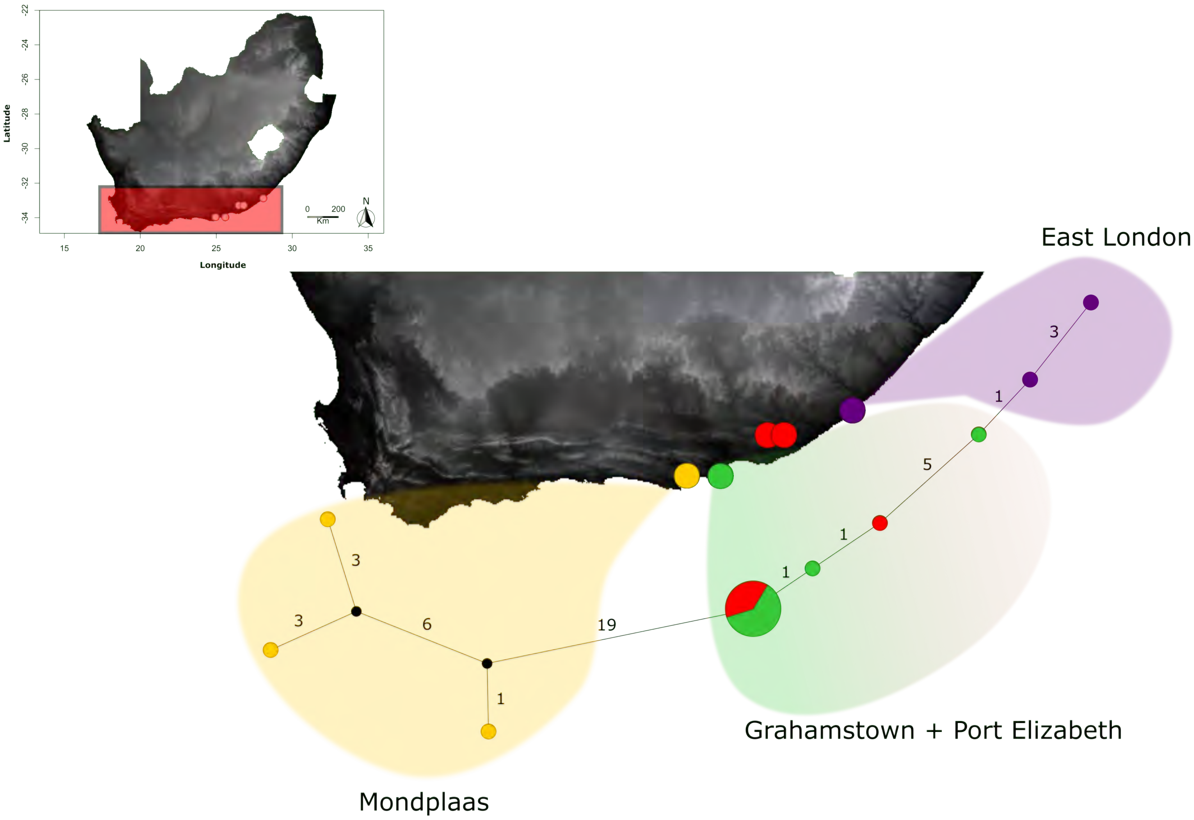


Figure 2.15: A COI TCS haplotype network for the Eastern Cape *Tetramesa* collected on *Eragrostis curvula*. The numbers between nodes indicate the number of mutations between haplotypes.

Table 2.3: Pairwise fixation index (FST) value comparisons (K2P model) for pronotal spot *Tetramesa* collected on *Eragrostis curvula* at A) a provincial level, and B) within the Eastern Cape. Significant comparisons are shown in bold ($p < 0.05$). EC = Eastern Cape, FS = Free State, MP = Mpumalanga, NC = Northern Cape, GHT = Grahamstown, PE = Port Elizabeth, EL = East London, MDPL = Mondplaas.

A) Provincial comparisons

	EC	**FS**	**MP**	**NC**
EC	0			
FS	**0.38**	0		
MP	0	0.23	0	
NC	0.17	0	0.17	0

B) Eastern Cape comparisons

	GHT	**PE**	**EL**	**MDPL**
GHT	0			
PE	0	0		
EL	**0.87**	**0.78**	0	
MDPL	**0.90**	**0.91**	0.79	0

2.4.4 Nuclear clock dating

The dated Bayesian 28S rRNA phylogeny showed the origin of the Eurytominae ingroup between the Middle Jurassic and Lower Cretaceous, at an estimated median date of 139.5 mya (node B; 95% HPD = 110.1 - 172.6 my, pp = 1) (For all references to node date estimates see Fig. 2.16 and Table 2.4). There is support for the Eurytomidae being a younger group relative to the outgroup families comprising the Chalcididae, Torymidae, and Megastigmidae (node A; median age = 287.7 mya, 95% HPD = 227.7 - 356.4 my), and the Rileyinae subfamily. Although these data are congruent with previous studies (e.g. Heraty and Darling (2009)), gaining support for these hypotheses is beyond the scope of the present study, and would require a substantially greater coverage of Chalcidoid sequence data to make further inferences.

The origin of the *Tetramesa* clade was placed between the Upper Cretaceous and the Eocene, at a median age of 67.1 mya (node E; 95% HPD = 47.4 - 91.8, pp = 1). The PNS and NPNS clades diverged ~ 50.2 mya (node F; 95% HPD = 36.3 - 67.9, pp = 0.95), and the northern and southern hemisphere PNS clades diverged ~ 44.5 mya (node G; 95% HPD = 31.9 - 59.6), although the support value for this node was low (pp = 0.55). The southern African clades began radiating ~ 34.3 mya (node H; 95% HPD = 24.6 - 46, pp = 0.98), with rapid diversification occurring ~ 5 - 15 mya (see the lineage through time plot inset in Fig. 2.16). The Northern Hemisphere *T. romana* and *T. bambusae* appear to have radiated during the same relative time frame as the southern African groups (~ 11.5 mya, node H, pp = 1).

Figure 2.16 shows the i) the estimated origin of stem-group Hymenoptera and Panorpoid orders (Grimaldi *et al.*, 2005), ii) the estimated origin and radiation of angiosperms based on molecular data (Li *et al.*, 2019), iii) records of the oldest known hymenopteran (Xyelidae family) fossils discovered in Australia (Riek, 1955; Yoshimoto, 1975), iv) possible origin of the Chalcidoidea (Heraty and Darling, 2009), v) proposed origin and radiation of angiosperms based on fossil data (Li *et al.*, 2019), but vi) a possible stem group may have originated in the Middle Triassic based on the discovery of angiosperm-like pollen grains, vii) some of the earliest chalcidoid fossils discovered in Ethiopia (Schmidt *et al.*, 2010), viii) period of chalcidoid radiation (Heraty and Darling, 2009), ix) Miocene grassland expansion in Africa (Jacobs, 2004; Uno *et al.*, 2016), and x) estimated dates of two *Eurytoma* and one *Decatoma* fossil/s (Yoshimoto, 1975).

The unidentified *Tetramesa* specimen collected in Maryland, USA (JN623677), was the first to diverge from the main clade ~ 67 mya (node E). Further information about this specimen is lacking (host plant use, native range), but it appears to represent a unique lineage that may comprise a more basal group.

Table 2.4: Divergence time estimates in millions of years (median ages) for the nodes shown in Figure 2.16, with 95% HPD confidence intervals.

Node	Median age	95% HPD CI	Node	Median age	95% HPD CI
A	287.7	227.7 - 356.4	H	34.3	24.6 - 46
B	139.5	110.1 - 172.6	I	18.3	7.3 - 31.5
C	128.5	102.1 - 158.9	J	11.5	4 - 21.2
D	104.4	78.9 - 134.4	K	19.7	16 - 27.4
E	67.1	47.4 - 91.8	L	31.9	28.8 - 37.4
F	50.2	36.3 - 67.9	M	19	9.3 - 30.7
G	44.5	31.9 - 59.6	N	23.8	22.9 - 28.5

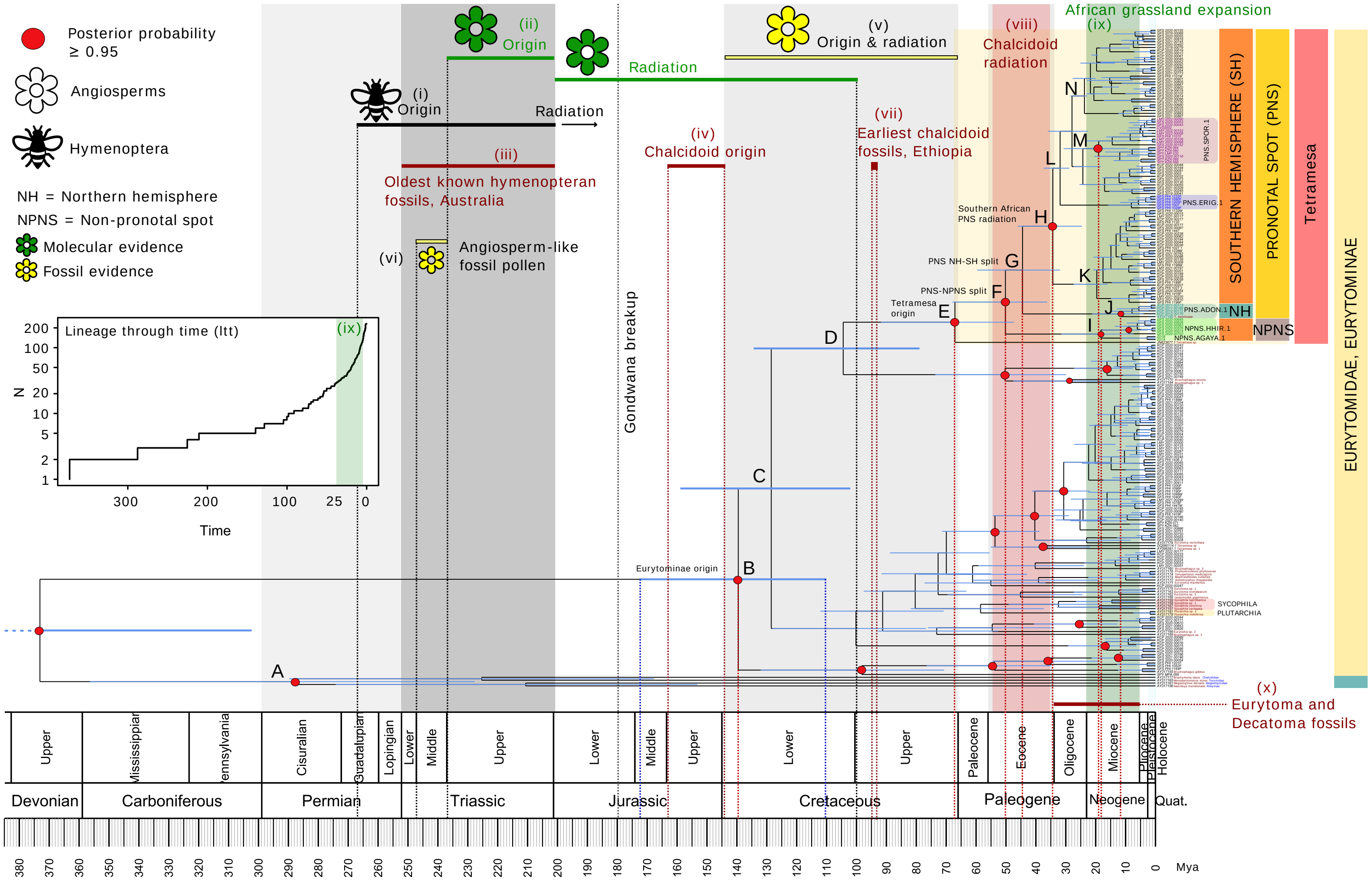


Figure 2.16: A Bayesian time-calibrated phylogeny for the nuclear 28S rRNA data set, showing important divergence events and points of origin, and the radiation of the *Tetramesa* (green shaded region). The horizontal blue lines through each node represent 95% HPD confidence intervals. See the associated text for more details. The lineage through time (ltt) plot shows the number of lineages (N) over millions of years.

2.5 Discussion

The Eurytomidae family is known to be polyphyletic from both molecular and morphological lines of evidence (Campbell *et al.*, 2000; Chen *et al.*, 2004; Lotfalizadeh *et al.*, 2007b), and so the suggestion of a major revision of the group would not be a new or surprising one (Gahan, 1922). The present phylogenetic analyses supported the proposition that the Eurytomidae family is a relatively young taxonomic group (agreeing with Zerova (1992)) that underwent rapid radiation during the Miocene. These results agreed with Chen *et al.* (2004) in (1) highlighting the inconsistency with current eurytomid, and more particularly *Tetramesa*, classification, (2) the incongruence of the nuclear and mitochondrial gene topologies (mitonuclear discordance), (3) the high incidence of unresolved clades observed in both the nuclear 28S and mitochondrial COI gene trees, (4) the polyphyly of the *Eurytoma* and *Bruchophagus* genera, and (5) the highly variable sequence divergences observed, particularly in the COI data set. Although the taxonomy of the Eurytomidae, and the *Tetramesa* in particular, still requires substantially more work, the results presented here have begun to fill some of the knowledge gaps and can be useful to biological control programmes seeking to use eurytomids as agents for invasive grasses.

2.5.1 Southern African *Tetramesa* phylogenetics

The nuclear 28S rRNA marker was effective in broadly separating what are likely true *Tetramesa* from other genera, where a suggested sequence divergence of ∼ 3 - 3.5% can be used for genus-level delimitation. Of the four 28S sequences identified as *Tetramesa* gleaned from GenBank, two fell within the *Tetramesa* clade (sourced from Chen *et al.* (2004) and Munro *et al.* (2011)) while the other two (Gillespie *et al.* (2005), and a sequence from unpublished material: `https://www.ncbi.nlm.nih.gov/nuccore/DQ080114`) fell in a NPNS polytomy that was more similar to some *Eurytoma* sequences, and with a divergence of as much as 7.1% from the *T. romana* group. This high nuclear divergence suggests that these NPNS GenBank specimens were either misidentified, or that the *Tetramesa* genus needs to be split using morphological and molecular tools, and ecological information as guides. Similarly, all the COI sequences deposited on BOLD that were identified as *Tetramesa* (collected in Canada and Germany) showed sequence divergences of nearly 20% compared to *T. romana* and *T. bambusae*, which is a clear indication that these specimens likely belong to a different genus. It is surprising that there are so few *Tetramesa* sequences available on GenBank and BOLD. This is a current gap that needs to be addressed, where further studies should delve further into the phylogeography of *Tetramesea* from different geographic regions across the world and their host plant complexes.

Online public databases such as GenBank (`https://www.ncbi.nlm.nih.gov/genbank/`) and BOLD (`https://www.boldsystems.org/index.php/databases`) are invaluable resources for taxonomic research, but there are a number of factors that can lead to erroneous data. These include the incorrect identification of voucher specimens, contamination, PCR errors, the sequencing of pseudogenes (e.g. numts), poor isolation techniques (e.g. in fungi), and endoparasites in insects (e.g. *Wolbachia*) (Bridge *et al.*, 2003; Vilgalys, 2003; Ashelford *et al.*, 2005; Seah *et al.*, 2017; Meiklejohn *et al.*, 2019). Specimens that are assigned to a genus and/or species based solely on COI Barcode Index Numbers (BINS) without formal morphological identification or the guidance of other biological information could hamper taxonomic progress (Collins and Cruickshank, 2013), particularly in complex groups that do not have verified reference data, such as the *Tetramesa*. This work has highlighted a number of northern hemisphere COI sequences gleaned from BOLD that were likely incorrectly assigned to the *Tetramesa* genus (Table S1).

Overall, the COI region indicated 13 potential host-specific taxonomic units (HSTUs) within the PNS group; nine of which were associated with *Eragrostis* species. Comparatively, the 28S data delimited six PNS HSTUs. Both gene trees supported the PNS *Eragrostis rigidior* and *Sporobolus* clades, and the NPNS *Hyparrhenia hirta* clade as being host-specific (of the two supported *H. hirta* clades, only one formed part of the *Tetramesa* group). The COI phylogeny further suggested that the PNS *E. rigidior* and *Sporobolus* clades comprised two distinct HSTUs, where the latter showed support for *Tetramesa* specific to *S. pyramidalis* and *S. africanus*.

In agreement with Chen *et al.* (2004), the nuclear 28S rRNA marker is preferred as an initial identification guide, because it yielded well-supported basal nodes, generally unanimous species delimitation results, and broader-scale taxonomic relationships that will be useful for genus-level taxonomic revisions. The COI marker should be used as second filter to focus on specific target groups identified in the nuclear gene tree, as it provided greater resolution within the PNS group and revealed some potential cryptic species and/or genetically distinct populations. Numerous polytomies, however, were a confounding factor in inferring how these groups are evolutionarily related. Additionally, the COI phylogeny did not produce the same monophyletic *Tetramesa* group as was seen using the 28S marker, and yielded surprisingly high sequence divergence values between the Northern Hemisphere *T. romana* and *T. bambusae* groups (10.8%). It was unexpected that the NPNS *H. hirta* and *A. gayanus* and the *T. romana* clade in particular did not cluster with the PNS group in the COI phylogeny as it did in the 28S phylogeny, and that the COI genetic divergences were unusually high for these groups. Future phylogenetic analyses should include additional markers such as CAD, ND1, 12S, 16S, LepWG, and/or AK, and specifically ultra-conserved elements (UCE) to resolve polytomies and to identify which clades represent true cases of rapid speciation.

It is known that the COI marker could yield unexpected results when delimiting species that have undergone a rapid, recent radiation, as the lack of recombination in the mitochondrial genome can lead to an overestimation of sequence divergences due to the accumulation and retention of mutations (Hupalo *et al.*, 2022). Comparatively, due to recombination, nuclear markers will thus more readily indicate renewed gene flow between previously-isolated populations (e.g. due to climatic cycles and habitat changes), which can result in conflicting phylogenies (Eyer *et al.*, 2017; Després, 2019) (Fig. 2.17). This dynamic is termed "mitonuclear discordance", and is more prevalent than previously realised (Toews and Brelsford, 2012; Ivanov *et al.*, 2018; Hupalo *et al.*, 2022). Other reasons for mitonucleaer discordance include horizontal gene transfer (Soucy *et al.*, 2015) (e.g. between *Wolbachia* (Rickettsiales, Rickettsiaceae) endosymbiotic bacteria and their hosts (Yang *et al.*, 2013)), the presence of numts (nuclear pseudogenes in mitochondrial DNA), which have been recorded in a number of hymenopteran studies (see for example Françoso *et al.* (2016); Francoso *et al.* (2019); Wang *et al.* (2020a)), and androgenesis (the mode of sexual reproduction whereby the male's nuclear genome is the sole source of genetic material in an embryo), which, although rare in the animal kingdom, is fairly common in hymenopterans (Schwander and Oldroyd, 2016). These factors may explain the unusually high sequence divergences found in the present eurytomid COI data, and the discordance with the nuclear gene tree. It is likely that these wasps can reproduce facultatively via thelytokous parthenogenesis

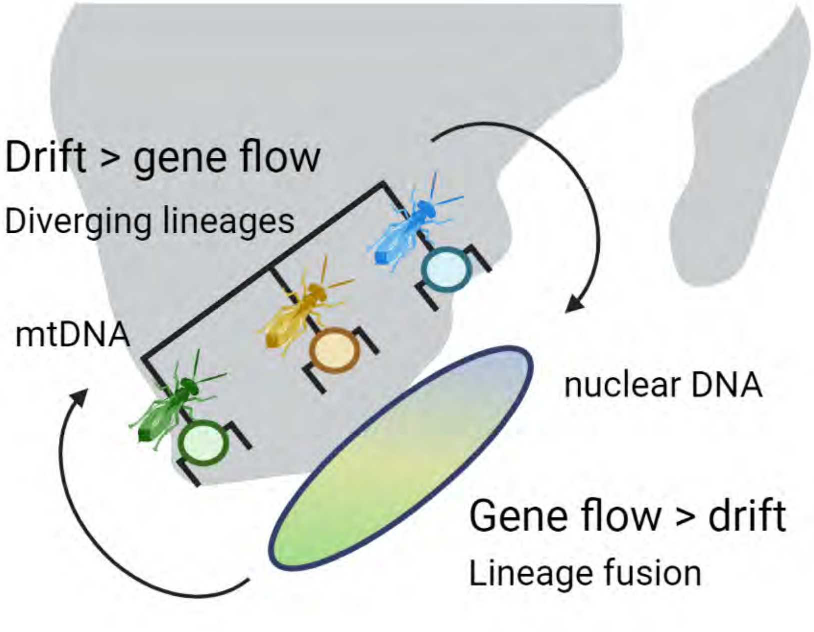


Figure 2.17: A schematic diagram illustrating how mitonuclear discordance can occur between two gene trees. The arrows denote climatic cycles that can lead to changes in the degree of gene flow between taxa, where mitochondrial markers often overestimate sequence divergences while nuclear markers more readily reveal renewed gene flow. Diagram adapted from Després (2019).

(i.e. a form of asexual reproduction where diploid daughters are produced from unfertilised eggs) (Moran and Goolsby, 2009). Compared to the default reproductive mode of arrhenotoky in the Hymenoptera (i.e. unfertilised eggs develop into haploid males and fertilized eggs develop into diploid females), thelytoky can lead to increased levels of homozygosity over time (Mateo Leach *et al.*, 2009). It is even possible that infection by different *Wolbachia* strains can cause reproductive isolation between intraspecific populations (e.g. in *Nasonia* wasps (Bordenstein *et al.*, 2001)). Future eurytomid phylogenetic studies should investigate a wider range of nuclear markers - both ribosomal and protein-coding - and also the relationship between *Wolbachia* and their *Tetramesa* hosts in order to determine the degree to which these endosymbionts might be affecting genetic diversity, and under what conditions the wasps change their mode of reproduction.

2.5.2 Radiation patterns and unresolved clades

Unresolved clades are not uncommon in hymenopteran phylogenies, and have been reported in a number of other families, including vespids (*Polistes* Latreille) (Perrard *et al.*, 2013; Santos *et al.*, 2015), colletids (*Colletes* Latreille) (Ferrari *et al.*, 2020), agrids (Malagón-Aldana *et al.*, 2022), and ichneumonids (Gauld and Dubois, 2006). Polytomies are usually indicative of insufficient data (in terms of sample size and/or appropriate markers and analysis methods) ("soft polytomies"), but in some cases signify rapid simultaneous speciation events ("hard polytomies") (Walsh *et al.*, 1999). Hard polytomies are often the result of the simultaneous isolation of multiple populations due to a vicariance event (e.g. glaciations, sea level rises, large-scale environmental changes), leading to allopatric or peripatric speciation (Hoelzer and Meinick, 1994). The rapid radiation of cichlids in Lake Malawi is a well-documented example of polytomous speciation, where it is estimated that hundreds of species arose within the last two million years following Pliocene rifting that formed Lake Malawi and the Kipengere (Livingstone) mountain range (Genner *et al.*, 2015; Scherz *et al.*, 2022). The Eurytomidae have been noted for the rapid rate of differentiation into new ecological niches leading to host specialisation (Zerova, 1992). Additionally, since phytophagous eurytomids are associated exclusively with angiospermous hosts, this differentiation has been postulated to have accompanied the diversification of angiosperms in the Southern Hemisphere (Gondwana) during the Lower Cretaceous (145 - 100.5 mya) based on fossil evidence (Zerova, 1992; Beaulieu *et al.*, 2013; van der Kooi and Ollerton, 2020).

Despite the caveats associated with phylogenetic dating using only molecular clock rates, the dating analysis of the 28S gene region supported the recent radiation of the Eurytomidae, with the *Tetramesa* clade originating between the Lower Cretaceous and the Eocene, and undergoing a rapid rate of diversification within the last 5 - 15 my. This rapid radiation likely explains the consistently unresolved clades in both the nuclear and mitochondrial results, suggesting a possible case of true "hard" polytomies in eurytomid genera. This can, however, only be confirmed with additional loci. The aim of the present dating analysis was not to obtain absolute dates, but rather to assist in explaining the broad patterns found in the eurytomid phylogenies, and to gain an overall understanding of the evolution of the group.

The estimated time ranges of the origin of the Eurytominae and *Tetramesa* coincide with the proposed Cretaceous radiation of the angiosperms (Zerova, 1992; Li *et al.*, 2019) and chalcidoid radiation during the Eocene (Heraty and Darling, 2009). The pattern of the rapid Miocene radiation seen in the PNS *Tetramesa* group has also been reported in other insects (Trewick and Morgan-Richards, 2005; Winkler *et al.*, 2009; Toussaint *et al.*, 2012; Aduse-Poku *et al.*, 2022; Perrichot *et al.*, 2022), and coincides with the expansion of grasslands and retraction of forest habitats in Africa (Jacobs, 2004; Osborne, 2008; Uno *et al.*, 2016). The expansion of C4 grasslands during the Neogene has been proposed to have been driven by global cooling, increased aridity, decreased atmospheric CO_2, and strong seasonality patterns that produced an environment prone to seasonal fires (Beerling and Osborne, 2006; Staver *et al.*, 2011). Additionally, it is hypothesised that abrupt climatic fluctuations during the Miocene (Zachos *et al.*, 2008) fragmented populations and drove lineage diversification.

There is support for a split between the NPNS and PNS *Tetramesa* $\sim$ 50.9 mya. Although the node was not strongly supported ($pp = 0.55$), the Northern and Southern Hemisphere *Tetramesa* may have diverged $\sim$ 45 mya, with the Southern Hemisphere group beginning to diversify $\sim$ 35 mya. It is not possible at present to ascertain whether the *Tetramesa* originated in the Northern or Southern Hemisphere due to both a lack of thoroughly curated Northern Hemisphere sequence data and reliable fossil evidence. These time-frames are, however, sufficiently large to suggest possible genus-level splits (Menezes *et al.*, 2020; Samacá-Sáenz *et al.*, 2022).

Since very little is known about the inheritance patterns and mitochondrial dynamics in the Eurytomidae (Chen *et al.*, 2004), it is recommended that nuclear clocks are investigated further for this group. Although the nuclear clock rate used in the present work, suggested by Papadopoulou *et al.* (2009) for tenebrionid beetles, might only provide rough estimates of divergence times, it appears that this approach is reliable, and that the results make sense in the context of what is already known from the fossil record. A number of other studies have utilised nuclear clocks for both deep-level and recent divergences (Wiegmann *et al.*, 2000; Steppan *et al.*, 2005; Schmitz *et al.*, 2007; Bertone *et al.*, 2008; Meredith *et al.*, 2008; Reidenbach *et al.*, 2009; Vea and Grimaldi, 2016; Kergoat *et al.*, 2018), suggesting that nuclear markers may sometimes be more useful and robust than mitochondrial genes (Steppan *et al.*, 2005; Sato *et al.*, 2016).

2.5.3 Biological control outlook

The NPNS clades appeared to generally display a much higher degree of generalist feeding behaviour relative to the PNS groups, and so biological control efforts should prioritise the latter, and those that form part of the NPNS *H. hirta* and *Andropogon gayanus* clade. This is based on the host-use patterns seen particularly in the COI phylogeny, where, with a few minor exceptions, each PNS clade was associated with a single host grass. Comparatively, the NPNS clades tended to show a greater degree of variability in host associations. The prospects for the biological control of the various target weeds are discussed below in the context of the phylogenies and inferences of host ranges. A promising species for *M. maximus* was found, but more samples from this target weed are needed before any firm conclusions can be drawn.

Eragrostis curvula

The PNS *E. curvula* wasps (morphospecies "*Tetramesa sp. 4*") belonged to the *Tetramesa* genus. No-choice host-specificity testing has shown that this wasp can complete its life-cycle on at least two other native African grasses; namely *E. planiculmis* and *E. plana* (Guy Sutton, unpublished data). These host-specificity test results align with the present phylogenetic work, and suggest that the nine PNS *Eragrostis* wasp HSTUs delimited in the present COI phylogeny (spanning *E. curvula*, *E. biflora*, *E. rigidior*, *E. trichophora*, and *E. plana*) likely belong to the same species, and may be in the process of speciation (i.e. incipient species). Testing on the *E. biflora* and *E. trichophora* wasps are currently also underway. The results of a Mantel test for all the PNS *E. curvula* specimens in the COI phylogeny showed a significant positive correlation between genetic divergence and geographic distance, which supports the existence of multiple intraspecific populations. The AMOVAs also supported a high degree of genetic structure within the PNS *E. curvula* wasps, and showed that variation was highest within populations when comparing across provinces, but among populations when comparing within the Eastern Cape only. There was, however, significant support for genetic differentiation between the Eastern Cape and Free State groups, suggesting a possible case of cryptic speciation. The results from within the Eastern Cape suggest that there are prominent intraspecific genetic gradients over relatively small spatial scales. *Tetramesa romana* appears to be able to disperse easily over large distances in the USA (Moran *et al.*, 2017), which might be facilitated by the flat landscapes on the continent. South Africa is comparatively mountainous, and so geographic barriers might be the cause of the genetic isolation observed in the Eastern Cape and the resulting radiation patterns. It may be that differences in *E. curvula* host forms are responsible for ecological barriers driving the genetic structuring observed (i.e. host-associated differentiation (HAD) that can lead to sympatric speciation). Similar patterns of HAD have been reported in numerous other specialist endophagous herbivores, such as the snakeweed grasshopper *Hesperotettix viridis* (Orthoptera: Acrididae) on *Solidago mollis* and *Gutierrezia sarothrae* (Asteraceae) lineages (Sword *et al.*, 2005), an assemblage of herbivores on *Solidago altissima* and *S. gigantea* (StiremanIII *et al.*, 2005), the seed weevil *Rhinusa antirrhini* Schoenherr (Coleoptera: Curculionidae) on *Linaria* hosts (Hernández-Vera *et al.*, 2010), and the corn leafhopper *Dalulus maidis* DeLong & Wolcott (Hemiptera: Cicadellidae) on *Zea mays* subspecies (Medina *et al.*, 2012).

Further work should focus on a population genetics analysis of both the PNS *E. curvula* *Tetramesa* wasps and their hosts from a wider geographic range to test whether host plant form may be driving genetic divergence. In the case of different *E. curvula* haplotypes in the native range, it will be vital to confirm matches to haplotypes in the invaded range to ascertain points of origin and select the most appropriate *Tetramesa* population/s as control agents. For example, Harms *et al.* (2021) identified a novel haplotype of the alien invasive water weed *Hydrilla verticillata* (L.f.) Royle, and found that the source population was from north-eastern China. This allowed the researchers to conduct targeted herbivore guild collections from this region.

Some microsatellite profiling has been done on *T. romana* in South Africa, where it was found that there are two genotypes present that are distinct from the French and Spanish genotypes; namely a strain common to the Limpopo and Mpumalanga provinces, and a strain common to the other seven provinces in the country (unpublished work, pers. comm. Costas Zachariades, Agricultural Research Council, KwaZulu-Natal). It would be valuable to add data obtained from ultra conserved elements and/or DDRADseq (double digest restriction-site associated DNA) to further investigate the genetic structure of the native South African PNS groups, and to include a climate matching exercise for each genotype in its native and invaded range.

The NPNS *E. curvula* wasp that was assigned the morphospecies name "*Tetramesa sp. 5*" does not appear to belong to the *Tetramesa* genus, based on the present nuclear 28S results. It is possible that the *Tetramesa* as it currently stands should be divided into two genera: one comprising the predominantly pronotal spot groups, and the other the non-pronotal spot clades. No-choice host specificity testing on *Tetramesa sp. 5* found that it could utilise at least three other native South African grasses; namely *E. planiculmis*, *E. plana*, and *E. rigidior*. Both *Tetramesa sp. 4* and *Tetramesa sp. 5* are currently being used in host-specificity tests on native Australian *Eragrostis* species, as this will be the ultimate deciding factor in their utility as biological control agents. It would also be vital to better understand the ecology of these species; including their dispersal and reproductive mechanisms, and phenology.

Sporobolus pyramidalis

The PNS *Sporobolus* wasps revealed two potential HSTUs, namely on *S. pyramidalis* (*Tetramesa sp. 1*) and *S. africanus*. Both field-based and no-choice tests have revealed that *Tetramesa sp. 1* is not able to utilise *S. africanus*, but the host-specificity of the *S. africanus* wasps should be investigated further.

Both the PNS and NPNS *Sporobolus* wasps (named morphospecies "*Tetramesa sp. 1*" and "*Tetramesa sp. 2*") have been shown to be host-specific in the field, where sp. 1 was found to be the more damaging of the two (Sutton *et al.*, 2021b). The present genetic results suggest that the NPNS *Tetramesa sp. 2* wasp does not belong to the *Tetramesa* genus. This wasp was identified as a *Tetramesa* species by expert taxonomists, which highlights the taxonomic confusion of this genus. It would be interesting to assess whether these two wasps arrive on the host plant at different times, and whether the earlier arrival of one species may lead to changes in induced host plant defences on the insect arriving later (see for example Poelman *et al.* (2008); Erb *et al.* (2011)). This can have important implications in the timing of agent releases.

The present genetic results suggest that the NPNS *Tetramesa sp. 2* wasp may be able to feed on *E. curvula* (two wasp samples out of the nine that were collected on *S. pyramidalis*), but field host range surveys by Sutton *et al.* (2021b) have found that the wasp was specific to only *S. pyramidalis* and *S. natalensis*. Laboratory-based host-specificity test have also been conducted on over 20 non-target species, including eight native South African *Sporobolus* species, with no non-target feeding recorded to date (Guy Sutton, pers. comm).

This scenario is an example of why it is imperative to have correctly identified each prospective agent in a biological control programme, and to have a solid understanding of their life histories and interspecific interactions in order to achieve the greatest level of damage. Since *Tetramesa sp. 2* may be able to use *E. curvula* as a marginal host in the field, and that the wasp is less damaging than *Tetramesa sp. 1*, it might be prudent to only use *Tetramesa sp. 1* for biological control.

Hyparrhenia hirta* and *Andropogon gayanus

The NPNS *Tetramesa* collected on *Hyparrhenia hirta* and *Andropogon gayanus* that formed a sister group to the PNS clade were host-specific and formed a monophyletic clade in both gene trees. This is not surprising, since *H. hirta* and *A. gayanus* are phylogenetically close, both being in the tribe Andropogoneae (Skendzic *et al.*, 2007), and structurally similar (i.e. both are tall-statured grasses) (Canavan *et al.*, 2019b).

The COI results suggested that this group may comprise three cryptic species, and could be considered as potential biological control agents because they are unique and host-specific. *Hyparrhenia hirta* has naturalised in the USA and many parts of Europe, but it is a particular problem in Australia, where it has become highly invasive (Chejara *et al.*, 2010). It has also been reported as a problematic weed in wheat fields in Pakistan (Hussain *et al.*, 2004). Similarly, *A. gayanus* has invaded the northern regions of Australia's tropical savannas (Rossiter-Rachor *et al.*, 2009). Invaded regions such as these could benefit from the *Tetramesa* biological control agents identified here. More intensive sampling and host-specificity testing would be required to gain further clarity on their suitability as control agents for these two invasive grasses.

2.6 Conclusion

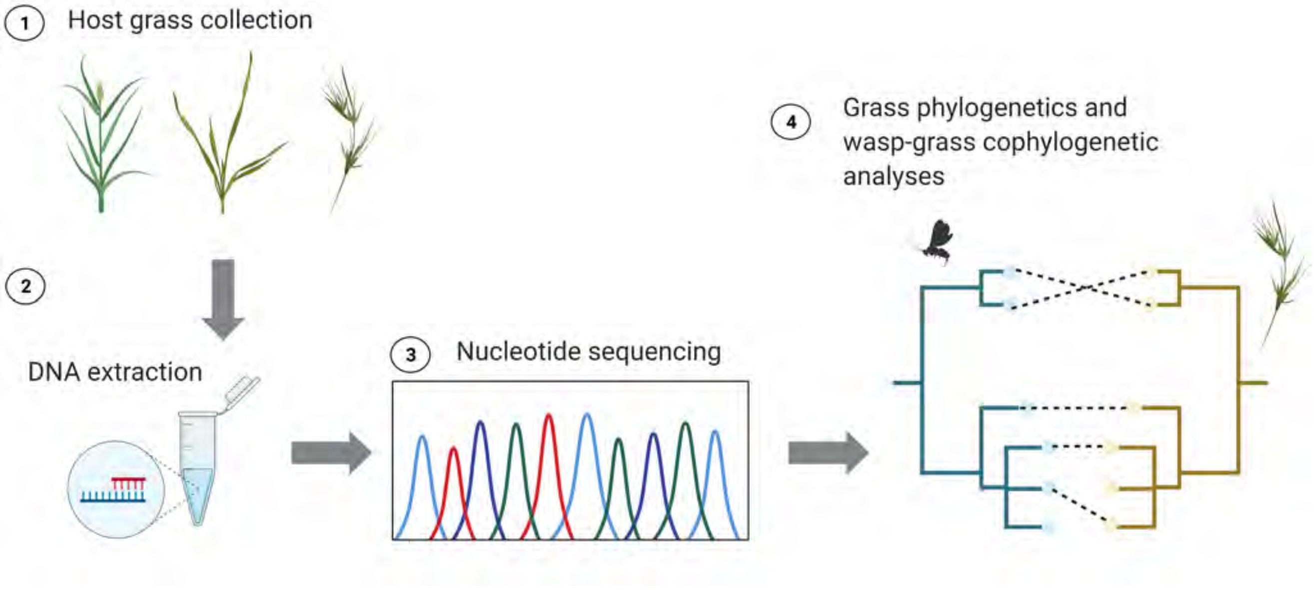
This chapter has presented the phylogenetic and species delimitation results of a nuclear (28S rRNA) and mitochondrial (COI) region for southern African eurytomids, focusing particularly on the *Tetramesa* genus. This is the first time that a comprehensive phylogenetic analysis has been applied to this group. The results presented here strongly support the need for a taxonomic revision of the *Tetramesa*, guided by evidence from nuclear sequence data, morphology, and ecological information (e.g. host-specificity, host plant genotype, biogeography).

The 28S region is recommended for reliable genus-level identification, where a divergence of 3 - 3.5% is suggested to determine which specimens are congeneric. For this nuclear region, all the PNS specimens fell within the proposed *Tetramesa* clade, while the only NPNS clades that formed part of the genus were those collected on *Andropogon gayanus* and *Hyparrhenia hirta*. Although there was evidence of mitonuclear discordance between the two gene trees, likely due to a combination of complex inheritance pathways and the recent diversification of the *Tetramesa* ($\sim$ 5 - 15 mya), the COI region is useful for assessing finer-scale relationships such as populations within host-specific taxonomic units (HSTUs). The COI region suggested that there were at least nine PNS *Tetramesa* HSTUs associated with *Eragrostis* ($n = 7$) and *Sporobolus* ($n = 2$) host grasses, and three NPNS HSTUs associated with *Andropogon gayanus* ($n = 1$) and *Hyparrhenia hirta* ($n = 2$). The Eastern Cape PNS *E. curvula* HSTUs showed a high degree of genetic structuring, suggesting that host plant genotype might be causing ecological barriers to gene flow, and driving host-associated differentiation (HAD) in these *Tetramesa* populations.

Recommendations for future research include: (1) incorporating multiple other nuclear regions into phylogenetic analyses to refine divergence thresholds for delimiting true *Tetramesa* from other eurytomid genera, (2) using nuclear phylogenetic results in conjunction with morphological and ecological data to revise the taxonomy of the group (with the possibility of creating subgenera), (3) conducting a population genetics analysis of South African *Eragrostis curvula* grasses across multiple provinces to determine whether genotypic differences may be driving genetic differentiation in their associated PNS *Tetramesa* herbivore assemblages, and (4) conducting host-specificity tests, impact assessments, and potential hybridisation trials with the different PNS *E. curvula* *Tetramesa* HSTUs to determine their efficacy as biological control agents.

Chapter 3

Cophylogenetic analyses



3.1 Introduction

Cophylogenetic studies analyse the associations and level of congruence between the phylogenies of interacting lineages, how they exert and respond to reciprocal selection pressures on each other, and whether these relationships indicate a significant degree of coevolution (Charleston and Libeskind-Hadas, 2014; Cruaud and Rasplus, 2016; Dismukes *et al.*, 2022). Cophylogenetic studies typically include interactions where at least one taxon has evolved non-independently of the other, such as in host-parasite, herbivore-plant, or host-pollinator relationships (Charleston and Perkins, 2006; Balbuena *et al.*, 2013) (see also Banks *et al.* (2006); Jousselin *et al.* (2008); Demastes *et al.* (2012); Doña *et al.* (2017); Nguyen *et al.* (2020)). Most cophylogenetic studies are asymmetrical in nature, where one taxon evolves at a slower rate than the other due to differences in generation times (Dismukes *et al.*, 2022). Examples of asymmetrical coevolution from the literature include studies of pocket gophers and penguins and their chewing lice (Banks *et al.*, 2006; Light and Hafner, 2007), chalcid wasps and their fig tree hosts (Jousselin *et al.*, 2008)), sunflower maggot flies and Asteraceae host species (Hippee *et al.*, 2021), platyhelminth parasites and their anuran hosts (Mulvey *et al.*, 2022), coral reef invertebrates and their endosymbionts (O'Brien *et al.*, 2021), and *Nosema* Nägeli microsporidia and bee hosts (Shafer *et al.*, 2009).

Other obligate mutualistic relationships between insects and endosymbiontic bacteria are well-studied systems for cophylogenetic analyses, where phylogenetic congruence has been shown, for example, in aphids and *Buchnera* Munson (Jousselin *et al.*, 2009), cockroaches and *Blattabacterium* Hollande and Favre (Garrick *et al.*, 2017), the Auchenorrhyncha and *Candidatus* Sulcia muelleri (Cao and Dietrich, 2022), and the Coccoidea and flavobacterial and enterobacterial endosymbionts (Rosenblueth *et al.*, 2012).

Cophylogenetic methods are either "global-fit" or "event-based" (Page, 2003; Dismukes *et al.*, 2022), where global-fit methods assess the overall congruence between two interacting phylogenetic trees, and event-based methods aim to assess how the processes of cospeciation, duplication, transfer (host-switching), and loss (failure to diverge and/or "missing the boat") have shaped evolutionary relationships (Charleston and Perkins, 2006; Charleston and Libeskind-Hadas, 2014) (Fig. 3.1). Duplication occurs when the herbivore or specialist natural enemy speciates but the host does not (possibly due to niche expansion). Duplication may include the process of host switching, where the herbivore adapts to a novel host. In some cases the herbivore may fail to diverge with the host, and either become a multi-host feeder that is able to exploit both diverging hosts while maintaining gene flow, or it may go extinct (possibly due to the host colonising a new habitat without the herbivore) (Charleston and Perkins, 2006).

Cophylogenetic methods can assist in understanding how host-specificity evolved and how it is maintained in herbivorous insect groups. For example, van Der Linden *et al.* (2021) showed how host plant chemical defence traits shaped the evolution of northwestern European butterflies (145 species across six families). The authors found that chemical defence profiles in host plants had a greater correlation with associated butterfly assemblages than genetic distance, suggesting that plant defensive chemistry was more of a driving factor in host-herbivore coevolution than a shared evolutionary history.

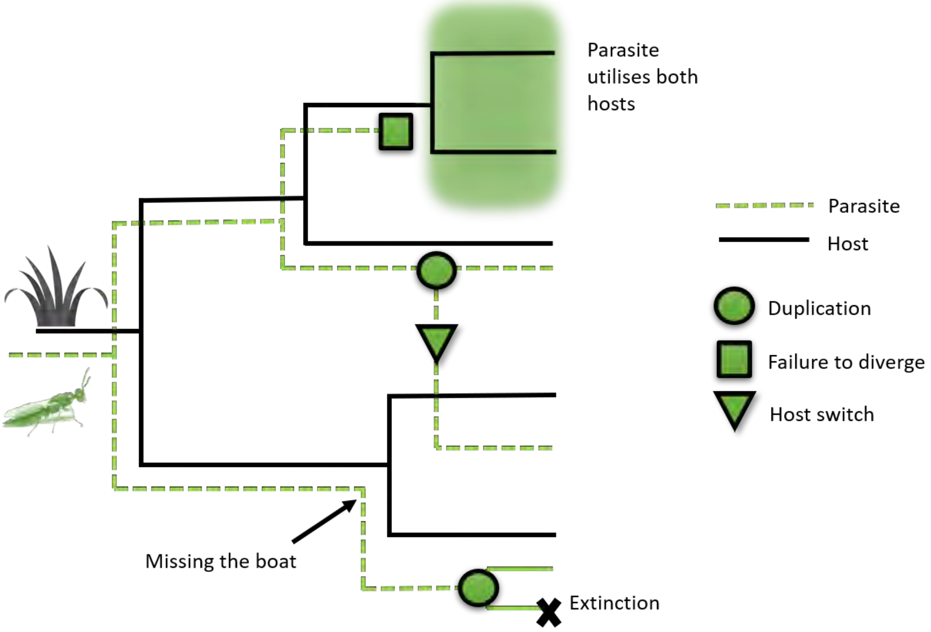


Figure 3.1: A hypothetical cophylogenetic scenario, showing the relationship between a herbivore (green dashed line) and host (black solid line) phylogeny. Adapted from Charleston and Perkins (2006).

In another example, Nyman *et al.* (2006) analysed the evolution of different feeding guilds, host-switching behaviour, and the host-specificity of herbivorous sawfly larvae (Hymenoptera: Tenthredinidae) (e.g. endophagous gallers and miners). Some of their key results suggested that (1) distinct endophagous feeding guilds have evolved independently multiple times from ectophagous ancestors, (2) that larvae are more likely to undergo a host shift than change their feeding behaviour/guild, (3) that host shifts are most likely to occur among plant taxa that are phylogenetically closely-related (congeneric and confamilial), but that sometimes a shift can occur to a more distant relative if the plant is an ecological analogue or has a broadly overlapping geographic distribution, (4) endophagous feeders display a higher degree of host-specificity, and (5) the speciation rates of endophages is likely to be slow on a host plant with a small distribution and low species diversity, while the converse is true on a speciose, widely-distributed plant.

Cophylogenetic analyses have rarely been applied in a biological control context, which is surprising considering the insights they can offer. To date, there are only three references to biological control which have focused on cophylogenetic relationships. These are firstly between the encyrtid wasp, *Anicetus* Howard, and their wax soft scale hosts, *Ceroplastes* Gray (Homoptera:

Coccidae) (Deng *et al.*, 2013). The parasitoids are often used as biological control agents of wax scales, as they are significant agricultural pests (e.g. on citrus and coffee) (Krull and Basedow, 2005). Secondly, between the Eucalyptus-feeding *Cardiaspina* and *Spondyliaspis* psyllids and a suite of their associated parasitoid wasps (Hall *et al.*, 2017), and thirdly between the entomopathogenic nematodes *Heterorhabditis* (Rhabditida) and their symbiotic bacteria *Photorhabdus* (Enterobacteriaceae) (Maneesakorn *et al.*, 2011). This symbiotic relationship has been exploited for the biological control of a number of soil-borne insect pests, for example the white grub sugarcane pest *Holotrichia serrata* Fab. (Coleoptera: Scarabaeidae) (Ehlers *et al.*, 2000).

Cophylogenetic tools can be very useful when host use has a strong phylogenetic signal because they can assist in predicting which taxa might be good biological control agents, identify which non-target taxa might be at risk, and support host-specificity data (see for example Hinz *et al.* (2008); Desneux *et al.* (2012); Kleinjan and Hoffmann (2013); Heimpel *et al.* (2021)). For example, cophylogenetic analyses could clarify some of the relationships between morphologically-identical cochineal lineages (*Dactylopius* spp.) and their cactus hosts, and can help in explaining the observed host preferences in these insects (see for example Hoffmann *et al.* (2002); Mathenge *et al.* (2015)). Other study systems could focus on the the diverse species assemblages of *Dasinuera* Rondani (Diptera: Cecidomyiidae) midges (Kolesik *et al.*, 2005) and *Melanterius* Erichson (Coleoptera: Curculionidae) weevils associated with the alien invasive Australian *Acacias* in South Africa (Impson and Hoffmann, 2019).

The aim of this chapter was to investigate the cophylogenetic relationships between potential *Tetramesa* biological control agents and their hosts, focusing particularly on a comprehensive coverage of the *Eragrostis* and *Sporobolus* genera. This is important in deciding which non-target grasses should be included in host-specificity testing for biological control programmes targeting invasive grasses. The chapter particularly focused on the host-specificity of *Tetramesa* sp. 4; the PNS wasp found on *E. curvula.* Evidence of non-target feeding would limit or preclude its use as a biological control agent on this grass.

3.2 Materials and Methods

3.2.1 Sample collection and sourcing

The *Eragrostis* and *Sporobolus* genera were prioritised because they are two of the main targets of biological control, particularly in Australia, with a particular focus on *E. curvula* and *S. pyramidalis*. Grasses were collected from the Free State, KwaZulu-Natal, Eastern Cape, Gauteng, and Limpopo provinces (Table S3), and preserved in sealed bags with silica gel beads.

All of the *Eragrostis* sequences (n = 701) from the four gene regions in Barrett *et al.* (2020) were downloaded from GenBank (`https://www.ncbi.nlm.nih.gov/genbank/`), and included in the multiple sequence alignment files in the present study; namely rps16-trnK (MK872656 - MK872800), rps16 (MK872570 - MK872655), rpl32-trnL (MK872402 - MK872569), and ITS (MK863080 - MK863244). The additional GenBank sequences listed in table S1 of Barrett *et al.* (2020) were also included in the alignment.

Sporobolus sequences (n = 349) for the same four genes were gleaned from Peterson *et al.* (2014). Previous phylogenetic analyses of *Sporobolus* species have shown varying topologies depending on the genetic marker used (Fig. 3.2). This chapter has used the nuclear and chloroplast sequence data from Peterson *et al.* (2014), as this is the most recent and thorough work carried out on the genus.

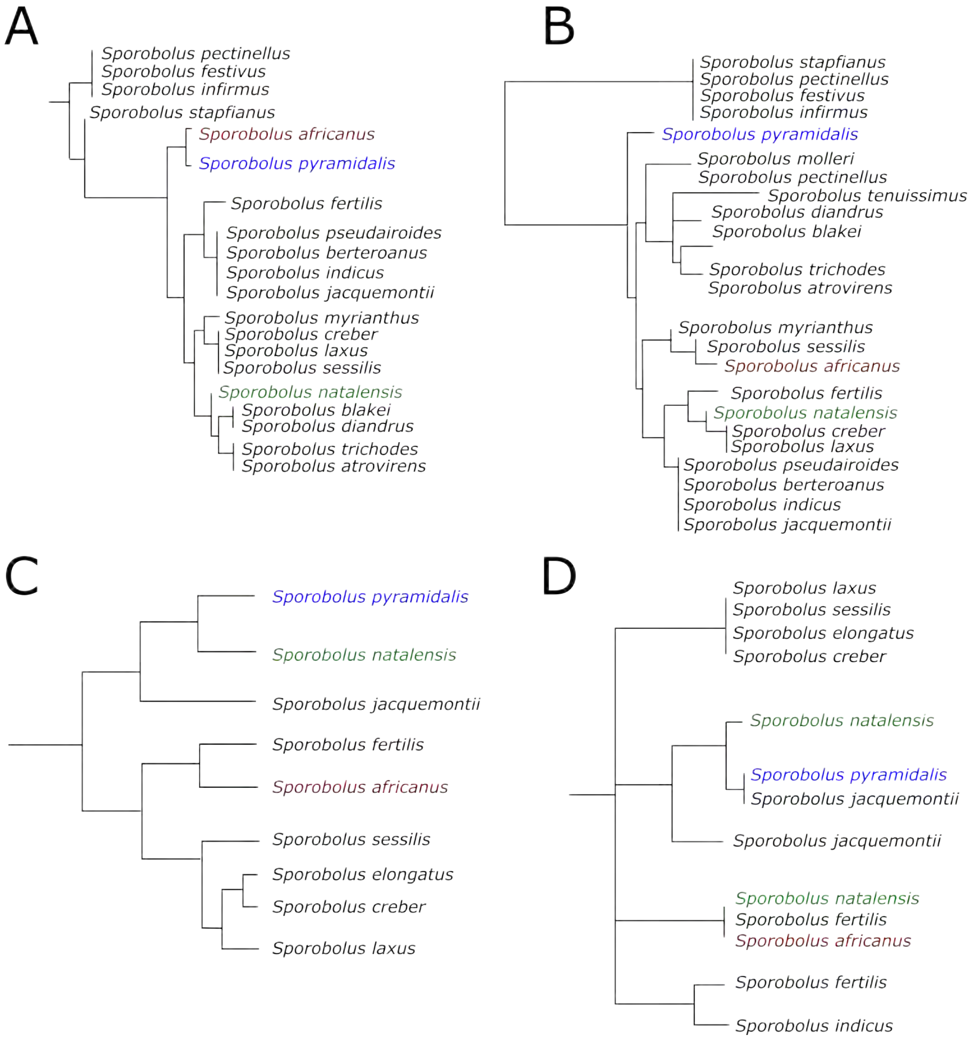


Figure 3.2: Summary phylogenetic relationships for selected *Sporobolus* species, particularly those closely related to *S. pyramidalis*. (A) nuclear ITS gene (Peterson *et al.*, 2014), (B) Chloroplast genes rpl32-trnL + rps16-trnK + rps16 (Peterson *et al.*, 2014), (C) RAPD markers (Shrestha *et al.*, 2005), and (D) ITS gene region (Shrestha *et al.*, 2003).

3.2.2 Grass DNA extraction

All grass samples were extracted using a PureLink^{TM} Genomic Plant DNA Purification Kit (ThermoFisher Scientific, catalogue number K183001). Samples were crushed in liquid nitrogen, and extracted according to the manufacturer's protocols. DNA extracts were eluted in a 150 μL Genomic Elution Buffer volume supplied with the kit, and stored at -20°C. The concentration and purity of DNA extracts were recorded using a Thermo Scientific NanoDrop 2000^{TM} spectrophotometer.

Grass Polymerase Chain Reaction

Each PCR reaction was carried out in a 25 μL volume, comprising PCR master mix (1x iTaq^{TM} Universal SYBR Green Supermix, Bio-Rad, catalogue number 1725121), 0.2 μM forward and reverse primer, PCR-grade water, and template DNA (5 - 20 ng/μL). The PCR protocol for the rpl32-trnL spacer, rps16-trnK spacer, and rps16 intron regions was as follows: 3 min denaturation at 95°C, followed by 35 cycles of 40 s at 94°C / 40 s at Ta° / 1 min 40 s at 72°C / and a final extension of 10 min at 72°C; where $\text{Ta}^{\circ}_{\text{rpl32-trnL}} = 53^{\circ}\text{C}$, $\text{Ta}^{\circ}_{\text{rps16-trnK}} = 52^{\circ}\text{C}$, and $\text{Ta}^{\circ}_{\text{rps16}} = 52^{\circ}\text{C}$. The protocol for the ITS region entailed: 4 min denaturation at 95°C, followed by 35 cycles of 30 s at 94°C / 30 s at 52°C / 1 min 30 s at 72°C / and a final extension of 10 min at 72°C.

Table 3.1: The primer pairs used to amplify target plant gene regions.

Region	**Name**	**Sequence (5'→ 3')**	**Reference**
rpl32-trnL	trnL(UAG)	CCTGGTTCTTTRATTGGTAATGATC	Shaw *et al.* (2007)
	rpL32-F	TAAACTTCTGGATGTCCAAAAA	
rps16-trnK	rpS16–900F	GGAGGATTTGGAAATTGATTAGTTCC	Peterson *et al.* (2010)
	3914PR	CCCGGTAAAATTAAAATATAAACTTC	
rps16 intron	rpS16F	ATTCCCGGACCACGCCTGGCTGA	Peterson *et al.* (2010)
	rpS16R	TCCTCCGCTTATTGATATGC	
ITS	ITS5a	ATTCCCGGACCACGCCTGGCTGA	Stanford *et al.* (2000)
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)

3.2.3 Cophylogenetic analyses

Separate chloroplast (rpl32-trnL + rps16-trnK + rps16) and nuclear (ITS) maximum likelihood phylogenies were generated using all the grass sequence data. This was carried out using IQ-TREE2 and MODELFINDER (see the references in Chapter 2 Section 2.2.6), where default parameters were applied in IQ-TREE, with 1000 ultrafast bootstrap repetitions. The optimal evolutionary BIC models were TPM3 + F + Γ for rpl32-trnL, TIM3 + F + Γ for rps-trnK, TIM + F + Γ for rps16, and SYM + I + Γ for ITS. Genetic-distance plots (uncorrected distances obtained in MEGA-11 (Kumar *et al.*, 2018) using the K2P model, 500 bootstrap repeats) were generated for all the *Eragrostis* species in the same clade as, and sister to, *E. curvula.*

The eurytomid COI sequences from Chapter 2 Section 2.2.6 were used for the cophylogenetic plots and tests. The COI tree was chosen because the target *Eragrostis* wasp clades were more resolved than in the 28S tree, and were thus more informative for uncovering host-use patterns. The `cophylo()` function in the `phytools` v1.0-3 R package (Revell, 2012) was utilised to generate cophylogenetic plots, and a global-fit cophylogenetic analysis was conducted using the `PACo()` function in the `paco` v0.4.2 R package (Hutchinson *et al.*, 2017). The PACo method (Procrustean Approach to Cophylogeny) uses the Procrustean superimposition tool to test the dependency of one phylogeny on another; typically the degree to which a group of organisms (e.g. parasites, herbivores, symbionts) track the evolution of their hosts (Hutchinson *et al.*, 2017). ParaFit (Legendre *et al.*, 2002) is one of the most commonly used cophylogenetic methods, but PACo outperforms it in both Type I error rates and statistical power (Balbuena *et al.*, 2013). For this reason, PACo was the preferred method in the present work.

The PACo analysis was prepared and run on reduced ML phylogenetic trees comprising representative COI sequences for each *Tetramesa* group (n = 18) and their associated grass species (n = 9). Representative 28S sequences were not used as they did not produce a phylogeny that was informative enough for the analysis (Fig. S4). The following parameters were applied: Cailliez correction, 1000 permutations, symmetric = false, and method = “r1”. The symmetric argument was set to false because it was assumed that the host grasses are driving eurytomid radiation, rather than a reciprocal relationship. The “r1” method was selected, as this null model algorithm treats the eurytomids as tracking the host grasses while also considering that host-specificity can influence cophylogenetic signal strength (Revell, 2012). Residuals for the pronotal and non-pronotal spot *Tetramesa* on *Eragrostis* hosts were compared using a one-tailed t-test to test whether the PNS samples had significantly lower residuals, and hence greater cophylogenetic congruence with their hosts relative to the NPNS groups.

An event-based cophylogenetic analysis using the Duplication-Transfer-Loss (DTL) model was performed in eMPRess software (Santichaivekin *et al.*, 2020), which is a replacement of its forerunner, Jane (Conow *et al.*, 2010). Event-based analyses allow for the assignment of cost scores to coevolutionary processes (Charleston and Libeskind-Hadas, 2014). Since eMPRess cannot run with missing associations, a reduced eurytomid COI and grass chloroplast dataset was generated so that every tip in the eurytomid tree had a matching host representative. The default cost-scheme was applied, where cospeciation = 0, duplication = 1, duplication with host switch = 2, loss = 1, and failure to diverge = 1 (Santichaivekin *et al.*, 2020).

Genetic distances

Uncorrected genetic p-distances (K2P, 1000 bootstrap repeats) were obtained in MEGA-11 (Kumar *et al.*, 2018) for all the *Eragrostis* species in Figure 3.5 A, relative to *E. curvula*. This was a useful way of comparing the data from field host range and no-choice host-specificity tests of *Tetramesa sp. 4* to host grass relatedness in order to estimate potential risk to native Australian grasses (Table 3.2).

Table 3.2: Summary of the field host range and no-choice host specificity tests of *Tetramesa sp. 4* (Guy Sutton unpublished data).

Host grass	Field host range	No-choice host-specificity tests
E. biflora	x	
E. plana	x	x
E. planiculmis		x
E. trichophora	x	

3.3 Results

3.3.1 Phylogenetic placements

Broad-scale patterns of host-use showed that the PNS eurytomids were associated with grasses in restricted clades, while the NPNS groups covered a much wider spectrum across the grass phylogeny (Fig. 3.3 and 3.4). The PNS *E. curvula* and *Sporobolus* wasps were restricted to grass clades E1 - E3 and S1 - S3, respectively. The ITS tree was generally more resolved than the chloroplast tree, with higher support values. In both the ITS (Log-likelihood = -40561.91) and chloroplast (Log-likelihood = -21463.33) phylogenies, (1) *E. plana* and *E. lehmanniana* grouped together in a clade and were sister to *E. tenuifolia*, and (2) *E. curvula* grouped in an all-African clade (Fig. 3.5 A and B). *Eragrostis planiculmis* formed a well-defined clade in both trees, but native Australian *E. trachycarpa* grouped closely with it in the ITS tree (Fig. 3.5 A). *Eragrostis trachycarpa* grouped instead with *E. parviflora* in the chloroplast tree, and formed a sister group to *E. planiculmis*.

Non-target *Eragrostis* grasses that fell within African clades included three Australian grasses (*E. parviflora*, *E. leptocarpa*, *E. trachycarpa*), two South American (*E. articulata*, *E. neesii*), one North and South American (*E. pectinacea*), and two widespread species (*E. cilianensis*, *E. pilosa*). African *E. tef*, *E. homomalla*, *E. rotifer*, and *E. planiculmis* in particular showed close relationships to non-native grasses (Fig. 3.5 A and B).

The genetic distances of all the *Eragrostis* species in Figure 3.5, relative to *E. curvula*, are shown in Figure 3.6. This K2P p-distance plot indicated, based on phylogeny, that all the species presented are potential hosts for the PNS *E. curvula Tetramesa sp. 4* wasp, since host-specificity tests have shown that the wasp can complete its lifecycle on both *E. plana* and *E. planiculmis*.

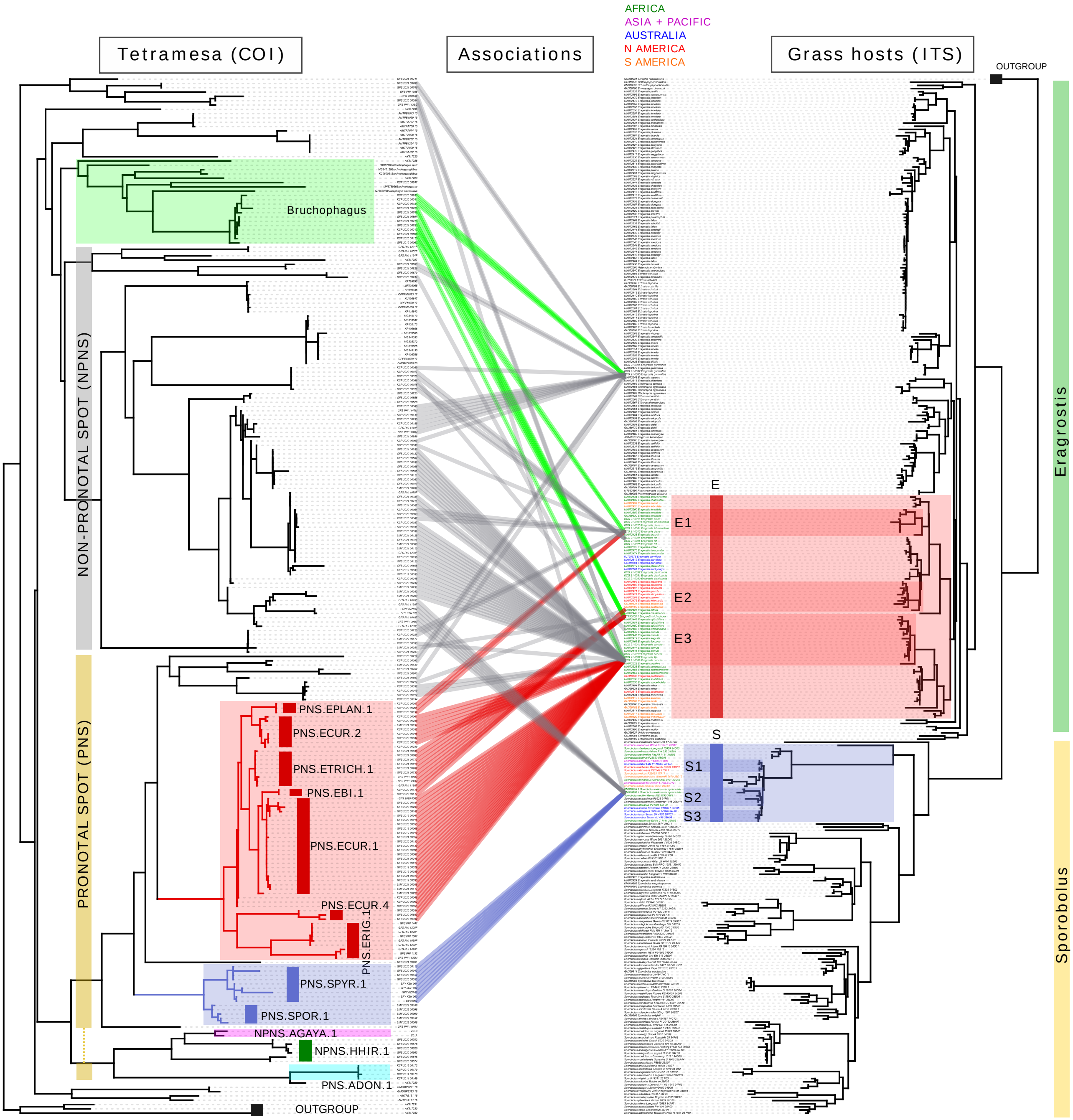


Figure 3.3: Maximum Likelihood phylogenies for the Eurytomidae (left; COI) and their host grasses (right; ITS). The coloured lines linking sequences represent herbivore-host associations. The clades marked "E" and "S" in the grass phylogeny are the clades that the pronotal spot *Eragrostis* and *Sporobolus* wasps might be able to utilise and should be prioritised for host-specificity testing. The text colour of the grass sequences of interest represent their native ranges (sourced from the Kew Plants of the World Online database: `https://powo.science.kew.org/results?`); green = Africa, purple = Asia and the Pacific, blue = Australia, red = North America, and orange = South America.

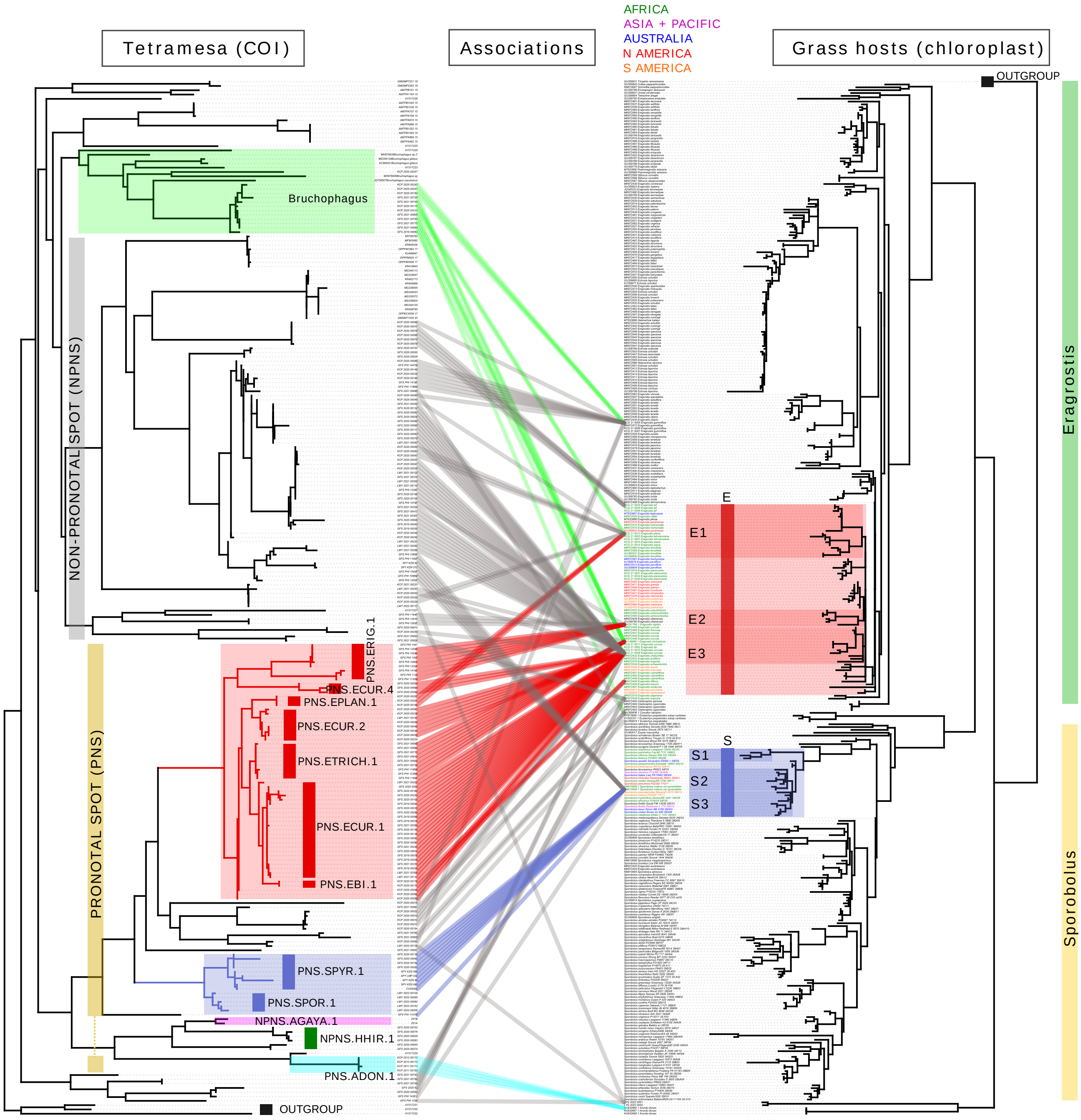


Figure 3.4: Maximum Likelihood phylogenies for the Eurytomidae (left; COI) and their host grasses (right; chloroplast rpl32-trnL, rps16-trnK, and rps16). The coloured lines linking sequences represent herbivore-host associations. The clades marked “E” and “S” in the grass phylogeny are the clades that the pronotal spot *Eragrostis* and *Sporobolus* wasps might be able to utilise and should be prioritised for host-specificity testing. The text colour of the grass sequences of interest represent their native ranges (sourced from the Kew Plants of the World Online database: https://powo.science.kew.org/results?); green = Africa, purple = Asia and the Pacific, blue = Australia, red = North America, orange = South America, black = unknown, widespread or cosmopolitan.

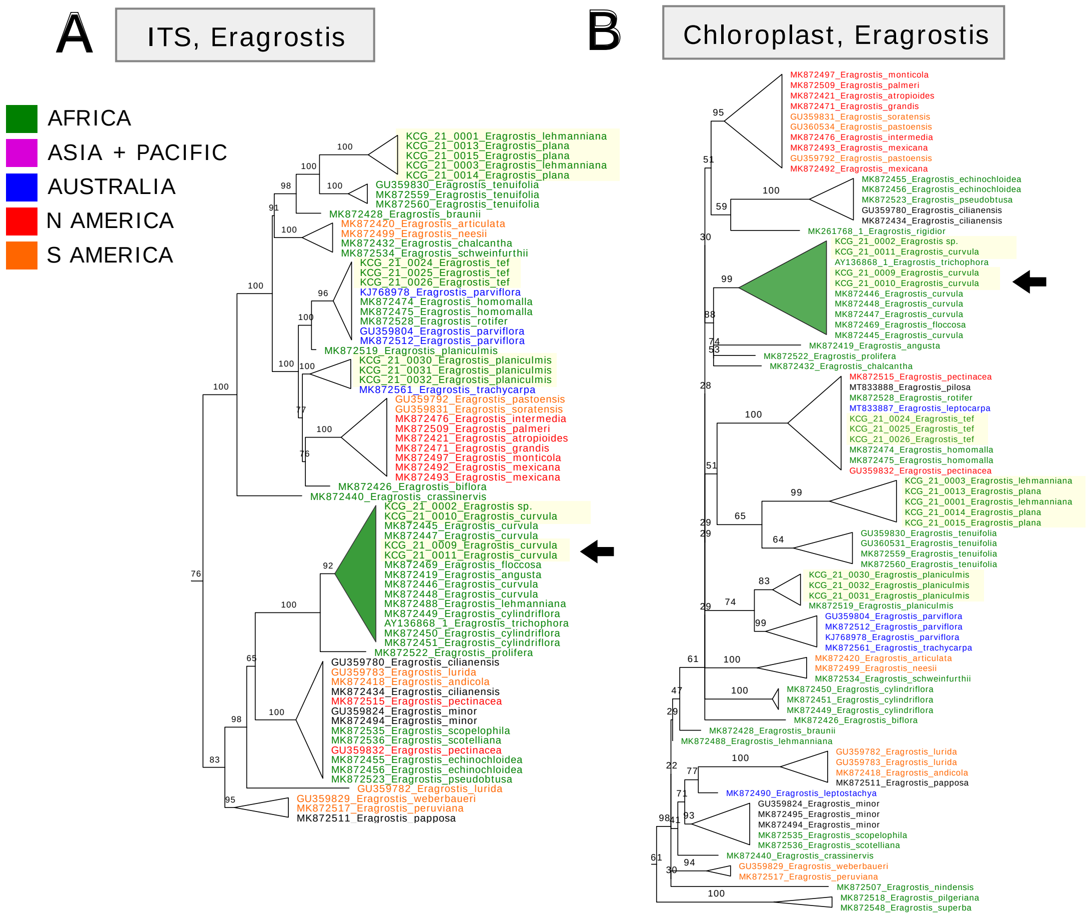


Figure 3.5: Extracts of the *Eragrostis* clades of interest from Fig. 3.4 (the sections marked "E"). (A) *Eragrostis* ITS, (B) *Eragrostis* chloroplast gene trees. The yellow highlights show the grass specimens included from the present study, and the arrows and green triangles indicate the position of target *E. curvula*. Specimen names are coloured by their native range, as indicated in the key. Values above the branches are bootstrap support values.

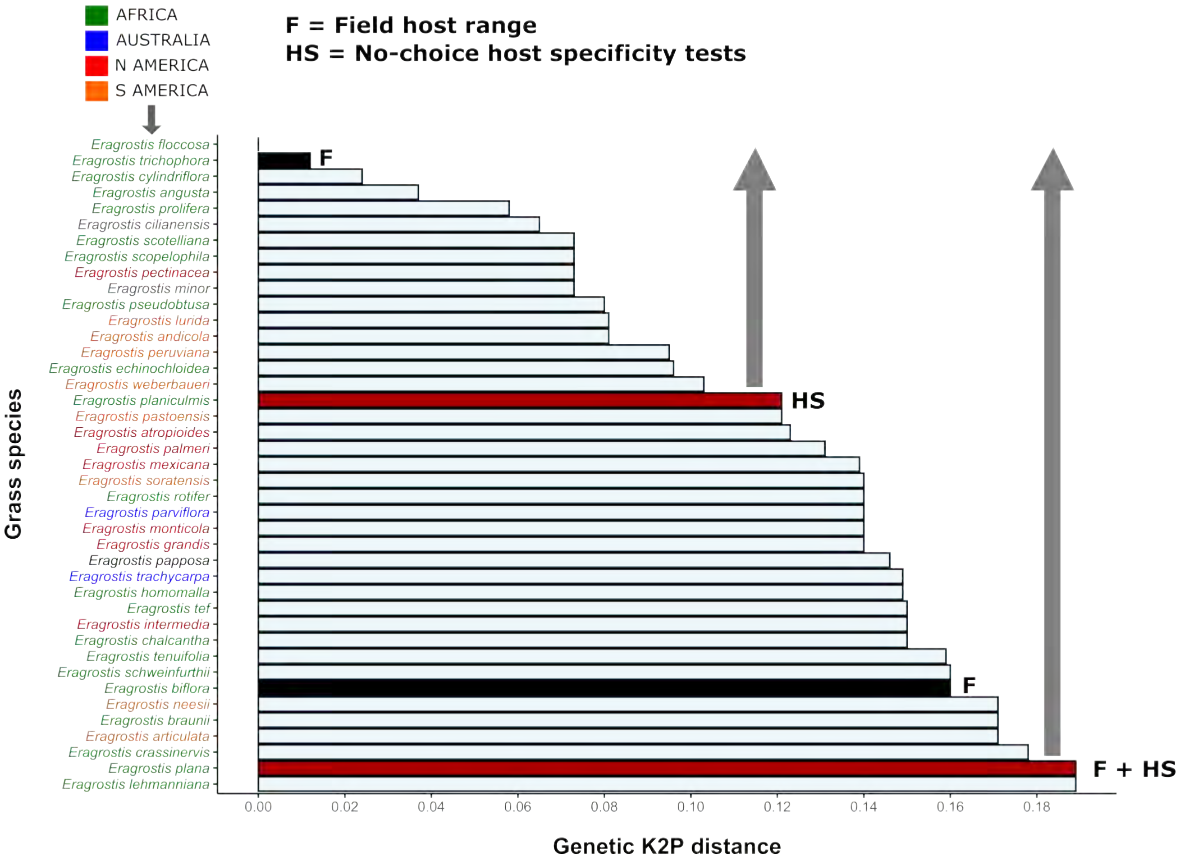


Figure 3.6: Genetic ITS p-distances (K2P, 1000 bootstrap repeats) for all the *Eragrostis* species shown in Figure 3.5 (A) relative to *Eragrostis curvula*. Species names are coloured according to their native range. The black and red bars indicate additional host plants that *Tetramesa sp. 4* can utilise based on field range observations and no-choice host-specificity testing. The arrows indicate that *Tetramesa sp. 4* may be able to utilise all the hosts above *E. plana* and *E. planiculmis*.

3.3.2 Cophylogenetic relationships

The PACo analysis returned a significant cophylogenetic signal between the *Tetramesa* and their associated host grasses (M^2_{XY} = 0.008, p = 0.048, n = 1000) (Fig. 3.7), where the PNS *E. curvula* wasps in particular showed significantly greater phylogenetic congruence relative to the NPNS groups (p = 0.032, t = 6.49, df = 1.26) (Fig. 3.8 and Fig. 3.9). In this sense, phylogenetic congruence is synonomous with evolutionary dependence, and means that the wasp's phylogenetic relationships have tracked that of its host grasses such that their topologies are congruent. The residuals of the 1000 simulated random network interactions and the observed network overlapped, such that none of the random instances showed greater phylogenetic congruence. This means that the null model of no phylogenetic congruence did not provide a better fit for the data, and could be refuted.

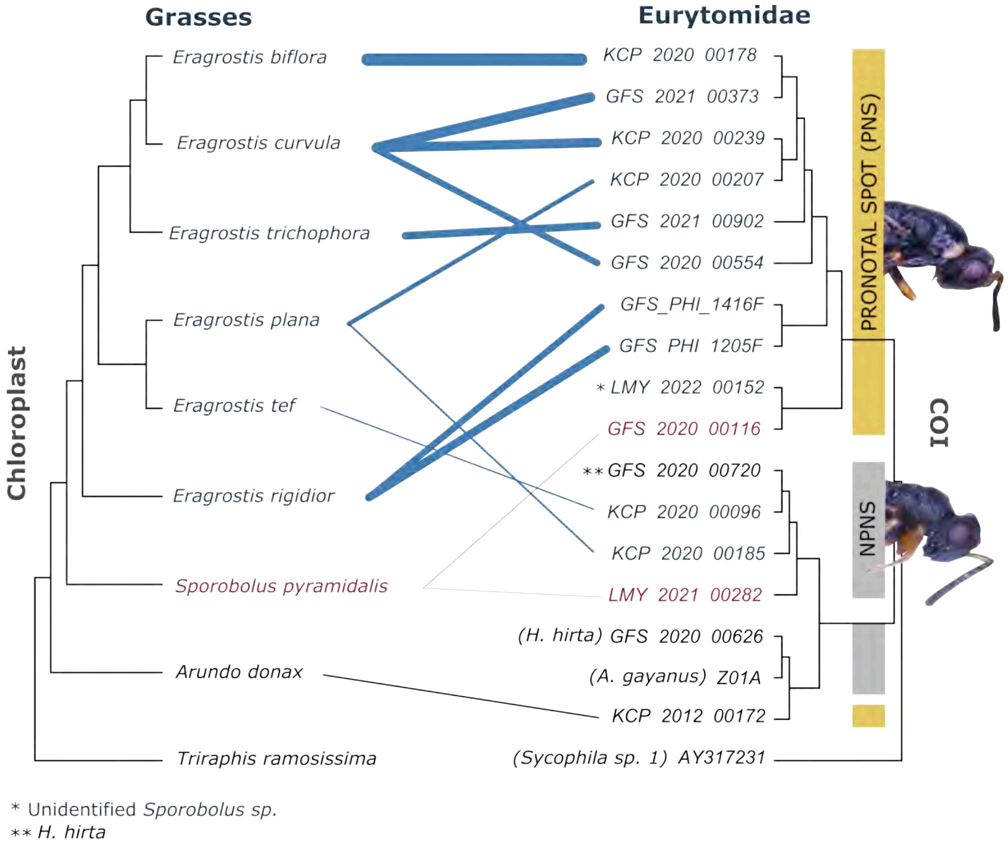


Figure 3.7: A cophylogenetic plot (grass chloroplast on the left (rpl32-trnL, rps16-trnK, and rps16), eurytomid COI phylogeny on the right) resulting from the R `PACo` analysis, where the thickness of the lines (thicker lines = smaller residuals) represent the relative strength of the association derived from residuals, where the thicker lines denote stronger support for phylogenetic congruence. The thickest line between *E. biflora* and its wasp represents a residual value of 0.0095, while the thinnest line between *Sporobolus pyramidalis* and its wasp represents a residual value of 0.052.

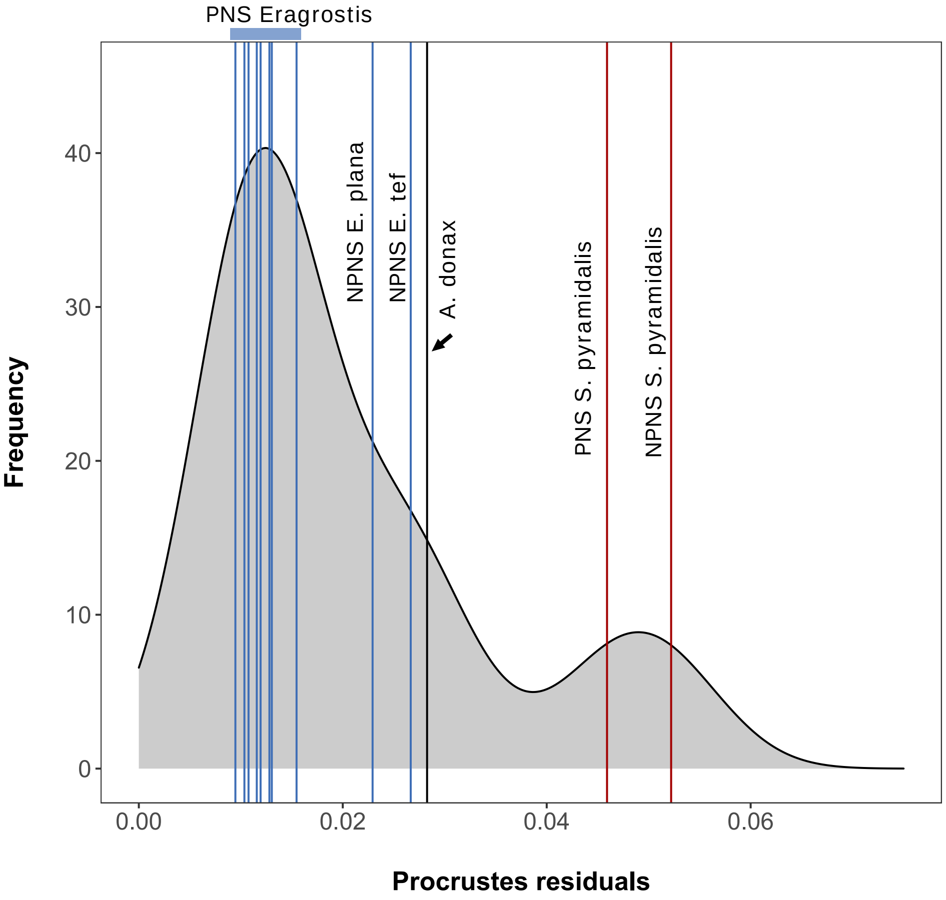


Figure 3.8: Residual plot for the cophylogenetic interactions shown in Figure 3.7. The smaller the residual value, the greater the support for phylogenetic congruence. The blue lines = *Eragrostis* wasps, the black line = *Tetramesa romana* on *Arundo donax*, and the red lines = *Sporobolus pyramidalis* wasps. PNS = pronotal spot, and NPNS = non-pronotal spot.

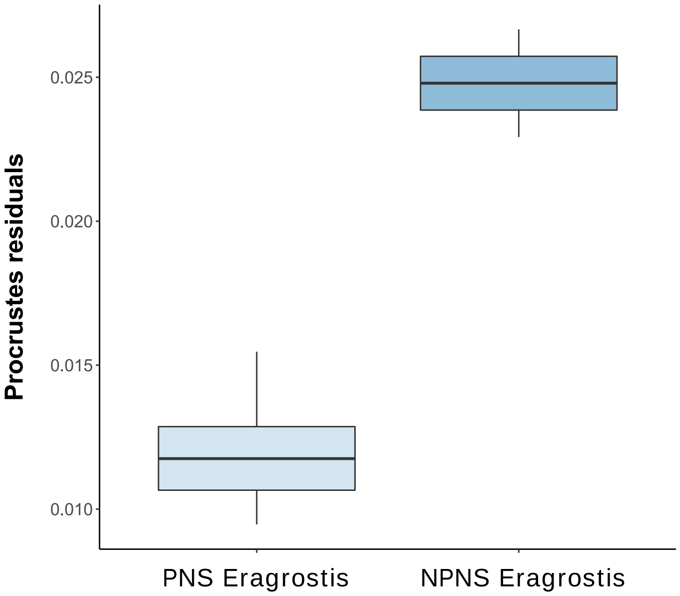


Figure 3.9: A residual box plot for the PNS and NPNS eurytomid interactions with *Eragrostis* hosts. The greater residual values in the NPNS group indicates lower support for phylogenetic congruence.

3.3.3 Event-based cophylogenetics

The eMPRess results showed a total of 7 cospeciations, 2 duplications, 3 transfers (host switches), and 4 losses (Fig. 3.10). The null hypothesis was refuted (i.e. that host grass and eurytomid herbivore tips were associated due to chance), with significant support that the grass-wasp pairs have coevolved (p = 0.0099) (Fig. 3.11). Only two of the three host switches were well-supported (probability = 75 for both), and were PNS *Tetramesa* from (1) *E. rigidior* to *E. curvula*, and (2) from *E. plana* to *E. curvula* (Fig. 3.10). There was only one duplication event on the terminal branches, which was recorded for PNS *Tetramesa* on *E. rigidior* (probability = 100). This means that the two *Tetramesa* wasp lineages diverged on the same host, which may be due to cycles of geographic isolation and re-contact (Banks *et al.*, 2006).

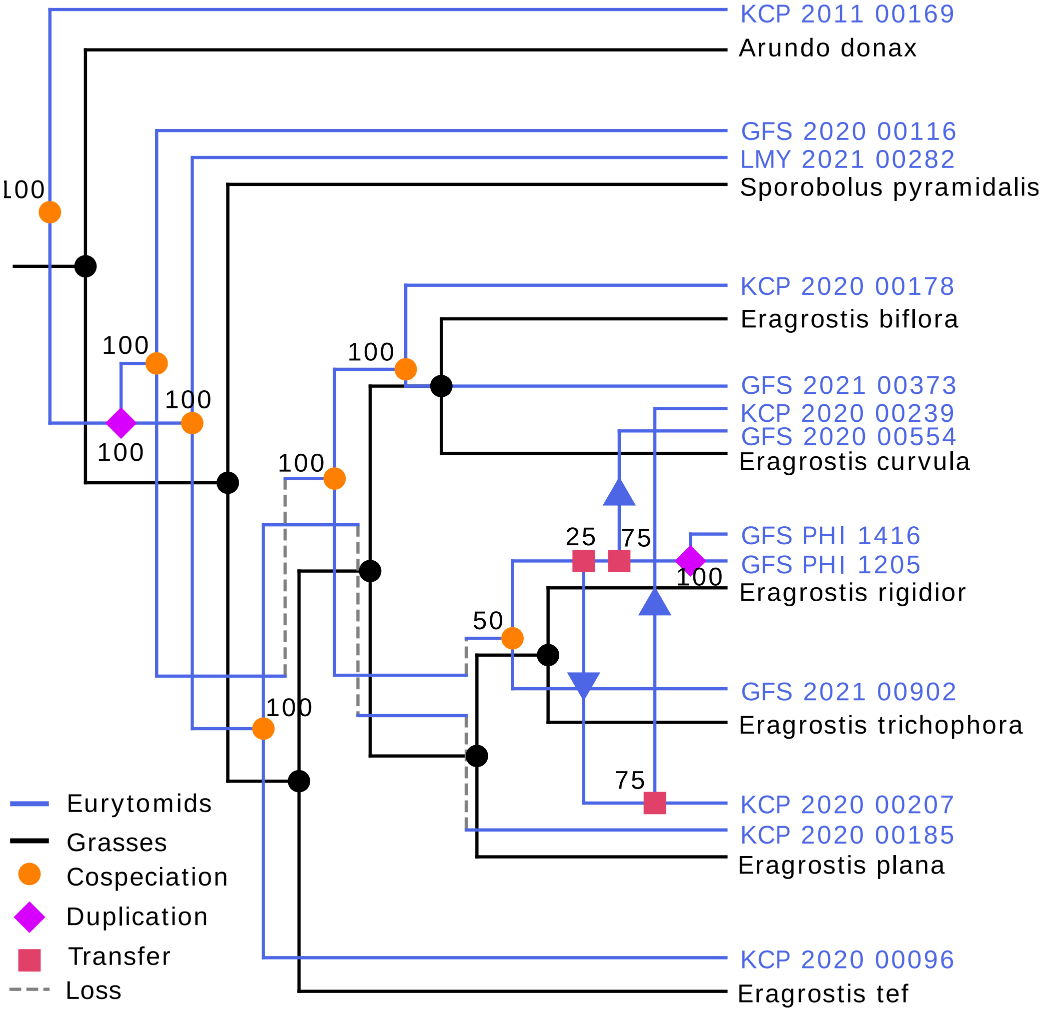


Figure 3.10: A Maximum Parsimony Reconciliation (MPR) phylogeny from eMPRess, showing the mapping of the eurytomid phylogeny into the grass phylogeny. Values shown at event-nodes indicate the probability of that event occurring.

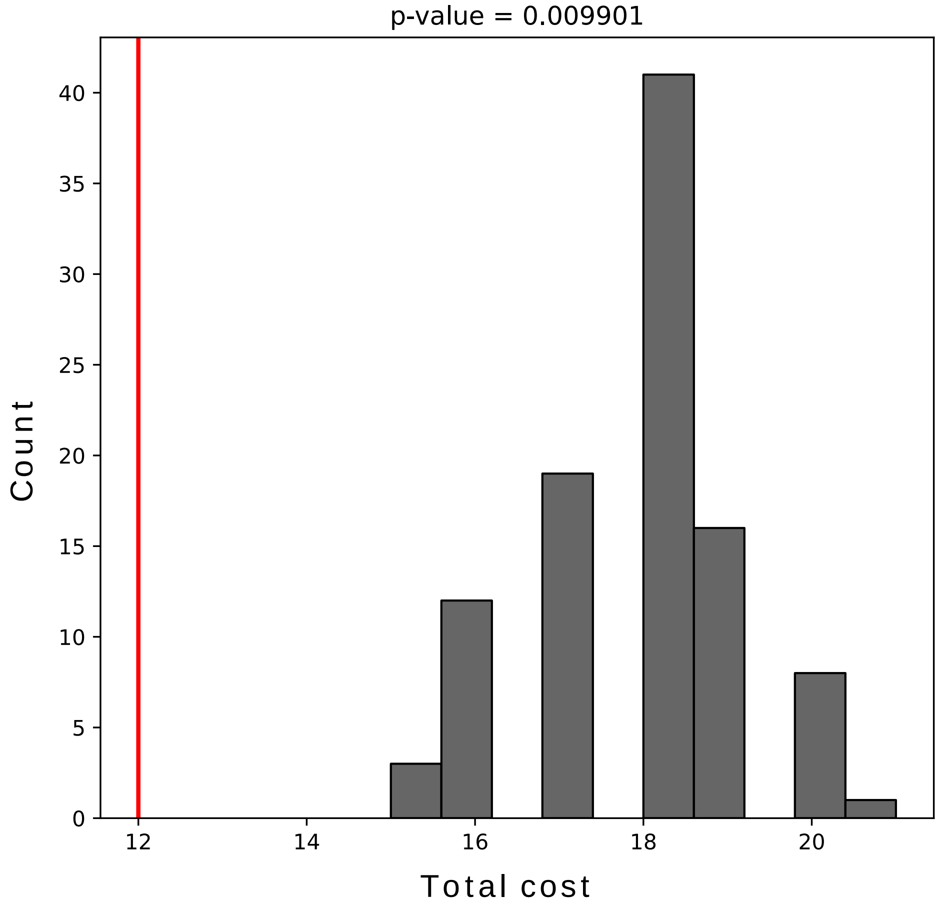


Figure 3.11: A p-value histogram for the grass-eurytomid relationships, where the red line indicates the optimal reconciliation cost of the coevolution trees, and the grey columns represent the optimal cost of the same trees constructed with tip associations permuted at random 1000 times.

3.4 Discussion

This chapter has used cophylogenetic methods to investigate evolutionary patterns between southern African eurytomids and their host grasses, focusing particularly on the PNS *Tetramesa* associated with *Eragrostis* and *Sporobolus* host species. The results have shown that (1) there are clear phylogenetic patterns in host-use, where the PNS *Tetramesa* are restricted to particular grass clades and display a higher degree of host-specificity than the other related eurytomids, (2) there is a strong cophylogenetic relationship between the PNS *Tetramesa* and their host grasses, as evidenced by this detailed study of those *Tetramesa* that utilise hosts within *Eragrostis*, (3) based on phylogenetic distance, the PNS *Tetramesa* on *E. curvula* (*Tetramesa* sp. 4) may be able to utilise numerous non-target *Eragrostis* species, which would preclude its use as a control agent, and (4) there is evidence to suggest host-switches from the geographically-restricted *E. plana* and *E. rigidior* onto the more widespread *E. curvula*. Based on this work, biological control practitioners are advised to prioritise PNS *Tetramesa* and their close sister groups (e.g. *Andropogon gayanus* and *Hyparrhenia hirta* wasps) when prospecting for potential grass agents. It is very likely that there are numerous other potential biological control agents, but the *Tetramesa* clade identified in this thesis appears to be the most likely group to find host-specific candidates.

Linking phylogenetic signal to a herbivore's potential host range underpins host-specificity test design in contemporary biological control programmes (Wapshere, 1974; Briese, 2003, 2005; Briese and Walker, 2008). Briese (2003) first suggested that the centrifugal phylogenetic method (WCFM) proposed by Wapshere (1974) was largely based on taxonomic circumscription (i.e Linnean taxonomic classification based on similarity) rather than on phylogenetic distance. Briese (2003) further warned that Linnean taxonomic rankings are not always indicative of evolutionary history (e.g. undetected paraphyly, polyphyly, and multiple monophyletic lineages/cryptic groups), which may have important implications for potential biological control agents with narrow host ranges. Molecular work on the *Acacia* genus, for example, has shown that the group is paraphyletic and was subsequently split into seven genera. There are a number of invasive South African "Acacia" species in Australia (e.g. *Vachellia xanthophloea* (Benth.) P. J. H. Hurter), and vice-versa (e.g. *A. mearnsii* De Wild), making the taxonomy of the group very important to understand for biological control initiatives. Most Australian species are more closely related to the mimosoid Ingeae tribe than to African species (Murphy, 2008; Bouchenak-Khelladi *et al.*, 2010; Taylor and Dhileepan, 2019). The taxonomic revisions resulting from the molecular work on the Acacia group have led to updated and more concise host test lists, for example those targeting prickly pear acacia, *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb., which was previously classified as *Acacia nilotica* (L.) Willd. ex Delile (Taylor and Dhileepan, 2019). Linking this back to the present work, there are still a few *Eragrostis* clades that have not been fully resolved yet (Barrett *et al.*, 2020), and so it is important that biological control practitioners keep abreast of phylogenetic revisions in order to design the most efficient and accurate host-specificity test lists.

The patterns of host-use and strong cophylogenetic signal within the PNS *Tetramesa* suggests a high level of host-specificity within the *Eragrostis* genus. This contradicts historical perceptions of grasses not supporting host-specific herbivore assemblages (Wapshere, 1990; Pemberton, 2002), and suggests that biological control programmes targeting invasive grass species hold promise. These findings also confirm the sentiments of Sutton *et al.* (2019). In context of the present phylogenetic results, however, it is unlikely that the PNS *Tetramesa* on *E. curvula* (*Tetramesa sp. 4*) is a suitable biological control agent for the grass. This is because the wasp is able to complete its lifecycle on at least two other native congeneric African grasses (*E. plana* and *E. planiculmis*), which have larger genetic distances to *E. curvula* than to a number of other non-target grass species native to Australia and North and South America. These included native Australian *E. scotelliana*, *E. parviflora*, *E. papposa*, and *E. trachycarpa* species.

An important caveat to predicting host-use using phylogenetic distance is that there may be a suite of other factors influencing plant-herbivore interactions, such as chemical and structural cues (McNaughton *et al.*, 1985; War *et al.*, 2012; Gershenzon and Ullah, 2022). In grasses, structural defences are typically in the form of silica phytolith depositions, trichomes, and high proportions of cellulose and lignin (Moore and Johnson, 2017). Chemical defences in grasses were believed to be poorly developed and ineffective (Redak, 1987), but later investigations revealed the presence of a wide variety of secondary metabolites including alkaloids, cyanogentic glycosides, benzoxazinones, phenolics, tannins, proteinase inhibitors, and terpenes (Redak, 1987). Some grasses release volatile organic compounds (VOCs) to attract natural enemies of herbivores, such as in tall fescue (*Festuca arundinacea* Schreb.) and Kentucky bluegrass (*Poa pratensis* L.), where these grasses attracted two species of tiphiid wasps (*Tiphia vernalis* Rohwer and *Tiphia popilliavora* Rohwer) that parasitise the invasive scarabid Japanese beetle, *Popillia japonica* Newman, and oriental beetle, *Anomala orientalis* Waterhouse (Obeysekara *et al.*, 2014). Some studies have found that symbiotic fungi produce alkaloids to protect their host grasses, for example *Neotyphodium uncinatum* Gams and *N. siegelii* fungi that produce loline alkaloids in fescue meadow grass (*Lolium pratense* Huds.) (Zhang *et al.*, 2009). These additional factors need to be considered before an agent is rejected by completing host-range testing on non-target grasses that are genetically closely related to the target weed. It might also be important that host range testing includes a variety of "biotypes" of the host plant, as this could influence insect performance. The apparent greater degree of oligophagy in the *E. curvula* PNS *Tetramesa* relative to some of the other PNS *Tetramesa* groups might be because the *Eragrostis* genus is a very difficult target compared to many other grasses because of how many close and structurally-similar relatives of the target species are present throughout the world. Many other grasses are more unique and have less closely-related species, making them better targets for biological control. It may also be that the wasp group comprises a suite of genetically-distinct populations with varying degrees of host-specificity (discussed in Chapter 2 with reference to host-associated differentiation (HAD)). These populations may have co-evolved with different *E. curvula* host forms, as phenological differences in host plants can result in the temporal isolation of herbivore populations, leading to reduced gene flow and ultimately sympatric speciation (Zhang *et al.*, 2019a). The HAD hypothesis would require further population-level investigations using methods such as fragment analysis or single nucleotide polymorphisms (SNPs). It is also possible that the wasp group is under strong selective pressure to expand its host range in favour of more widely-distributed hosts to ensure a constant and reliable food source, or it may be a predator-avoidance strategy. These ideas will be discussed further in the following paragraphs.

The event-based cophylogenetic analyses supported two host shifts of PNS *Tetramesa*; namely from *E. plana* and *E. rigidior* to *E. curvula*. As discussed earlier, it is possible that these host shifts are coupled to resource availability, as *E. curvula* has a much wider distribution across South Africa than the other two species; therefore offering a more reliable food source, and allowing for range expansion (Fig. 3.12). It is also possible that the alternative hosts are more nutritious, have lower defences, exhibit chemical cues that make them more apparent to herbivores, or a combination of these factors. Host switching to *E. curvula* allows for *Eragrostis rigidior* and *E. plana* wasps to expand their distribution further south and east, respectively, which may also be further driven by climate change and land-use patterns (Masubelele *et al.*, 2014; Moncrieff *et al.*, 2015). Although there is evidence of a host shift, it could also be the result of spillover feeding. Additionally, although host shifts can occur naturally in a short time, it is very unlikely, and if no-choice host specificity testing is conducted, predictions of the chances of it occurring could be estimated (Paynter *et al.*, 2020).

Strong selection pressures can exist for some herbivores to utilise a more abundant host in favour of a rare one in order to reduce competition (Okamoto *et al.*, 2019). Augustyn *et al.* (2017), for example, found that host shifts in the South African leafhopper *Cephalelus uncinatus* (Hemiptera: Cicadellidae), a specialist on restios in the Cape Floristic Region, was driven by the high spatial turnover of host plant availability across the insect's distribution. It is also possible that natural enemy avoidance can drive host shifts, such that herbivores are under selection pressure to move into enemy-free space (Bernays and Graham, 1988; Zvereva *et al.*, 2010). Adapting to different hosts in this way could be an example of a bet-hedging strategy, where the PNS *Tetramesa* associated with *Eragrostis* species are optimising their fitness across an unpredictable landscape (Olofsson *et al.*, 2009). It would be interesting to measure the relative fitness of *Tetramesa sp. 4* across different *Eragrostis* hosts to test the bed-hedging hypothesis.

Eragrostis curvula is more structurally complex than *E. rigidior* and *E. plana*, which may provide more niches to exploit for predator avoidance. Field observations have indicated that the *Tetramesa* on *Eragrostis* and *Sporobolus* are heavily parasitised, predominantly by *Ormyrus* Westwood species (Hymenoptera: Ormyridae) (Guy Sutton, pers. comm.). Additionally, Witt and McConnachie (2004) suggested that the difficulty in rearing *Sporobolus* wasps might be due to parasitism. Information about the parasitoids associated with the *Tetramesa* is sparse, where only the Palearctic species in the *Eurytoma appendigaster* Swederus, *Bruchophagus atra* Walker, and *B. phlei* Erdös groups have been recorded (Lotfalizadeh *et al.*, 2007b), and more recently *Eurytoma amicophaga* Lotfalizadeh on *Tetramesa amica* Lotfalizadeh associated with medusahead (*Taeniatherum caput-medusae* L.). It would be valuable to undertake further research into the natural enemy assemblages associated with the southern African *Tetramesa*, as understanding these ecological networks may help in further explaining host-use patterns and possible differential feeding damage that could influence agent selection.

The duplication event on *E. rigidior* indicated a case of sympatric intrahost speciation (i.e. two diverging lineages on the same host), corroborating the species delimitation results in Chapter 2. It is not known whether these two PNS *E. rigidior* wasp taxa occupy different microhabitats/microenvironments on the host to reduce competition (see for example Tooker and Giron (2020)), or whether there are differences in their adult emergence times, leading to temporal separation (e.g. Zhang *et al.* (2019a)).

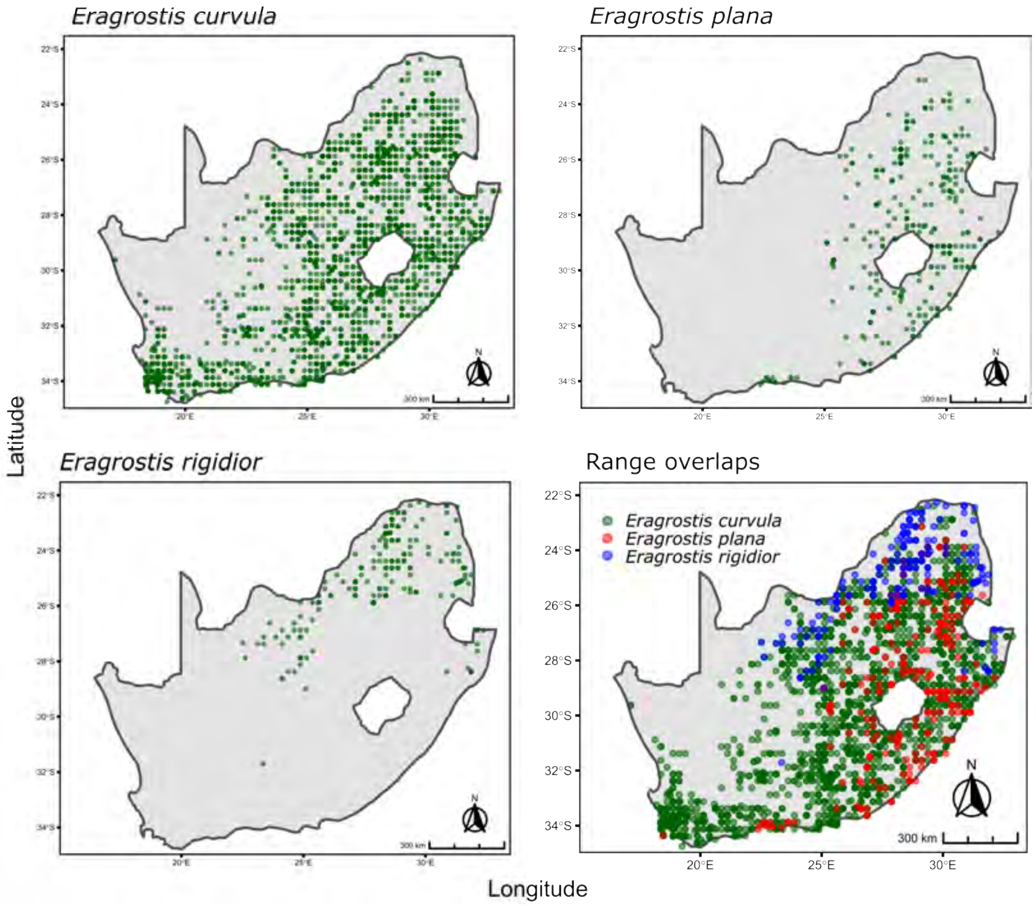


Figure 3.12: Distributions of *Eragrostis curvula*, *E. plana*, and *E. rigidior* in South Africa, with the bottom right map showing an overlap of the distributions. Data were sourced from the GBIF (Global Biodiversity Information Facility) database using the R spocc (SPecies OCCurrence) package (`https://github.com/ropensci/spocc`).

3.4.1 Conclusion

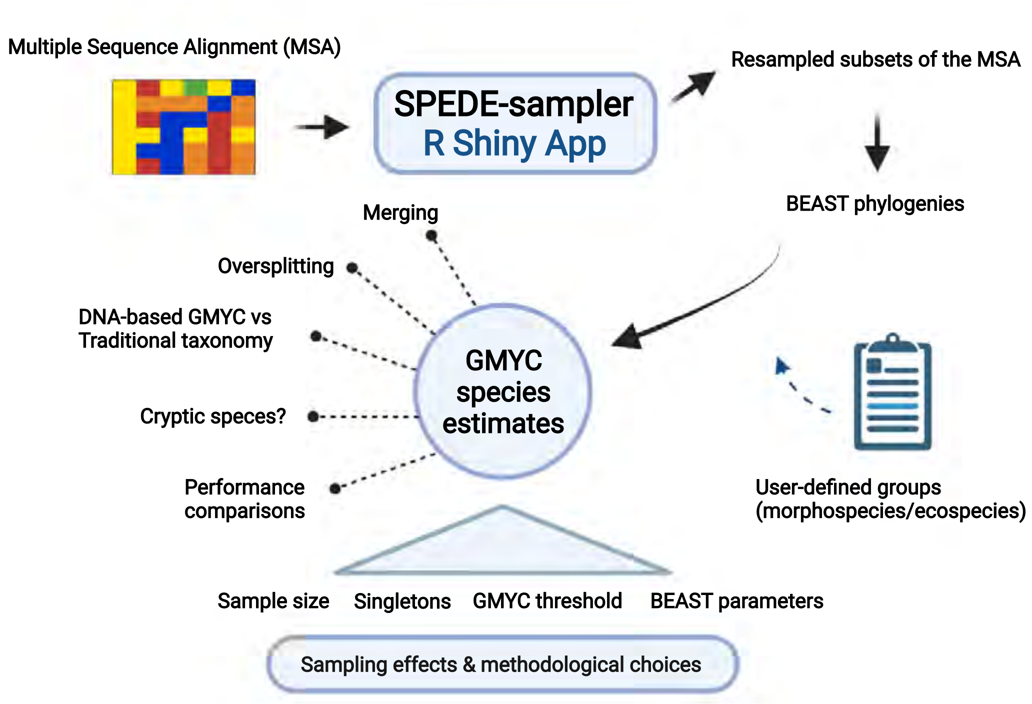
The data from this chapter has highlighted the importance of combining phylogenetic inferences with ecological and biological information in order to predict the potential host range of candidate *Tetramesa*. Phylogenetic distance alone may not always be a sufficient measure, as host use can be influenced by a number of selection pressures from the host plant itself, predators and parasitoids, or intraspecific competition. It is important to consider as many of these factors as possible when deciding on the suitability of a potential biological control agent.

This work has also shown that both global-fit and event-based cophylogenetic tools can be very valuable in formulating host test lists and providing a better understanding of the ecology of the target system. Contrary to previous critics of grass biological control, the present data suggests that there are potential *Tetramesa* taxa that may be useful biological control agents for invasive African species, and that species discovery is expected to increase substantially with greater sampling effort.

Chapter 4

SPEDE-sampler software

Graphical Overview



4.1 Introduction

This thesis has shown that the taxonomy of the *Tetramesa* is complex, that the group has radiated relatively recently, and that morphological species assignments are difficult and error-prone, and can lead to underestimates of species diversity. In Chapter 2, a number of species delimitation tools were used to assist in delineating different taxa from the genetic data. However, recent studies have shown that many factors can lead to biases in species delimitation methods.

The GMYC model is a widely-applied ultrametric tree-based tool for species delimitation that implements maximum likelihood statistics to single-locus genetic data (predominantly mitochondrial) (Pons *et al.*, 2006; Fontaneto *et al.*, 2007; Fujisawa and Barraclough, 2013). Model assumptions include that (1) species are monophyletic, (2) there is no intraspecific geographic structuring, and (3) there are no extinctions (Fujisawa and Barraclough, 2013). The method has become very popular in ecology because it does not require prior knowledge of the target study group; which makes it a particularly useful tool for studies involving species for which taxonomic knowledge is limited or non-existent (Talavera *et al.*, 2013).

The performance of the GMYC species delimitation model has been shown to be affected by a number of methodological and computation factors (Esselstyn *et al.*, 2012; Hamilton *et al.*, 2014; Tang *et al.*, 2014; Dellicour and Flot, 2015; Blair and Bryson Jr, 2017; Fonseca *et al.*, 2021; Magoga *et al.*, 2021). The model is subject to lower performance when there are few species (O'Meara, 2010), singletons (which can result in over-estimations of species numbers) (Fujisawa and Barraclough, 2013), and/or recent, rapid divergences (Reid and Carstens, 2012). Species numbers may regularly be overestimated due to the sensitivity of delimitation algorithms to intraspecific population structure, which can be exacerbated by incomplete sampling (Papadopoulou *et al.*, 2009; Sukumaran and Knowles, 2017). In the *Aphonopelma* Pocock tarantula genus, for example, Hamilton *et al.* (2014) found that the number of GMYC species varied "alarmingly" due to incomplete or biased sampling. Instead of improved performance with greater sampling, the authors found larger variation in species richness estimates. Tang *et al.* (2014) highlighted the effect that branch smoothing (correcting for rate heterogeneity so that clock-like, ultrametric phylogenies are produced) can have on the aberrant lumping or splitting of groups due to variability in branch lengths, and how this can drastically alter interpretations. In another example involving *Hipposideros* Gray bats, Esselstyn *et al.* (2012) found that the accuracy and precision of the GMYC method declined when effective population size (Ne) and speciation rate (i.e rapid divergence) increased.

The performance of the GMYC model has been predominantly tested on simulated data where the effects of factors are controlled, and the model's assumptions are not violated (Papadopoulou *et al.*, 2009; Esselstyn *et al.*, 2012; Fujisawa and Barraclough, 2013; Talavera *et al.*, 2013). Most applications of the GMYC using empirical data will, however, likely violate these assumptions. This highlights a large knowledge gap in its performance when using datasets that have unknown species boundaries, are subject to under-sampling bias and unequal sampling effort, or a combination of these factors.

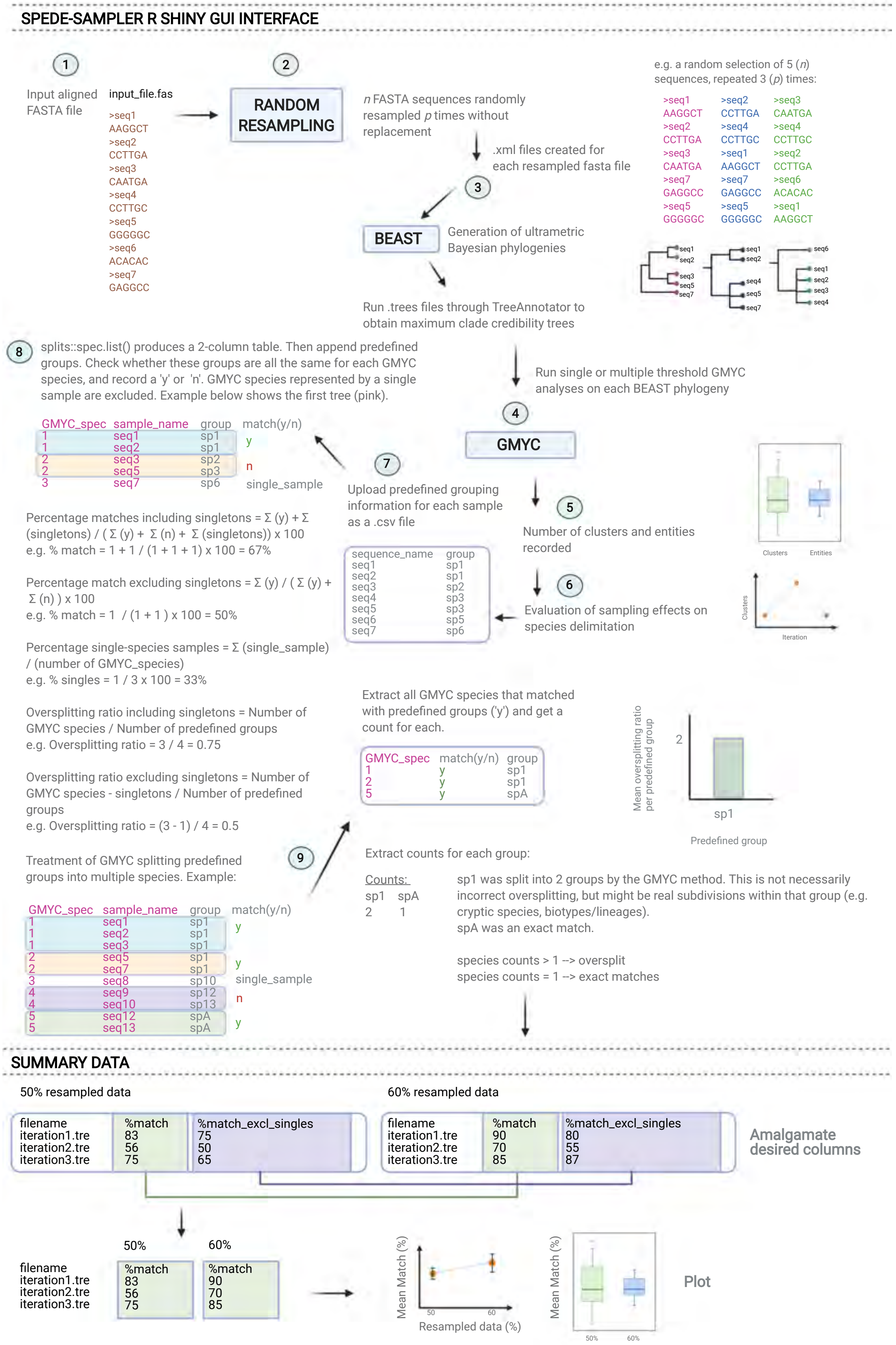
In this chapter, I present "SPEDE-sampler", an R shiny application that allows users to easily assess potential biases and limitations of the GMYC delimitation model when used on their data sets. SPEDE-sampler is used here to assess the uncertainty and potential biases in the species delimitation results presented in Chapter 2 and highlight potentially undiscovered species diversity. Due to the small number of sequences available for some eurytomid morphospecies and links to the literature on the potential effects of sample size and singletons on GMYC results, particular emphasis was paid in this chapter to assessing the effect of these factors on *Tetramesa* GMYC delimitations. The results are discussed in the context of developing biological control programmes against invasive grasses and improving the taxonomy of the *Tetramesa* genus.

4.2 Materials and Methods

SPEDE-sampler is an application that takes DNA sequences as input and assesses potential species diversity while considering sampling effects such as data size and singletons, and computational parameters. It does this using a simulation approach, which has the benefit of allowing for the calculation of uncertainty. The SPEDE-sampler software has been published in Molecular Ecology Resources (van Steenderen and Sutton, 2022), and is available in the associated GitHub repository (`https://github.com/CJMvS/spede-sampler`) with a worked example. The repository needs to be downloaded and unzipped, and the code in Box A needs to be run in R in order to launch the application. An overview of the software will be discussed in the following sections.

4.2.1 Workflow

The workflow of the application begins with the uploading of an aligned multiple sequence alignment (MSA) file that is subsetted and then randomly resampled a desired number of



>seq1
AAGGCT
>seq2
CCTTGA
>seq3
CAATGA
>seq4
CCTTGC
>seq5
GGGGGC
>seq6
ACACAC
>seq7
GAGGCC

n FASTA sequences randomly resampled *p* times without replacement

.xml files created for each resampled fasta file

e.g. a random selection of 5 (*n*) sequences, repeated 3 (*p*) times:

>seq1	>seq2	>seq3
AAGGCT	CCTTGA	CAATGA
>seq2	>seq4	>seq4
CCTTGA	CCTTGC	CCTTGC
>seq3	>seq1	>seq2
CAATGA	AAGGCT	CCTTGA
>seq7	>seq7	>seq6
GAGGCC	GAGGCC	ACACAC
>seq5	>seq5	>seq1
GGGGGC	GGGGGC	AAGGCT

Generation of ultrametric Bayesian phylogenies

Run .trees files through TreeAnnotator to obtain maximum clade credibility trees

Run single or multiple threshold GMYC analyses on each BEAST phylogeny

Number of clusters and entities recorded

Evaluation of sampling effects on species delimitation

Upload predefined grouping information for each sample as a .csv file

sequence_name	group
seq1	sp1
seq2	sp1
seq3	sp2
seq4	sp3
seq5	sp3
seq6	sp5
seq7	sp6

splits::spec.list() produces a 2-column table. Then append predefined groups. Check whether these groups are all the same for each GMYC species, and record a 'y' or 'n'. GMYC species represented by a single sample are excluded. Example below shows the first tree (pink).

GMYC_spec	sample_name	group	match(y/n)
1	seq1	sp1	y
1	seq2	sp1	
2	seq3	sp2	n
2	seq5	sp3	
3	seq7	sp6	single_sample

Percentage matches including singletons = Σ (y) + Σ (singletons) / (Σ (y) + Σ (n) + Σ (singletons)) x 100
e.g. % match = 1 + 1 / (1 + 1 + 1) x 100 = 67%

Percentage match excluding singletons = Σ (y) / (Σ (y) + Σ (n)) x 100
e.g. % match = 1 / (1 + 1) x 100 = 50%

Percentage single-species samples = Σ (single_sample) / (number of GMYC_species)
e.g. % singles = 1 / 3 x 100 = 33%

Oversplitting ratio including singletons = Number of GMYC species / Number of predefined groups
e.g. Oversplitting ratio = 3 / 4 = 0.75

Oversplitting ratio excluding singletons = Number of GMYC species - singletons / Number of predefined groups
e.g. Oversplitting ratio = (3 - 1) / 4 = 0.5

Treatment of GMYC splitting predefined groups into multiple species. Example:

GMYC_spec	sample_name	group	match(y/n)
1	seq1	sp1	y
1	seq2	sp1	
1	seq3	sp1	
2	seq5	sp1	y
2	seq7	sp1	
3	seq8	sp10	single_sample
4	seq9	sp12	n
4	seq10	sp13	
5	seq12	spA	y
5	seq13	spA	

Extract all GMYC species that matched with predefined groups ('y') and get a count for each.

GMYC_spec	match(y/n)	group
1	y	sp1
2	y	sp1
5	y	spA

Extract counts for each group:

Counts:

sp1	spA
2	1

sp1 was split into 2 groups by the GMYC method. This is not necessarily incorrect oversplitting, but might be real subdivisions within that group (e.g. cryptic species, biotypes/lineages).
spA was an exact match.

species counts > 1 --> oversplit
species counts = 1 --> exact matches

50% resampled data

filename	%match	%match_excl_singles
iteration1.tre	83	75
iteration2.tre	56	50
iteration3.tre	75	65

60% resampled data

filename	%match	%match_excl_singles
iteration1.tre	90	80
iteration2.tre	70	55
iteration3.tre	85	87

	50%	60%
filename	%match	%match
iteration1.tre	83	90
iteration2.tre	56	70
iteration3.tre	75	85

Figure 4.1: Illustrated workflow for the SPEDE-sampler R Shiny application. Resampling of FASTA sequences →creation of BEAST phylogenies →GMYC analyses →results →plotting. Figure created with BioRender.com

times without replacement (Fig. 4.1 steps 1 and 2). For example, a MSA file of 500 sequences might be uploaded, randomly subsetted to 50% of the data, and repeated ten times. This will yield ten FASTA files comprising a random assortment of 250 sequences in each. The user has the option of uploading an Excel .CSV file containing predefined grouping information for each sequence, which can be used to ensure that at least one representative sequence for each predefined group is included in each resampled file.

Box A

```r
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
BiocManager::install("Biostrings")
library(Biostrings)
install.packages("shiny")
library(shiny)
setwd("path/to/spede_sampler_R-main")
shiny::runApp(appDir = getwd())
```

Each of these FASTA files is then used to generate an .XML file for analysis in BEAST (Bouckaert *et al.*, 2019), using the R package "beautier" (Bilderbeek, 2018a) (Fig. 4.1 step 3). The user can set up the .XML file in the SPEDE-sampler application, with the option of selecting a site and clock model, clock rate, tree prior, associated rate distributions, and an MCMC value. For large MSA files, it is advisable to run BEAST on the CIPRES Science Gateway platform (`http://www.phylo.org/`) for faster performance.

The resulting .TREES files produced by BEAST need to be inputted to TreeAnnotator in order to obtain maximum clade credibility (MCC) trees. The user can set a percentage burnin, and select from different node height options. The resulting MCC trees are then used as input for GMYC analyses (Fig. 4.1 steps 4 - 9). Tracer is available via the "tracerer" R package (Bilderbeek, 2018b) to check effective sample size (ESS) scores and for MCMC convergence. LogCombiner is available in SPEDE-sampler as an optional means of reducing the size of the .TREES files by resampling states at a lower frequency.

The user can optionally upload a .CSV file containing morphospecies, ecotypes, or other relevant predefined grouping information for each sequence in the BEAST-generated phylogenies. The GMYC method does not require prior grouping information, but this feature is available in SPEDE-sampler in order to compare DNA-based species delimitation to traditional taxonomy. The GMYC species estimates are compared to these predefined groups in order to assess a match rate, and to what degree groups have been "oversplit" by the GMYC method. Comparing DNA-based GMYC estimates to existing morphologically or ecologically-defined species in this way can very useful in deciding whether the taxonomy is likely outdated, and contains possible cryptic species.

The user can choose between a single (Pons *et al.*, 2006) or multiple GMYC threshold (Monaghan *et al.*, 2009) approach. Applying a multiple threshold method may be useful in large datasets where there is significant variation in intra and inter-specific genetic divergences. Generally, however, a single-threshold approach is recommended as it is less likely to oversplit (Fujisawa and Barraclough, 2013; Talavera *et al.*, 2013; Blair and Bryson Jr, 2017).

Once the GMYC analysis is complete for all BEAST tree files, the application records the estimated number of entities and clusters (the number of delimited groups comprising two or more samples, including and excluding singleton sequences, respectively), and optionally compares the match rate of user-defined groups to estimated GMYC species groups. Additionally, the application assesses (1) the effect that singletons have on species delimitation, (2) the number of GMYC merges and exact matches, and (3) the frequency with which the GMYC method "oversplits" species relative to predefined groups. These oversplitting ratios highlight which species groups contain potential undiscovered biodiversity. Box B contains a list of terms and definitions as applied to the usage of SPEDE-sampler.

Box B

Clusters The number of delimited groups comprising two ore more samples, excluding singletons.

Entities The number of delimited groups comprising two ore more samples, including singletons.

Exact match An instance during scoring when all the samples belonging to a particular user-defined group (morphospecies or other user-defined group) correspond to the same GMYC species.

Split match An instance during scoring when the samples belonging to a particular user-defined group (morphospecies or other user-defined group) are split into two ore more GMYC species groups. This indicates the possibility of the underestimation of species richness by the user.

Match (y/n) A means of denoting, in the work-through of the R code, whether each GMYC species comprises one unanimous user-predefined group.

Merge An instance during scoring when two or more user-defined groups are merged into one GMYC species. This indicates the possibility of an overestimation of species richness by the user.

Oversplitting The outcome where the GMYC model has estimated more species than those estimated by the user (= "discordant splitting"). This could mean either (1) the incorrect splitting into too many species, or (2) the genuine presence of undiscovered biodiversity or cryptic species.

Undersplitting The outcome where the GMYC model has estimated fewer species than those estimated by the user. This could mean either (1) the incorrect merging into too few species, or (2) the genuine presence of lower biodiversity than expected (e.g. variations in intraspecific morphological characters that are mistaken for being interspecific).

Splitting ratio The ratio of the total number of estimated GMYC species to the total number of user-defined groups in the dataset. A value greater than one indicates oversplitting, while a value less than one denotes undersplitting. A value of one means perfect agreement between the GMYC and the user's estimates.

(Overall) percentage match The overall proportion of successful matches (records of "y") in a dataset. This includes cases of both exact matches and split matches, and is calculated with and without singletons.

Singleton A species represented by only one individual/genetic sequence.

4.2.2 Calculation of GMYC metrics

The R Shiny application generates a summary table of each sample name, its designated GMYC species group number, the corresponding user-predefined group, and a score of "y" (yes) or "n" (no) to denote whether the GMYC species designations and the user's predefined groups consistently match (Fig. 4.1 step 8 and Fig. 4.2). In a similar approach to Magoga *et al.* (2021), an “n” outcome is recorded as a “merge”, where the GMYC has lumped two or more groups defined by the user (i.e. the user has overestimated species richness) (“merge type I”). A merge is recorded even if one or more of these groups is represented by a singleton (“merge type II”). A “y”/match outcome can take two forms, namely (1) “split”, where the GMYC has split one user-defined group into two or more groups (i.e. the user has underestimated species richness), or (2) “exact ”, where the user-defined groups match the GMYC estimates exactly (Fig. 4.2). Overall percentage match scores, including (m_i) and excluding (m_e) singletons are subsequently calculated as shown in the equations below.

$$m_\mathrm{i} = \frac{\sum y + \sum singletons}{\sum y + \sum n + \sum singletons} \times 100$$

$$m_\mathrm{e} = \frac{\sum y}{\sum y + \sum n} \times 100$$

Overall percentage match scores include both match scenarios (i.e. “split” and “exact” matches), which is different from the exact match score calculation. Exact match scores are calculated per group (i.e. morphospecies or other user-defined group), as the number of times that a particular group is scored as an exact match, averaged across all GMYC runs. The oversplitting ratio is calculated as the ratio of the estimated number of GMYC species (including and excluding singletons) to the number of user-predefined groups, and the percentage of singletons is calculated as the ratio of the sum of the number of singletons to the number of GMYC species. Box C provides a hypothetical example of how SPEDE-sampler calculates these metrics in practice, using the scenario shown in Figure 4.2.

The user can explore and download a variety of summary plot outputs, including the fluctuations in the number of clusters (excluding singletons) and entities (including singletons) across tree iterations, boxplots for the overall number of clusters and entities across all iterations, particular input trees with GMYC support values, changes in percentage matches across tree iterations, and boxplots and barplots for groups that were oversplit or merged. Each plot can be downloaded in .PNG, .SVG, or .PDF format, with customisable dimensions and resolutions where applicable.

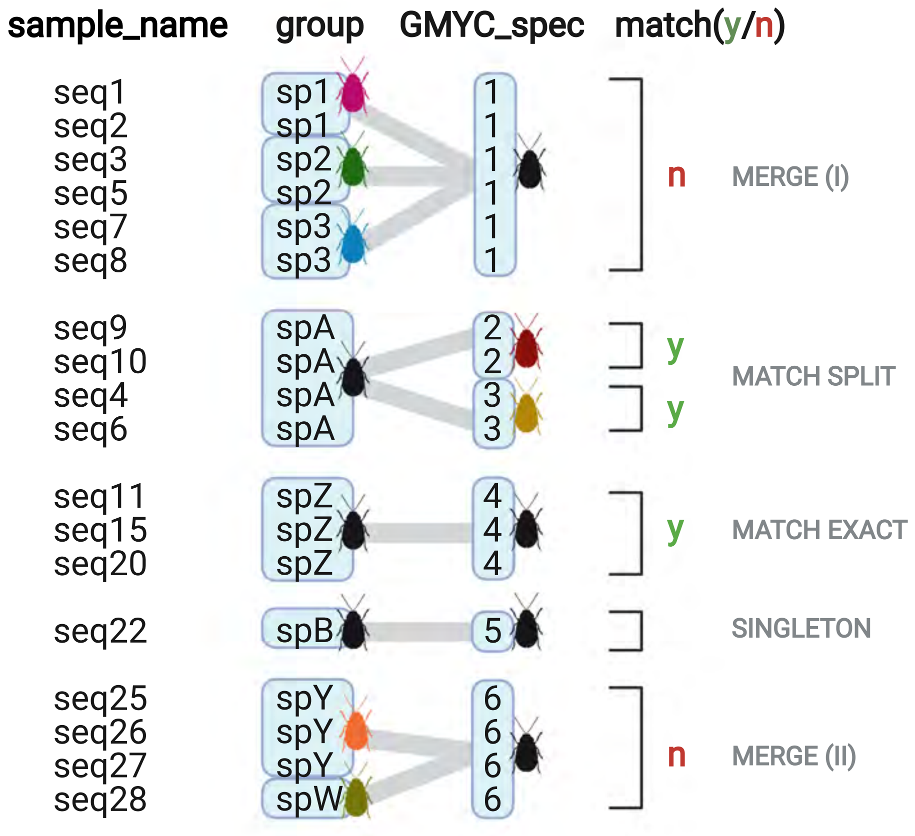


Figure 4.2: A detailed diagrammatic explanation of how SPEDE-sampler determines cases of merges, splits, exact matches, and singletons in a hypothetical example of one GMYC analysis (i.e. one BEAST phylogeny input). A merge occurs when the user has overestimated the number of groups, and the GMYC has lumped them into one (merge type I). A merge is recorded even if one group is a singleton (for example spW in merge type II). A match can take two forms; (1) a split, where the user has underestimated the number of groups, or (2) an exact match, where a user-defined group and a GMYC species delimitation corroborate exactly. Singletons occur when there is one sequence representing a GMYC species. Figure created with BioRender.com

Box C

Using Figure 4.2 as an example of the output of one GMYC analysis:

There are six GMYC species and 8 user-defined groups.
(1) Species 1 (sp1), 2 (sp2), and 3 (sp3) would be recorded as a merge (**merge type I**), while species A (spA) would be flagged as being oversplit by a factor of 2 by the GMYC model. Species A may present a case of two previously undiscovered cryptic species, or merely intraspecific population structuring. Species Z (spZ) would be recorded as an exact match. In a hypothetical scenario, if a total of three GMYC analyses were run, where species Z was recorded as an exact match in two of the runs, then Species Z would have a 67% **exact match** score (i.e. exact match score = Σ(exact match count)/(the number of input files) x 100 = 2/3 x 100 = 67%).

(2) Overall, with species Z being the only user-predefined group with an exact GMYC match, the **exact match incidence** in this GMYC run = (the number of user-defined groups with a recorded exact match)/(the number of user-defined groups) = 1/8 = 13%.

(3) Species B (spB) would be recorded as a singleton.

(4) Species Y (spY) and species W (spW) would be recorded as a merge, even though spW is a singleton (**merge type II**). The user should pay attention to these cases, as they might be potential taxonomic misidentifications.

(5) The **splitting ratio** including singletons would be calculated as: (the number of GMYC species)/(the number of user-defined groups) = 6/8 = 0.75. The oversplitting ratio excluding singletons would be: (the number of GMYC species - the number of singletons)/(the number of user-defined groups) = (6-1)/8 = 0.63. Oversplitting ratios < 1 indicate that there is a high incidence of overall merging by the GMYC due to an overestimation of species richness by the user. Oversplitting ratios > 1 indicate that species richness has been underestimated by the user, and that there may be cryptic species in the mix. Ratios that equal one indicate that the number of user-defined groups and the number of GMYC species are the same.

(6) The overall **percentage match**, including singletons (m_{i}) = Σ(y) + Σ(singletons)/(Σ(y) + Σ(n) + Σ(singletons)) = (3 + 1)/(3 + 2 + 1) = 67%. Excluding singletons, the percentage match (m_{e}) = Σ(y)/(Σ(y) + Σ(n)) = 3/(3 + 2) = 60%. In this case, singletons are causing a 7% inflated percentage match estimate.

(7) The **percentage of singletons** = Σ(singletons)/(number of GMYC species) = 1/6 = 17%.

4.2.3 Sampling effects on *Tetramesa* species delimitation

Using the SPEDE-sampler application, both the PNS *Tetramesa* 28S and COI datasets were separately analysed (Table 4.1), where they were randomly resampled 10 times without replacement for subsets of 25, 50, 75, and 100% of the sequence data. An Excel file with 14 morphospecies assignments was uploaded as per the software manual. The option of keeping at least one representative of each predefined morphospecies group in the random subsets was selected. A random seed was set for each resampling event. BEAST-compatible .XML files were subsequently generated using the GTR site model, strict clock (clock rate = 1), Yule tree prior with a uniform birth rate distribution, and the MCMC set to ten million generations with sampling after every 1000. The .xml files were uploaded to BEAST2 on the CIPRES Science Gateway portal for faster performance. BEAST log files were uploaded to Tracer in the SPEDE-sampler application to check for MCMC convergence. TreeAnnotator was then used to generate maximum clade credibility (MCC) trees, where the burnin was set to 25%, and heights to "median". The resulting MCCC trees were used as input for the GMYC analyses, where the single-threshold option was selected, and a random seed set.

4.2.4 Host-specificity indices

Host-specificity indices were calculated for the GMYC species in the full COI data set, based on the methods in Abram *et al.* (2021). This entailed the calculation of two metrics: (1) taxonomic host specificity (S_{TD}), which is a measure of the average pairwise taxonomic distinctness of host species used by a herbivore and (2) phylogenetic species variability (PSV), which indicates the degree to which the host species of a herbivore are phylogenetically related. The S_{TD} value greater than 1 indicates an increasing degree of divergence among species. PSV values range between 0 and 1, where 1 indicates maximum variability, and 0 denotes that there is only one host, and therefore no variability.

The host ranges for each delimited GMYC species was used as input, based on the host plant that each specimen was collected on. The no-choice host-specificity test results on *Tetramesa sp. 4* were included in the host range data (i.e. non-target feeding on *E. plana* and

E. planiculmis in addition to target *E. curvula*; Guy Sutton pers. comm). The supplementary R script provided in Abram *et al.* (2021) was applied as per the authors' instructions.

Table 4.1: The number of genetic sequences per morphospecies for the nuclear 28S and mitochondrial COI genes, used as input for SPEDE-sampler.

Morphospecies	Host plant	28S	COI
Tetramesa (A. gayanus)	*A. gayanus*	0	2
Tetramesa romana	*Arundo donax*	4	4
Tetramesa sp. 1	*Sporobolus pyramidalis*	18	13
Tetramesa sp. 4	*Eragrostis curvula*	39	37
Tetramesa sp. 7	*Hyparrhenia hirta*	0	6
Tetramesa sp. 8	*Cynodon dactylon*	2	0
Tetramesa sp. 10	*E. trichophora*	9	7
Tetramesa sp. 11	*E. trichophora*	2	2
Tetramesa sp. 12	*Eustachys paspaloides*	1	0
Tetramesa sp. 13	*E. superba*	1	1
Tetramesa sp. 17	*E. gummiflua*	1	2
Tetramesa sp. 18	*E. gummiflua*	2	1
Tetramesa sp. 23	*E. biflora*	2	3
Tetramesa sp. 24	*E. capensis*	1	2
Tetramesa sp. 25	*E. plana*	3	3
Tetramesa sp. 26	*E. rigidior*	8	8

4.3 Results

4.3.1 Nuclear 28S

The average number of clusters (species diversity estimate excluding singletons) and entities (species diversity estimate including singletons) in the full data set (100% data size) was 5.9 ± 3.7 and 6.9 ± 3.7, respectively (Fig. 4.3 A1, A2). Mean splitting ratios were less than a value of one across all data set sizes (Fig. 4.3 B), with an average of 0.5 ± 0.27 and 0.42 ± 0.26 in the full data set including and excluding singletons, respectively. The average percentage of singletons decreased as data size increased, dropping from 42.4% ± 15.3 in the 25% data set size to 19.8% ± 9.4 in the full data set (Fig. 4.3 C). The diversity of two morphospecies was underestimated, namely *Tetramesa* sp. 1 and 4, with mean splitting ratios of 2.7 ± 1.2 and 3.5 ± 2.1, respectively (Fig. 4.3 D). Mean percentage matches including singletons were consistently higher on average across data sizes (52.1% ± 10.7; full data set) compared to when singletons were excluded (40.5% ± 10.1; full data set) (Fig. 4.3 E). The disparity between the two measures was most prominent in the 25% data set size. Overall, five cases of exact matches were reported (35.7% of user-defined morphospecies), comprising *Tetramesa* sp. 1. sp. 4, sp. 10, sp. 26, and *T. romana*, and five cases of merges. The merged groups were [*Tetramesa* sp. 4, 8, 10, and 17] in GMYC group 1, [*Tetramesa* sp. 4 and 17] in GMYC group 2, [*Tetramesa* sp. 4 and 10] in GMYC group 3, [*Tetramesa* sp. 1, 4, 10, 12, and 13] in GMYC group 4, and [*Tetramesa* sp. 1, 4, 8, 10, 11, 18, 23, 24, 25, 26] in GMYC group 6.

4.3.2 Mitochondrial COI

The average number of clusters and entities in the full data set (100% data size) was 15 ± 0 and 18 ± 0, respectively (Fig. 4.4 A1, A2). This was in the region of 2.5-fold more than was reported for the 28S data. Mean splitting ratios were greater than a value of one across all data set sizes when singletons were included (Fig. 4.4 B), with an average of 1.29 ± 0 in the full data set. The mean oversplitting ratio excluding singletons only exceeded a value of one in the full data set (Fig. 4.4 B). The percentage of singletons decreased as data size increased, starting at 66.5% ± 12.9 in the 25% data set size, down to 16.7% ± 0 in the full data set (100%) (Fig. 4.4 C). The diversity of three morphospecies was underestimated, namely *Tetramesa* sp. 1, sp. 4, and sp. 7, with mean splitting ratios of 2 ± 0 across all three (Fig. 4.4 D). Mean percentage matches including and excluding singletons were consistently higher than 60% across data sizes, except in the 25% data set when singletons were excluded (mean = 35.5% ± 15.11) (Fig. 4.4 E). Overall, four cases of exact matches were reported (28.6% of user-defined morphospecies), comprising *Tetramesa* romana, *Tetramesa* sp. 25 and 26, and the *Tetramesa* wasp collected on *Andropogon gayanus* that formed a sister group to *T. romana*. Five cases of merges were reported (35.71% of user-defined morphospecies), and were [*Tetramesa* sp. 4 and 23] in GMYC group 3, [*Tetramesa* sp. 10 and 11] in GMYC group 4, [*Tetramesa* sp. 4 and 24] in GMYC group 6, [*Tetramesa* sp. 18 and 26] in GMYC group 9, and [*Tetramesa* sp. 4, 23, and 24] in GMYC group 11.

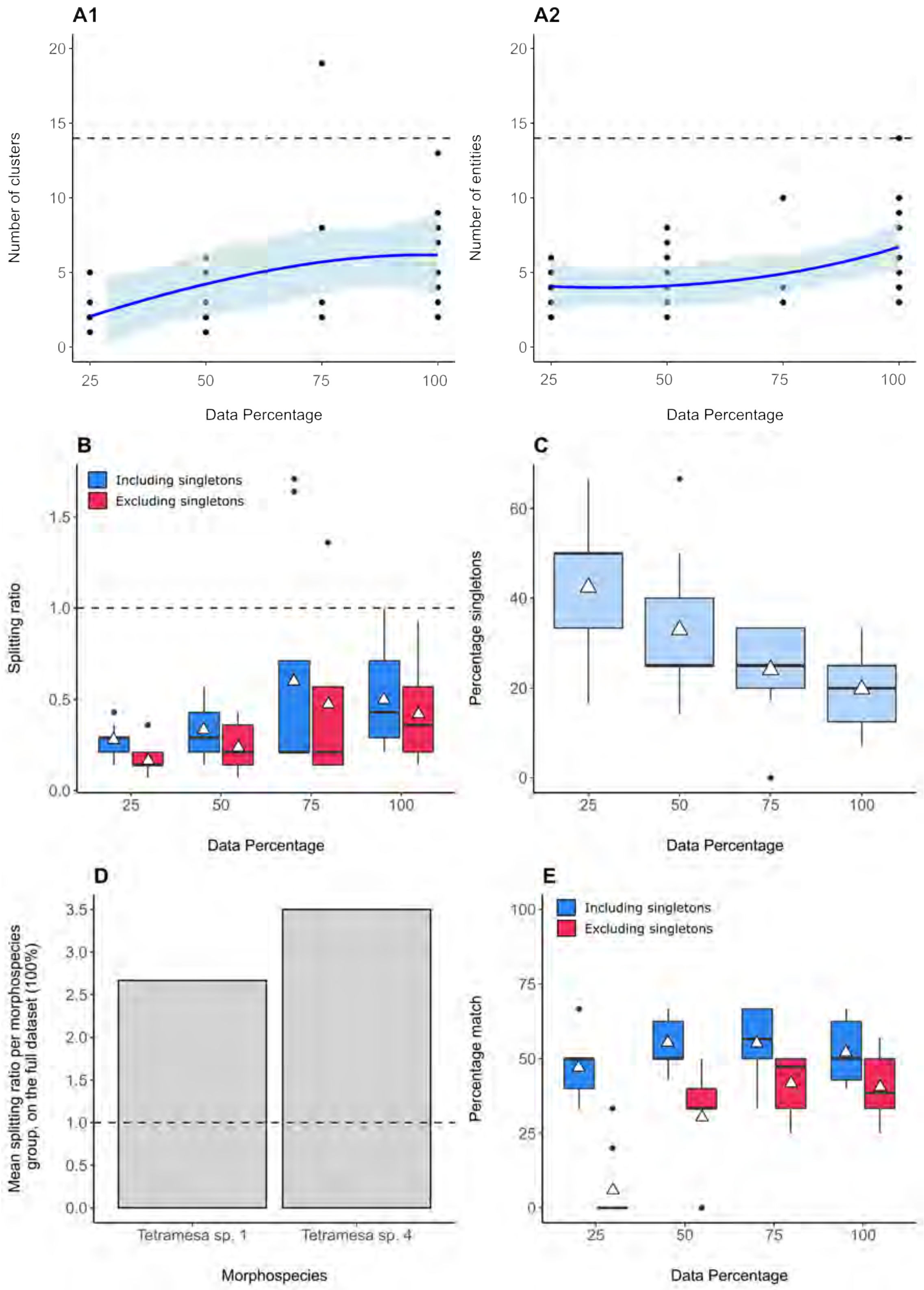


Figure 4.3: SPEDE-sampler results for 93 28S sequences. (A1 and A2) The number of clusters and entities across subsetted data set sizes. The light blue band represents a 95 confidence interval. The dotted lines at y = 14 is the number of predefined morphospecies. (B) A boxplot of the splitting ratios across dataset sizes, including (blue) and excluding (red) singletons. The dotted line at y = 1 indicates the expected ratio if no splitting occurred. (C) A boxplot of the percentage of singletons across data set sizes. (D) The mean splitting ratios of morphospecies groups that exceeded a ratio of 1 (dotted horizontal line). (E) A boxplot of the percentage matches between predefined morphospecies and GMYC species, including (blue) and excluding (red) singletons. White triangles represent means.

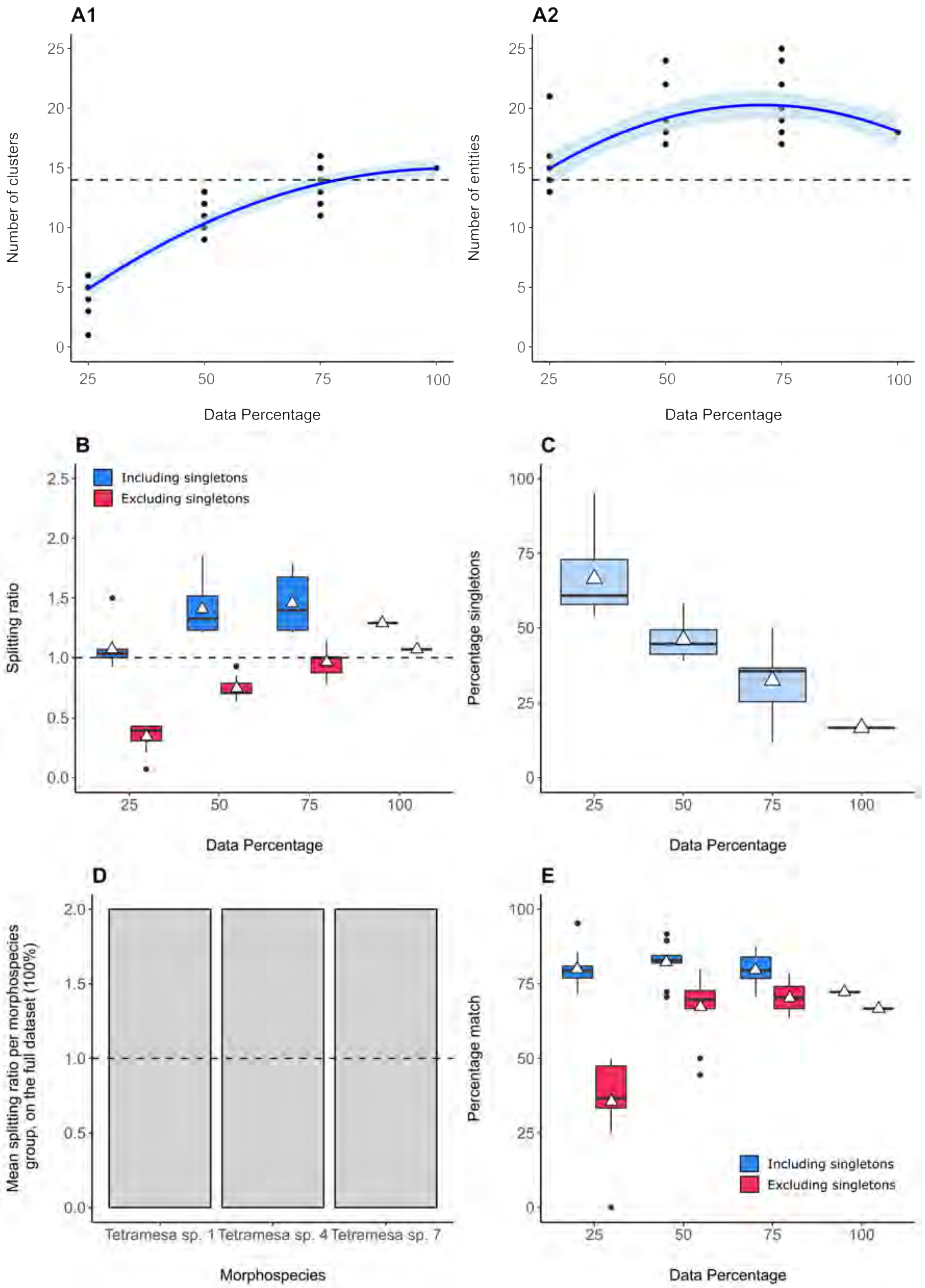


Figure 4.4: SPEDE-sampler results for 91 COI sequences. (A1 and A2) The number of clusters and entities across subsetted data set sizes. The light blue band represents a 95% confidence interval. The dotted lines at y = 14 is the number of predefined morphospecies. (B) A boxplot of the splitting ratios across dataset sizes, including (blue) and excluding (red) singletons. The dotted line at y = 1 indicates the expected ratio if no splitting occurred. (C) A boxplot of the percentage of singletons across data set sizes. (D) The mean splitting ratios of morphospecies groups that exceeded a ratio of 1 (dotted horizontal line). (E) A boxplot of the percentage matches between predefined morphospecies and GMYC species, including (blue) and excluding (red) singletons. White triangles represent means.

4.4 Discussion

4.4.1 Sample size and population structure

Both the nuclear and mitochondrial genes showed that an increase in taxon sampling (i.e. the number of wasp sequences per morphospecies) led to (1) an increase in splitting ratios, and (2) a decrease in the percentage of singletons. The percentage match scores between GMYC species and predefined groups/morphospecies plateaued once the data size approached 50% and above. This was likely caused by the large number of representatives of *Tetramesa sp. 1* and *Tetramesa sp. 4*, which suggests that half the number of sequences for these groups could have been used ($\sim$ n = 15) to obtain similar results. Species richness (the number of clusters and entities) in both genes did not vary drastically with an increase in data size, but rather showed a steady upward trajectory.

The increase in species richness with more data contrasts the findings of Hamilton *et al.* (2014), who reported a large degree of variation in GMYC species richness across data sizes in a study of the North American tarantula genus *Aphonopelma*. Phylogenetic construction method could be a factor in explaining the differences between the current results and Hamilton *et al.* (2014), as they created maximum likelihood phylogenies (compared to a Bayesian approach in SPEDE-sampler), and converted them into ultrametric trees using the "chronopl" and "multi2di" functions in the R `ape` package (Paradis *et al.*, 2004). Talavera *et al.* (2013) found that this approach led to poorer performance in correctly identifying morphospecies, and that if ML phylogenies are to be used, that PATHD8 (Britton *et al.*, 2007) or r8s (Sanderson, 2003) software is more reliable. Variation in species richness estimates could be exacerbated by (1) sensitivity to intraspecific population structure, (2) effects of incomplete lineage sorting or recent, rapid radiations within the group, and (3) an artefact of one or more violations of the GMYC model's assumptions, or a combination of these factors. The results from Chapter 2 have shown that points (1) and (2) above apply to the *Tetramesa* data, particularly regarding the intraspecific population structure in the pronotal spot (PNS) wasps associated with *Eragrostis curvula*, and the relatively recent radiation patterns found for the genus.

The results from both sets of genes flagged *Tetramesa sp. 1* (PNS *Sporobolus pyramidalis*) and *Tetramesa sp.4* (PNS *Eragrostis curvula*) as being undersplit (i.e. that they may be more diverse than the morphospecies assignments suggest), with the addition of *Tetramesa sp. 7* (NPNS *Hyparrhenia hirta*) in the COI results only. The GMYC assumption of the absence of geographical substructuring within a data set is almost certainly violated in real-world scenarios, and so unbalanced sampling across distribution ranges may also contribute to variation in GMYC results (Talavera *et al.*, 2013). Additionally, sufficient loci are important to offset biases inherent in each locus. Intraspecific geographical structuring could be mistaken for species-level divergences and therefore lead to inflated species richness estimates. *Tetramesa sp. 4* on *E. curvula*, for example, revealed a degree of provincial sub-structuring between specimens collected in the Eastern Cape and Free State provinces (Table 4.3). Bergsten *et al.* (2012) showed how the identification success of barcode queries decreased as the geographical scale of sampling increased. This is an important factor to consider in the sampling design and data analysis of species delimitation studies. Corroborating the results presented in Chapter 2, the diversity underestimations of *Tetramesa* sp. 1, 4, and 7 are likely due to geographic structuring, and coevolutionary relationships with their hosts (discussed in Chapter 3). The underestimation of species diversity can have important implications for biological control, as there may be cryptic species that display varying degrees of feeding damage, host-specificity, and/or climatic suitability.

4.4.2 Singletons

The average species diversity (i.e. entities) in the COI data appeared to be more affected by the presence of singletons than the 28S data set, where the number of COI entities tended to exceed the number of predefined morphospecies across all data sizes. GMYC merging (i.e. when splitting ratios are less than one, indicating that species have been undersplit) occurred across all data sizes in the 28S results whether or not singletons were included, while merges only occurred in the COI results when singletons were excluded. This was despite both genes having a similar range of singletons (Table 4.2). Additionally, the average percentage match scores in both genes were inflated when singletons were included, although the discrepancy was particularly large only in the 25% data set where the singleton percentage was highest. Small sample sizes are therefore not ideal, particularly because of the high sensitivity to singletons.

It is known that the GMYC model can accommodate a moderate number of singletons, but that skewed results have been observed when too many are included (Lohse, 2009; Lim *et al.*, 2012; Puillandre *et al.*, 2012; Ahrens *et al.*, 2016). There are, however, contrasting reports in the literature regarding this effect. Talavera *et al.* (2013), for example, reported that although a higher proportion of singletons negatively affects biological meaningfulness, their GMYC success rate did not decrease even with a singleton incidence of 95%. Similarly, Ceccarelli *et al.* (2012) reported that despite their COI and cytochrome b data comprising 64% and 67% singletons, respectively, GMYC species richness estimates corroborated their morphological identifications.

It is clear that the effects of singletons, and any other potential sampling effects, need to be assessed on a case-by-case basis. It is also important that other independent lines of evidence are acquired to complement single-locus genetic data, such as additional genetic markers, geographical, behavioural, and morphological information where applicable (Carstens *et al.*, 2013).

Table 4.2: SPEDE-sampler summary results from the full datasets (100% sequence data) for the PNS *Tetramesa* 28S and COI sequences. SR = splitting ratio, + singletons = including singletons, - singletons = excluding singletons. Standard deviations are shown where appropriate.

Gene	**28S**	**COI**
Number of sequences	93	91
Singletons (%)	19.8 ± 9.4	16.7 ± 0
GMYC clusters	5.9 ± 3.7	15 ± 0
Max. GMYC clusters	13	15
Min. GMYC clusters	2	15
GMYC entities	6.9 ± 3.7	18 ± 0
Max. GMYC entities	14	18
Min. GMYC entities	3	18
User-defined groups	14	14
GMYC Exact matches, (%)	5, (35.7%)	4, (28.6%)
GMYC Merges, (%)	5, (35.7%)	5, (35.7%)
Match (+ singletons) (%)	52.1 ± 10.7	72.22 ± 0
Match (- singletons) (%)	40.5 ± 10.1	66.7 ± 0
SR (+ singletons)	0.5 ± 0.27	1.29 ± 0
SR (- singletons)	0.42 ± 0.26	1.07 ± 0

Sampling effects

Singletons can be particularly pronounced in a variable marker such as mitochondrial COI (e.g. Fig. 4.4 B), and that despite COI being the "barcoding gene", it needs to be complemented with other gene regions; preferably nuclear markers. As discussed earlier in this thesis, the *Tetramesa* may reproduce via facultative thelytokous parthenogenesis (unfertilised eggs produce females), which may have an effect on mitochondrial gene diversity. Observations of the *Tetramesa sp. 1* wasps suggest that they are arrhenotokous (unfertilised eggs produce males) (Guy Sutton pers. comm.). Species delimitation in organisms that reproduce asexually can be challenging because every individual is reproductively isolated from all other individuals; making it difficult to ascertain meaningful genetic boundaries (Lin *et al.*, 2017; Havill *et al.*, 2021). It would be interesting to explore how these different modes of reproduction affect inheritance, and how this may influence phylogenetic interpretations.

A small sample size can lead to a high incidence of singletons (e.g. Fig. 4.4 C), and so future surveys need to be carefully planned to ensure that a sufficient number of representative genetic samples are collected. It is advisable to collect at least three to five samples per morphospecies. For recently radiated groups, particularly those that produce parthenogenetically, it is vital that integrative taxonomy that includes morphological and ecological information, be used. Inter-breeding experiments can be useful in assessing mating barriers (e.g. Paterson *et al.* (2016)); which are already underway using *Tetramesa* sp. 1, sp. 2, sp. 4, and sp. 5.

4.4.3 Relevance to grass biological control

The SPEDE-sampler results presented here have a number of practical outcomes that can benefit a grass biological control programme aiming to utilise *Tetramesa* as possible agents. The results are particularly useful to identify potentially distinct host-specific taxonomic units (HSTUs) and intraspecific herbivore strains, and to gain a better understanding of the effect that geographic sub-structuring can have in driving population structure. The following paragraphs summarise each putative *Tetramesa* group. The distributions for each of these HSTUs is provided in Table 4.3 and Fig. 4.5, with references to the COI phylogeny and sequence divergences presented in Chapter 2 where relevant.

Delimited genetic groups

***Tetramesa sp. 1* A and B** The PNS *Tetramesa* on *Sporobolus pyramidalis* (sp. 1A) collected between 2017 and 2021 and the presumed *S. africanus* (sp. 1B) collected in 2022 in the Eastern Cape are two distinct HSTUs that form sister clades in the COI phylogeny, with a sequence divergence of 3.9%. The distribution range of *Tetramesa sp. 1A* spans three provinces, where it appears that gene flow is high enough to maintain one homologous genetic group.

Tetramesa sp. 1B is a novel group that requires host-specificity testing mirroring the work done on *Tetramesa sp. 1A* by Sutton *et al.* (2021b). As discussed in Chapter 1, Sutton *et al.* (2021b) have already found that *Tetramesa sp. 1A* is suitably host-specific and damaging to *S. pyramidalis* and *S. natalensis* in the field, and that this wasp has been recently imported into quarantine in Australia for further host-specificity testing.

***Tetramesa sp. 4* A - E** There are five distinct PNS *Tetramesa* associated with *Eragrostis curvula* (sp. 4A - 4E), but these are more likely intraspecific populations that have been split based on either geographic sub-structuring or possibly as a result of host-associated differentiation (HAD), discussed in Chapter 3. The sequence divergences between these groups ranged between 3.1 and 11.7%.

The five distinct *Tetramesa sp. 4* groups are good candidates for further investigation, where the damage impact of each entity should be tested, singly and in combination, on *E. curvula.* The *Tetramesa sp. 4* species complex showed a greater degree of host variability than most of the other delimited *Tetramesa* groups, ranging between three and five different host species. This group may therefore not be good candidates for biological control, but further host-specificity testing is required on non-target species in the invaded range of interest. Assessing the performance of intraspecific populations might be helpful in identifying a more damaging strain to use for biological control.

***Tetramesa sp. 7* A and B** The two distinct *Tetramesa* on *Hyparrhenia hirta* collected in the Eastern Cape in 2020 appear to be sympatric cryptic species that form sister clades in the COI phylogeny, and are more closely related to *T. romana* than to the other *Tetramesa* groups. The sequence divergence between *Tetramesa sp. 7* A and B was 12.6%, which suggests that these are cryptic species. These wasps are one of two morphospecies that were NPNS, but fell within the PNS *Tetramesa* clade in the nuclear 28S phylogeny. *Tetramesa sp. 7* A and B appear to be good biological control agent candidates for *H. hirta*, but further sampling and host-specificity testing is required..

Tetramesa sp. 10 The PNS *Tetramesa* on *E. trichophora* was collected in KwaZulu-Natal in 2021, and is the only wasp observed to date to form a noticeable gall on the stem of the host grass. This group was most closely related to *Tetramesa sp. 4A* on *E. curvula* in the COI phylogeny, where the sequenced divergence was 2.8% between the two groups. This low sequence divergence suggests that host-specificity testing of these two wasps on *E. curvula* and *E. trichophora* is important, as each wasp may be able to utilise both host plants, which could preclude their use as biological control agents. No-choice host specificity testing should be completed as it seems unlikely, although not impossible, that the same species of wasp would have such different feeding modes on two different host plant species; namely galling *E. trichophora* and mining the stems of *E. curvula*.

Tetramesa sp. 25 The PNS *Tetramesa* on *E. plana* was collected in the Free State in 2022, and was most closely related to *Tetramesa sp. 4C* on *E. curvula*; where the sequence divergence was 4.1% between the two groups. Although this sequence divergence is not as small as between the *Tetramesa sp. 4* and *Tetramesa sp. 10* wasps, it would also be worth carrying out host-specificity tests on these two groups, as they may also be able to utilise each other's host grasses.

***Tetramesa sp. 26* A and B** The two *Tetramesa* on *E. rigidior* collected in KwaZulu-Natal in 2021 appear to be sympatric cryptic species that formed sister clades in the COI phylogeny. The sequence divergence between these two groups was 7.1%. *Tetramesa sp. 26A* appears to be able to use *E. gummiflua* as an additional host, and may therefore not be an appropriate biological control agent. *Tetramesa sp. 26B*, however, may be useful as it was specific to only *E. rigidior*. Further sampling focusing on these species is required.

***Tetramesa sp. gayanus* A** The *Tetramesa* on *A. gayanus* was collected in northern Zimbabwe in 2022, and is most closely related to *Tetramesa sp. 7A* on *H. hirta* (9.8%). Although there were only two specimens in this group, it would be worth conducing additional surveys on *A. gayanus* in Zimbabwe, as there is likely to be a large degree of species diversity on the grass.

Tetramesa romana The *Tetramesa romana* specimens included in this study were all collected on *Arundo donax* in the Free State in 2011 and 2012, and therefore likely form part of the more widely-distributed genotype that was found to be distinct from the original French and Spanish strain, and from those in the Limpopo and Mpumalanga provinces (Ramanand *et al.*, 2018). It may be worth conducing an analysis of the molecular diversity of *T. romana* across its native range in Europe, as some *T. romana* genotypes have been found to be more successful than others, likely influenced largely by climatic suitability (Goolsby *et al.*, 2014). Being able to predict which populations are likely to successfully establish across different climatic regions may benefit the control of *A. donax* in South Africa.

4.4.4 Conclusion

This chapter has shown how the number of genetic sequences per morphospecies group can influence the percentage of singletons (which decreases with larger sample sizes) and splitting ratio values (which increases with larger sample sizes) resulting from the single-threshold GMYC species delimitation method. It also showed that the mitochondrial COI gene was more sensitive to the presence of singletons than the nuclear 28S region, particularly when sample sizes were small. Lastly, the SPEDE-sampler software presented here was useful in identifying morphospecies groups that may contain cryptic species, or where species diversity may have been overestimated. The software is broadly applicable across taxa, where single-locus genetic data are available. The present work has illustrated its usefulness in a biological control context, where the early identification of cryptic species and distinct intraspecific populations is vital.

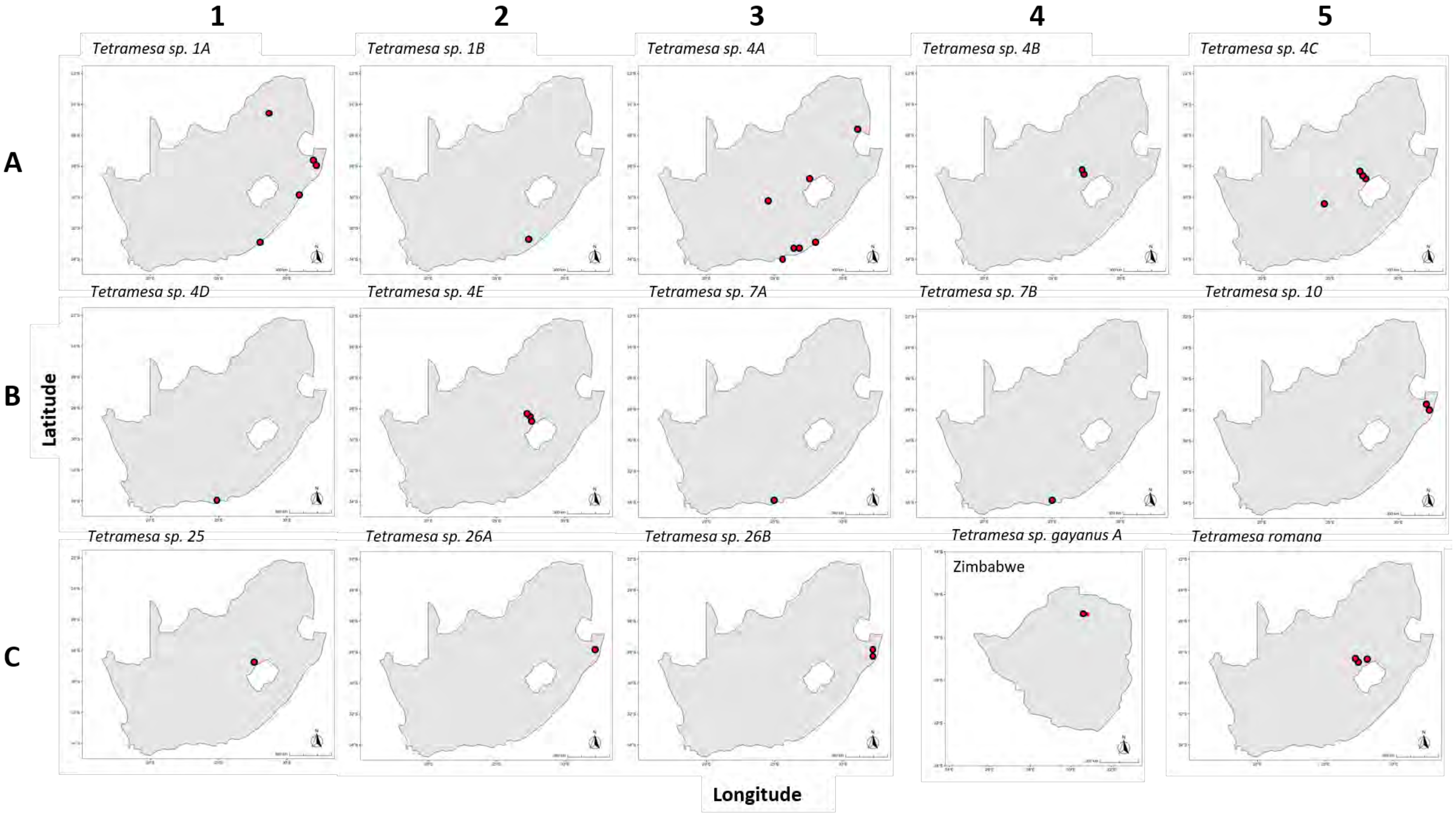


Figure 4.5: Distribution ranges for each proposed *Tetramesa* GMYC species, based on their collection sites.

Table 4.3: A summary table of *Tetramesa* GMYC species delimitations. Host SpR = host species richness, Host S_{TD} and Host PSV_t = an index of taxonomic host specificity and phylogenetic species variability, respectively (Abram *et al.*, 2021). The map panel index numbers refer to Fig. 4.5, the clade names and numbers refer to those used in Chapter 2 Fig. 2.9, and the images refer to the reference microscope images in Fig. S1. Province acronyms: EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, LP = Limpopo, MP = Mpumalanga, NC = Northern Cape, ZIM = Zimbabwe.

GMYC species	**Host plant/s**	**Map panel; Province/s**	**Clade name [number]**	**Host SpR**	**Host S_{TD}**	**Host PSV_t**	**Image**
Tetramesa sp. 1 A	*Sporobolus pyramidalis*	A1; EC, KZN, LP	PNS.SPOR.PYR [10]	1	1	0	S1 G
Tetramesa sp. 1 B	*Sporobolus africanus (?)*	A2; EC	PNS.SPOR [11]	1	1	0	S1 H
Tetramesa sp. 4 A	*Eragrostis curvula, E. biflora, E. plana, E. planiculmis*	A3; EC, FS, MP, NC	PNS.ECUR.1 [1]	4	1	0.091	S1 A
Tetramesa sp. 4 B	*Eragrostis curvula, E. plana, E. planiculmis*	A4; FS	PNS.ECUR.3 [6]	3	1	0.091	
Tetramesa sp. 4 C	*Eragrostis curvula, E. plana, E. planiculmis, E. capensis*	A5; FS, NC	PNS.ECUR.2 [4]	4	1	0.091	
Tetramesa sp. 4 D	*Eragrostis curvula, E. plana, E. planiculmis*	B1; EC	PNS.ECUR.4 [9]	3	1	0.091	
Tetramesa sp. 4 E	*Eragrostis curvula, E. biflora, E. capensis, E. plana, E. planiculmis*	B2; FS	P1 [12]	5	1	0.091	
Tetramesa sp. 7 A	*Hyparrhenia hirta*	B3; EC	HHIR.2 [22]	1	1	0	
Tetramesa sp. 7 B	*Hyparrhenia hirta*	B4; EC	HHIR.3 [24]	1	1	0	
Tetramesa sp. 10	*Eragrostis trichophora*	B5; KZN	PNS.ETRICH.1 [3]	1	1	0	S1 C
Tetramesa sp. 25	*Eragrostis plana*	C1; FS	PNS.EPLAN [5]	1	1	0	S1 D
Tetramesa sp. 26 A	*Eragrostis rigidior, E. gummiflua*	C2; KZN	PNS.ERIG.1 [7]	2	1	0.091	S1 E
Tetramesa sp. 26 B	*Eragrostis rigidior*	C3; KZN	PNS.ERIG.2 [8]	1	1	0	S1 F
Tetramesa sp. gayanus A	*Andropogon gayanus*	C4; ZIM	A.GAYA [23]	1	1	0	S1 N
Tetramesa romana	*Arundo donax*	C5; FS	ROM [25]	1	1	0	S1 O

Chapter 5

General Discussion

South Africa is a larger donor than receiver of invasive grasses (Visser *et al.*, 2016), but very little is known about the herbivorous arthropod assemblages associated with them. Grass biological control programmes have been limited in the past due to concerns about the apparent lack of host-specific natural enemies and the potential non-target damage that could arise on agriculturally-important cereals and grains. Recently, perceptions towards grass biological control have changed, and with this, the interest in the herbivores associated with African grasses has increased.

This thesis aimed to conduct a phylogenetic analysis of the herbivorous eurytomid wasps on native African grasses that are invasive elsewhere, focusing particularly on the diversity, host-use, and cophylogenetic patterns within the *Tetramesa* genus. These outcomes are important for both the biological control of invasive grasses, and the taxonomy of this historically-complex and under-sampled hymenopteran group in the Southern Hemisphere.

This final chapter discusses the suitability of using *Tetramesa* wasps as biological control agents of invasive southern African grasses. It then addresses the importance of the correct taxonomic identification of herbivore assemblages in a target weed's native range. The importance of understanding a herbivore's evolutionary history and how this can be informative in developing a successful biological control programme is then covered, and finally how sampling effects (e.g. data size and the proportion of singletons) can influence species delimitation results.

5.1 Are the *Tetramesa* suitable biological control agents of invasive southern African grasses?

In general, very little taxonomic work has focused on Afro-tropical insects, particularly micro-Hymenoptera such as the *Tetramesa* (van Noort *et al.*, 2015; Berry and van Noort, 2016; Olmi *et al.*, 2016; Hopkins *et al.*, 2019). This thesis has provided new insights into the *Tetramesa* assemblages on native African grasses by investigating their diversity, evolutionary history, and host-specificity. The simple answer to the question posed in the section heading is: yes, but some groups are more suitably host-specific than others, and care must be taken to use an integrative taxonomic approach rather than relying solely on one line of evidence for identification. The following sections explore which *Tetramesa* are suitable biological control agents based the phylogenetic results generated in this thesis, and discuss them in the context of the published literature.

5.1.1 Herbivorous eurytomids on African grasses

The taxonomic status of both the hymenopteran Eurytomidae family and the *Tetramesa* genus has been the subject of much debate since the early 1900s (Chapter 1). The family itself is not monophyletic (Chen *et al.*, 2004), very limited genetic data are available for members of the *Tetramesa*, and the morphological similarities between *Tetramesa* species makes identification very challenging (Chapter 1). African *Tetramesa* species only represent about 2% of the ∼ 200 described species globally (van Noort, 2020). This paucity of information poses a large knowledge gap in invasion biology and biological control because many invasive grass species are African in origin (Visser *et al.*, 2016). The present work is the first time that genetic sequences for native southern African *Tetramesa* have been generated and used to estimate taxonomic boundaries and assess coevolutionary herbivore-host relationships. The data suggest that there is a large diversity of undiscovered African eurytomid taxa, and that there are up to 15 putative *Tetramesa* MOTUs associated with grasses in the *Eragrostis* (MOTUs = 10), *Sporobolus* (MOTUs = 2), *Hyparrhenia hirta* (MOTUs = 2), and *Andropogon gayanus* (MOTUs = 1) genera surveyed (Chapter 2). These *Tetramesa* taxa are new to science, and require formal species descriptions and more extensive investigations into their life-histories. Most of the *Tetramesa* identified here were of the pronotal spot (PNS) morphotype; where only two MOTUs (the wasps on *A. gayanus* and *H. hirta*) did not have this feature (NPNS). The remaining NPNS clades fell outside the *Tetramesa*, and generally displayed broader host-ranges.

Tetramesa MOTUs displayed varying degrees of host-specificity, where those associated with *E. curvula* (*Tetramesa sp. 4*) tended to have broader host ranges than the two *Sporobolus* wasp groups (*S. pyramidalis* and the putative *S. africanus* host). Additionally, the *E. curvula Tetramesa sp. 4* complex showed evidence of geographic sub-structuring between the Eastern Cape and Free State provinces, and distinct populations within the Eastern Cape itself. These distinct populations may be the result of host-associated differentiation (HAD), discussed in Chapter 2.

A prominent caveat in species delimitation is the difficulty in differentiating between interspecific and intraspecific variation (e.g. due to recent speciation events or intraspecific geographic sub-structuring) (Sukumaran and Knowles, 2017; Derkarabetian *et al.*, 2019). In a study of species delimitation in the *Cataglyphis bicolor* Fab. (Hymenoptera: Formicidae) complex, for example, Eyer *et al.* (2017) found four distinct species groups, where it was proposed that one of these complexes, *C. niger* André, comprised at least three geographically-structured sub-groups that might be cryptic species. As with some of the *Tetramesa* complexes in the present study, it remains uncertain whether these three ant groups in Eyer *et al.* (2017) are in fact distinct species, or an artefact of intraspecific geographic variation. There are countless other examples of, and discussions about, this dilemma in the literature (e.g. Freudenstein *et al.* (2017); Gratton *et al.* (2016); Sukumaran and Knowles (2017); Yang *et al.* (2019); Chambers and Hillis (2020); Huang (2020)), which is best remedied with an integrative taxonomic approach (Carstens *et al.*, 2013). The dating analysis conducted in Chapter 2 provided evidence of the recent radiation of the *Tetramesa*, which would help in explaining the observed mitonuclear discordance between the mitochondrial and nuclear gene trees, and the high degree of substructuring in the mitochondrial COI phylogeny. Delimiting species that have radiated recently remains a major taxonomic challenge, and is discussed in more detail later in this chapter.

The phylogenetic data presented in this thesis suggest that the *Tetramesa* on *Sporobolus* *pyramidalis* (*Tetramesa sp. 1*) and *S. africanus* are suitably host specific for biological control, which is supported by the field-based surveys in Sutton *et al.* (2021b). *Tetramesa sp. 4* on *E. curvula*, however, may feed on non-target *Eragrostis* species in an invaded range. This is due to the wasp's ability to complete its lifecycle on native African *E. plana* and *E. planiculmis* (Guy Sutton pers. comm., unpublished data), which are genetically more distant from *E. curvula* than some native Australian *Eragrostis* species (e.g. *E. scotelliana*, *E. parviflora*, *E. papposa*, and *E. trachycarpa*, discussed in Chapter 3). It is recommended that this wasp undergoes further host-specificity testing, focusing on Australian native species analogous to the South African species that are known hosts, before being considered as a biological control agent for invasive *E. curvula*. It is not currently known whether the different *Tetramesa sp. 4* populations uncovered here cause differing levels of damage, whether they can hybridise, or whether they have differential thermal tolerances or other biological requirements that may affect establishment.

Although these results suggest precluding *Tetramesa* sp. 4 from further consideration for biological control, future work could focus on finer-scale analyses of the *Tetramesa sp. 4* complex in particular, using techniques such as DDRADseq, and the incorporation of climate matching to estimate in which regions of an invaded range these insects may have the highest chance of establishment. It would be valuable to utilise the population genetic methods used by Ramanand *et al.* (2018) for *T. romana* genotyping and apply that to the specimens in this thesis. Cross-breeding experiments would also be useful to complement species boundaries estimates, and even detect possible hybrids (e.g. the Paterson *et al.* (2016) example discussed in Chapter 1 Section 1.5.5). Additionally, the *Tetramesa* wasps on *E. plana*, *E. trichophora*, *A. gayanus*, and *H. hirta* require host-specificity testing, as these groups show promise as potential host-specific biological control agents. Greater sampling effort is required for *Tetramesa* on *A. gayanus* and *P. maximum*, as these are particularly problematic weeds in Australia and Texas (USA), respectively (Mercadier *et al.*, 2009; Rossiter-Rachor *et al.*, 2017).

5.2 Taxonomic and phylogenetic challenges

The phylogenetics of the *Tetramesa* have proven to be complex, although this is not surprising for a genus within a non-monophyletic family. Both the nuclear and mitochondrial gene trees revealed a number of unresolved clades, and brought into question the accuracy of sequence identifications on public genetic databases such as GenBank and BOLD. As this study progressed, it became evident that the *Tetramesa* genus requires a taxonomic revision that is based on the integration of morphological, behavioural, genetic, and ecological data, and that the revision should include Southern Hemisphere representatives. This section further discusses the challenges of *Tetramesa* species delimitation and taxonomy, and proposes a way forward for future genetic work.

5.2.1 Identification

Specimens within each of the two predominant morphotypes presented here (the pronotal and non-pronotal spot forms) are morphologically indistinguishable to a non-expert (Fig 5.1 A and B), which places a heavy reliance on genetic methods for identification and uncovering new taxonomic groups. A prominent issue that came to light in this work was that the only *Tetramesa* COI barcode sequences available on BOLD were assigned to the genus based solely on the Barcode Index Number (BIN) system (Ratnasingham and Hebert, 2013), without any formal morphological identification or associated biological information.

The BIN system is an automated approach in delineating taxonomic groups (MOTUs) based on COI sequence variation and similarity to known taxa on the BOLD database. The BIN method, and other automated delimitation algorithms (Chapter 2), are undoubtedly valuable tools in biodiversity assessments and as taxonomic aides, but they can be prone to being applied beyond the scope for which they were intended; much like a "one-size-fits-all" approach to taxonomy using only a single line of evidence (see for example the reviews by Will and Rubinoff (2004); DeSalle *et al.* (2005), and Shen *et al.* (2013)). This is particularly important to take note of when there is little to no verified genetic data for a taxonomic group, because incorrect identifications can create a flawed baseline and hinder taxonomic efforts (Collins and Cruickshank, 2013). It

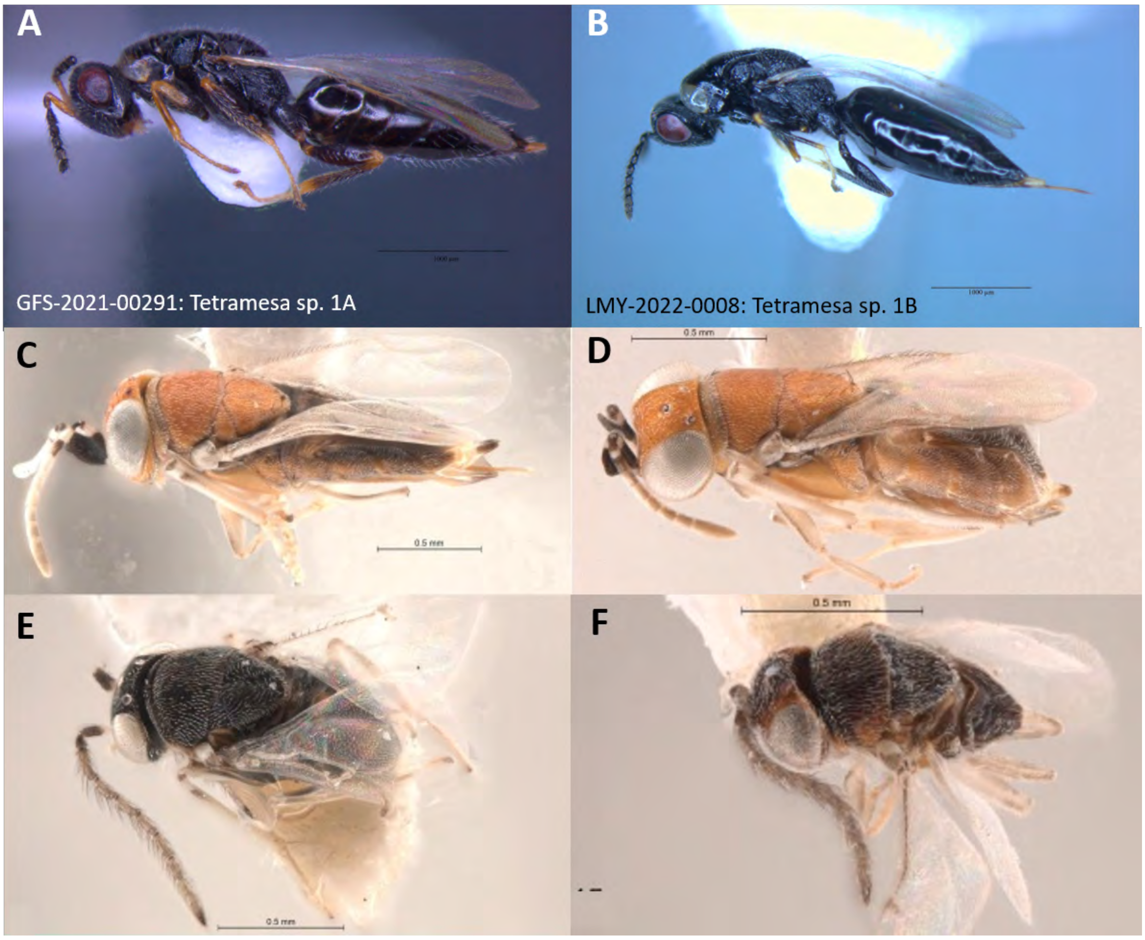


Figure 5.1: An image plate showing the morphological similarities between different wasp species, and the ease of misidentification. (A) *Tetramesa sp. 1A* from *Sporobolus pyramidalis* and (B) *Tetramesa sp. 1B* from *Sporobolus africanus* (present study), (C) *Anagyrus callidus* female, (D) *A. kamali* female, (E) *A. callidus* male, and (F) *A. kamali* male. Images C - F taken from Foster *et al.* (2021).

would be of great value to establish an online database for the *Tetramesa* as a collaborative effort between expert taxonomists, phylogenetecists, and biological control researchers; perhaps as a complement or addition to WaspWeb for Afro-tropical Hymenoptera (`http://www.waspweb.org/Afrotropical_wasps/index.htm`). Because identification accuracy can decrease when intraspecific variation increases with a larger geographic sampling range (Bergsten *et al.*, 2012; Gaytán *et al.*, 2020), it would be important to include specimens along a larger longitudinal gradient where possible. This would also offer insights into whether the genus originated in the Northern or Southern Hemisphere, and could lead to an interesting biogeographical study.

Misidentifying insect agents or target weeds can seriously impede the progress of a biological control programme because researchers may search for natural enemies in the wrong areas, select inappropriate agents, find that establishment rates and/or damage impacts are low or unsuccessful, have trouble in post-release monitoring, and observe potential non-target effects, which could be devastating to the reputation and safety of biological control as a discipline (Gordh, 1977). The misidentification of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) wasps for the control of the sugarcane borer *Diatraea saccharalis* Fab. (Lepidoptera: Crambidae) in Barbados, for example, led to the release of over 300 million *Trichogramma fasciatum* Perkins wasps over a 20 year period that were meant to be *T. minutum* Riley (Simmonds, 1972; Gordh, 1977; Pinto and Stouthamer, 1994). The programme was ineffective, and had to be terminated. Similarly, the parasitoid *T. chilonis* Ishii for the control of *Crocidolomia pavonana* F. (Lepidoptera: Crambidae), a pest of *Brassica* crops, was much more successful in Samoa than in other parts of Asia (Foster *et al.*, 2021). Foster *et al.* (2021) concluded that the wasp was either misidentified or comprises several undescribed cryptic species. A final example of misidentifications of hymenopteran agents is that of *Anagyrus pseudococci* Girault (Hymenoptera: Encyrtidae) complex, used to control mealybugs (Hemiptera: Pseudococcidae) worldwide (Noyes, 2012). Andreason *et al.* (2019) reported that the newly-described wasp species *A. callidus* (Triapitsyn, Andreason & Perring) for the control of the pink hibiscus mealybug (*Maconellicoccus hirsutus* Green) in California, USA, had been misidentified as *A. kamali* Moursi (Fig. 5.1 C - F). The wasp was mass-reared and released for three years (2014 - 2017) before genetic sequencing revealed that the insect had been misidentified.

With the recent advancement in both phylogenetic and machine learning methods, it may be feasible to use image recognition techniques in conjunction with genetic barcoding to decrease the likelihood of misidentifications in biological control programmes. With sufficient image data to serve as a training set, image recognition by means of computer vision is a very powerful tool and can be used to distinguish between taxa that may otherwise require an expert taxonomist (Valan *et al.*, 2019, 2021; Tariq *et al.*, 2022). Generating large image databases of the different *Tetramesa* taxa identified in this work may be worth pursuing for those that are considered for biological control. The present project has already started building up an image database, but it requires substantially more images.

This work has also highlighted the important role that expert taxonomists play in biological control research and invasion biology, and that although genetic barcoding and other phylogenetic methods are invaluable, the importance of classical taxonomy must not be underplayed. Ebach *et al.* (2011) warned that taxonomists are slowly being replaced by technologies such as DNA barcoding, and that the discipline is losing expertise. Will *et al.* (2005) went as far as calling DNA barcoding a "noisome weed", although this is arguably biased in itself. Ebach *et al.* (2011) argued that relying heavily on genetic technology is contributing to what the authors term a "two-fold taxonomic impediment"; to both the accuracy and speed at which taxonomic knowledge becomes available, and to the lack of funding and the generation of new research. Wheeler (2005), Rubinoff *et al.* (2006), and Pearson *et al.* (2011) expressed similar views, and called for an emphasis on integrative taxonomy that makes use of multiple-character systematics to discover, identify, and delimit taxa.

Having well-defined named species is integral to the practice of biological control, and is also important in biodiversity assessments and conservation efforts. The difficulty in delineating the *Tetramesa* and assigning appropriate terms to clades (i.e. is a clade of interest a species, intraspecific population, biotype, lineage, molecular operational taxonomic unit (MOTU), or tribe, for example?) certainly calls for increased taxonomic training - particularly for Afro-tropical insect groups - and supports the need for an integrative approach. The existence of intraspecific populations and cryptic species complexes within the *Tetramesa* will have important implications for biological control programmes, because these taxonomic groups may have differing damage impacts, establishment success rates, and climatic requirements that may need to be considered.

5.2.2 Evolutionary history

Biological control agents, as with any life-form, are not static entities. Each taxonomic group is at a different stage along an evolutionary trajectory. De Queiroz (1998)'s concept of taxa being more like metapopulation lineages than "species" is apt, where metapopulations are defined as groups of local populations that inhabit discrete habitats at a certain time (Hanski, 1998).

Taxa that have radiated recently pose a challenge to delimitation methods because they may not have reached a state of reproductive isolation or monophyly yet (Shaffer and Thomson, 2007) (Fig. 5.2). Rapid radiations are fairly common in parasitoid Hymenoptera, which is hypothesised to be due to host-switching to access novel ecological niches (Smith *et al.*, 2008; Endara *et al.*, 2018; Peters *et al.*, 2018; Klopfstein *et al.*, 2019). The nuclear dating analysis in Chapter 2 of this thesis suggested that the *Tetramesa* are also a recent radiation, only diversifying about 5 - 15 mya. This time frame coincides with the expansion of grasslands displacing forested habitats in Africa (Jacobs, 2004), and it is likely that various host-switches took place from grasses with a smaller geographic range to more abundant species (Chapter 3). The recent radiation and host-switching behaviour of the *Tetramesa* should be further investigated, because these properties may exclude them as biological control agents. The pattern of recent radiation, in addition to the effects of endosymbiotic *Wolbachia* bacteria on reproduction and inheritance, may assist in explaining (1) the unresolved clades reported in the present work, (2) the mitonuclear discordance between the COI and 28S gene trees, (3) the unexpectedly high COI sequence divergences between conspecific groups, (4) some of the host-specificity patterns observed in the *Eragrostis* and *Sporobolus* grass genera, and (5) the diversification of *Tetramesa* on *Eragrostis* species (which may be cryptic species complexes), with evidence of host-associated differentiation (HAD)(discussed in Chapter 2).

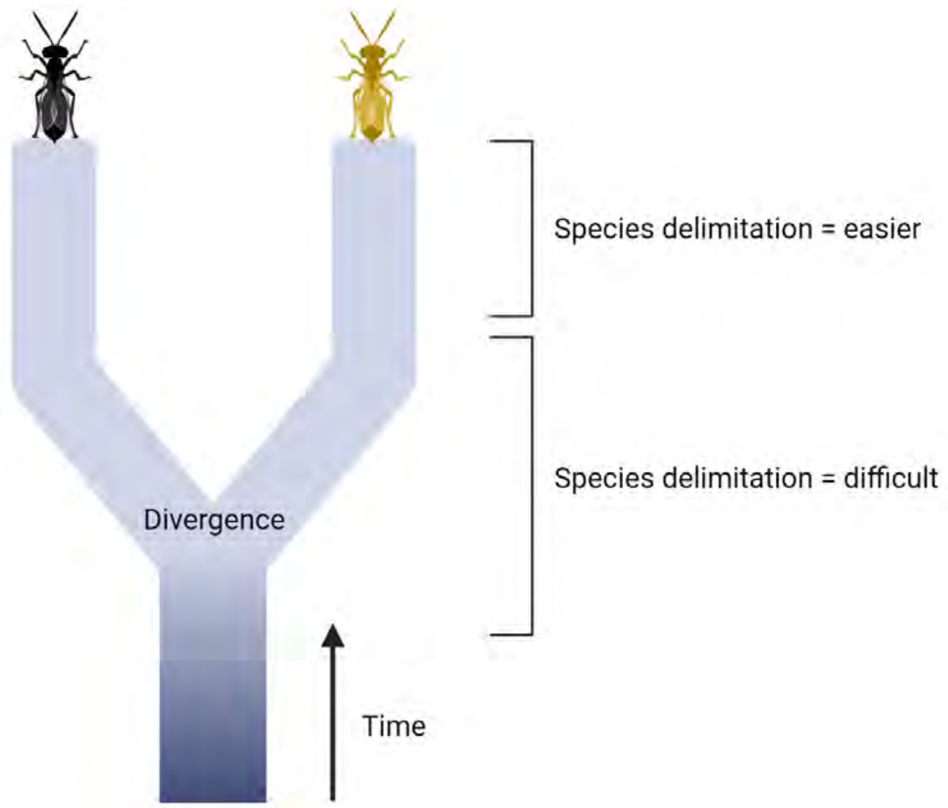


Figure 5.2: A diagram showing the divergence of species through time, where species delimitation becomes easier with older lineages that have reached a state of monophyly. Adapted from De Queiroz (1998).

It is surprising that biological control research studies have rarely incorporated cophylogenetic analyses and molecular dating exercises into the implementation pipeline. Chapters 2 and 3 have shown the value of these methods; particularly how the results can assist in developing grass species lists for host-specificity testing, predicting which *Tetramesa* agents may not be suitably host-specific for release, and explaining taxonomic oddities and polytomies in phylogenetic trees.

Future studies may benefit from delving into the *Wolbachia* associations within *Tetramesa* taxa. Since *Wolbachia* are known to be able to drive speciation in their hosts by generating a state of reproductive isolation (e.g. by inducing parthenogenesis) (Bordenstein *et al.*, 2001; Elias-Costa *et al.*, 2019), different *Wolbachia* strains may be useful in separating broader host species complexes using cophylogenetic analyses. An investigation of *Dactylopius tomentosus* Lamarck (Hemiptera: Dactylopiidae) lineages, for example, used *Wolbachia* sequences to complement a COI phylogeny, which assisted in the improved identification of three intraspecific lineages (Mathenge *et al.*, 2015). Similarly, in a study of the parasitoid wasp genus *Diplazon*

Nees von Esenbeck (Hymenoptera: Ichneumonidae), Klopfstein *et al.* (2016) found two species that shared identical COI haplotypes and *Wolbachia* strains, while the ITS2 region suggested that the same two species were not even closely related.

It would also be valuable to investigate the induced and constitutive chemical and structural defences present in target African grasses, as this might shed more light on *Tetramesa* host-specificity and feeding behaviour. McNaughton and Tarrants (1983) and McNaughton *et al.* (1985) conducted investigations into the use of silica as a structural defence by grasses in Tanzania, and found that herbivory led to an increase in silica content. This was also dependent on the quantity of silica available in the soil, which could be an important factor in geographically-separated grass populations that have adapted to different soil profiles. More recently, Gyan *et al.* (2020) found that *Eragrostis tef* responded to herbivory by the aphid *Rhopalosiphum padi* L. by up-regulating the production of volatile organic compounds (VOCs) and deterrent metabolites, and a switch from quantitative (carbon-rich) to more qualitative (nitrogen-rich) metabolites. Understanding the biochemistry of host plants can be very useful in understanding the possible differential feeding damage by different biological control agent taxa, and may assist in selecting herbivores that are more adapted to overcoming plant defences, and identifying climates and habitat types that may be more conducive to a control programme.

5.3 Sampling effects in phylogenetics and species delimitation

Every stage of a scientific investigation is inevitably open to errors and biases, from the data collection phase (e.g. prioritising natural enemy collection sites in the native range of a target weed (McCulloch *et al.*, 2022)) to laboratory protocols (e.g. polymerase chain reaction (PCR) and sequencing errors (Kunin *et al.*, 2010)) and choice of statistical analyses (e.g. alignment and tree-building method (Kapli *et al.*, 2020)). An appropriate sampling design (e.g. accounting for sample size and geographic distribution) lays the groundwork for any phylogenetic study (Austerlitz *et al.*, 2009; Zhang *et al.*, 2010; Bergsten *et al.*, 2012), where each set of requirements is usually taxon-specific. Additionally, the choice of analysis method and parameter settings can influence the interpretation of results, which is illustrated well in species delimitation output using different tools (e.g. tree or distance-based) (Carstens *et al.*, 2013; Rannala, 2015; Dellicour and Flot, 2018).

Researchers utilising DNA barcoding and applying species delimitation methods to their data need to be cognisant of the caveats associated with each step of an analysis protocol, and that the species concept debate will always persist because it is often impossible to place discrete boxes around life-forms that are continuously adapting to change under various selection pressures. Chapter 2 showed how multiple species delimitation methods can be used to gain a consensus output, and how more biologically-meaningful taxonomic groupings can be assigned with the addition of ecological (e.g. host plant affiliation, and gall formation in the PNS *Tetramesa* on *E. trichophora*) and geographical (e.g. local to provincial scales) information.

Sampling effects such as the number of genetic sequences used per morphospecies group, the abundance of singletons, and the choice of genetic marker can play a significant role in the splitting and lumping behaviour of a delimitation algorithm such as the widely-used GMYC model (Talavera *et al.*, 2013; Hamilton *et al.*, 2014). Chapter 4 showed how a similar abundance of singletons had a greater effect on species diversity estimates in the COI marker than the 28S region, and how singletons can lead to overestimates of species diversity; particularly when data sizes are small. These are useful indicators for optimising a phylogenetic study and making meaningful inferences from the data. The SPEDE-sampler software presented in this thesis (Chapter 4) is broadly applicable across taxonomic groups for single-locus genetic data, and is an additional valuable tool in the biological control researcher's toolkit.

5.4 Conclusion

Correctly identifying potential insect agents at every step of a biological control pipeline is vital to its safety and success. This thesis has shown how molecular tools that include DNA barcoding, species delimitation, and cophylogenetic analyses can assist in the initial screening for potential new insect taxa, determining host-insect evolutionary histories, and estimating the host ranges and suitability of potential biological control agents.

Despite the previous trepidation about applying biological control to invasive grasses, this work has shown that some of the *Tetramesa* taxa identified here hold promise as potential agents due to their host-specificity and diversity on African grasses. This thesis has highlighted the difficulties in phylogenetic analyses when mitonuclear discordance is uncovered, morphospecies groups are identical, and sampling effects influence species delimitation output. The application of integrative taxonomy is key, where the present project has opened the door to further the discovery of African eurytomid diversity, and the advancement of control programmes for invasive African grasses in other regions of the world.

References

Abella, S. R., 2009. Post-fire plant recovery in the Mojave and Sonoran Deserts of western North America. *Journal of Arid Environments*, 73(8):699–707. doi:10.1016/j.jaridenv.2009.03.003.

Abram, P. K., Labbe, R. M., and Mason, P. G., 2021. Ranking the host range of biological control agents with quantitative metrics of taxonomic specificity. *Biological Control*, 152: 104427. doi:10.1016/j.biocontrol.2020.104427.

Aduse-Poku, K., van Bergen, E., Sáfián, S., Collins, S. C., Etienne, R. S., Herrera-Alsina, L., Brakefield, P. M., Brattström, O., Lohman, D. J., and Wahlberg, N., 2022. Miocene Climate and Habitat Change Drove Diversification in *Bicyclus*, Africa's Largest Radiation of Satyrine Butterflies. *Systematic Biology*, 71(3):570–588. doi:10.1093/sysbio/syab066.

Ahrens, C. W., James, E. A., Miller, A. D., Scott, F., Aitken, N. C., Jones, A. W., Lu-Irving, P., Borevitz, J. O., Cantrill, D. J., and Rymer, P. D., 2020. Spatial, climate and ploidy factors drive genomic diversity and resilience in the widespread grass *Themeda triandra*. *Molecular Ecology*, 29(20):3872–3888. doi:10.1111/mec.15614.

Ahrens, D., Fujisawa, T., Krammer, H.-J., Eberle, J., Fabrizi, S., and Vogler, A. P., 2016. Rarity and incomplete sampling in DNA-based species delimitation. *Systematic Biology*, 65(3):478–494. doi:10.1093/sysbio/syw002.

Al-Barrak, M., 2006. Host choice in *Tetramesa petiolata* (Walker) (Hymenoptera: Eurytomidae). *Journal of Entomology*, 3:55–60.

Al-Barrak, M., Loxdale, H., Brookes, C., Dawah, H., Biron, D., and Alsagair, O., 2004. Molecular evidence using enzyme and RAPD markers for sympatric evolution in British species of *Tetramesa* (Hymenoptera: Eurytomidae). *Biological Journal of the Linnean Society*, 83(4):509–525. doi:10.1111/j.1095-8312.2004.00408.x.

Alonso-Zarazaga, M. and Sanchez-Ruiz, M., 2002. A revision of the *Trichosirocalus horridus* species complex, with description of two new species (Coleoptera, Curculionidae, Ceutorhynchinae). *Australian Journal of Entomology*, 41:199–208.

Andersen, J. C. and Wagner, D. L., 2016. Systematics and biological control. *Integrating Biological Control into Conservation Practice*, pages 105–129. doi:10.1002/9781118392553.ch6.

Anderson, F. E., Gallego, L., Sánchez, R. M., Flemmer, A. C., Hansen, P. V., McLaren, D., and Barton, J., 2017. Plant/pathogen interactions observed during host range testing of the rust fungus *Uromyces pencanus*, a classical biological control agent for Chilean needle grass (*Nassella neesiana*) in Australia and New Zealand. *Biocontrol Science and Technology*, 27(9):1096–1117. doi:10.1080/09583157.2017.1384795.

Andreason, S. A., Triapitsyn, S. V., and Perring, T. M., 2019. Untangling the *Anagyrus pseudococci* species complex (Hymenoptera: Encyrtidae), parasitoids of worldwide importance for biological control of mealybugs (Hemiptera: Pseudococcidae): Genetic data corroborates separation of two new, previously misidentified species. *Biological Control*, 129:65–82. doi:10.1016/j.biocontrol.2018.09.010.

Anguita-Salinas, S., Barahona-Segovia, R. M., Poulin, E., and Zuniga-Reinoso, A., 2017. Genetic and morphological evidence for a new cryptic species of *Ectinogonia* (Coleoptera: Buprestidae) from central Chile. *Zootaxa*, 4303(2):284–292. doi:10.11646/zootaxa.4303.2.8.

Antonini, G., Audisio, P., De Biase, A., Mancini, E., Rector, B., Cristofaro, M., Biondi, M., Korotyaev, B., Bon, M., Konstantinov, A., *et al.*, 2008. The importance of molecular tools in classical biological control of weeds: two case studies with yellow starthistle candidate biocontrol agents. In *Proceedings of the 12th International Symposium on Biological Control of Weeds. Wallingford, UK: CAB*, pages 263–269, 2008.

Ashelford, K. E., Chuzhanova, N. A., Fry, J. C., Jones, A. J., and Weightman, A. J., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Applied and environmental microbiology*, 71(12):7724–7736. doi:10.1128/AEM.71.12.7724-7736.2005.

Augustyn, W. J., Anderson, B., Van der Merwe, J. F., and Ellis, A. G., 2017. Spatial turnover in host-plant availability drives host-associated divergence in a South African leafhopper (*Cephalelus uncinatus*). *BMC Evolutionary Biology*, 17(1):1–14. doi:10.1186/s12862-017-0916-0.

Austerlitz, F., David, O., Schaeffer, B., Bleakley, K., Olteanu, M., Leblois, R., Veuille, M., and Laredo, C., 2009. DNA barcode analysis: a comparison of phylogenetic and statistical classification methods. *BMC Bioinformatics*, 10(14):1–13. doi:10.1186/1471-2105-10-S14-S10.

Ayres, D. R., Zaremba, K., and Strong, D. R., 2004. Extinction of a common native species by hybridization with an invasive congener. *Weed Technology*, 18(sp1):1288–1291. doi:10.1614/0890-037X(2004)018[1288:EOACNS]2.0.CO;2.

Bailey, D. W., Mosley, J. C., Estell, R. E., Cibils, A. F., Horney, M., Hendrickson, J. R., Walker, J. W., Launchbaugh, K. L., and Burritt, E. A., 2019. Synthesis paper: targeted livestock grazing: prescription for healthy rangelands. *Rangeland Ecology & Management*, 72(6):865–877. doi:10.1016/j.rama.2019.06.003.

Balbuena, J. A., Míguez-Lozano, R., and Blasco-Costa, I., 2013. PACo: a novel procrustes application to cophylogenetic analysis. *Public Library of Science One*, 8(4):e61048. doi:10.1371/journal.pone.0061048.

Ballard, J. W. O. and Whitlock, M. C., 2004. The Incomplete Natural History of Mitochondria. *Molecular Ecology*, 13(4):729–744. doi:10.1046/j.1365-294X.2003.02063.x.

Banks, J., Palma, R., and Paterson, A., 2006. Cophylogenetic relationships between penguins and their chewing lice. *Journal of Evolutionary Biology*, 19(1):156–166. doi:10.1111/j.1420-9101.2005.00983.x.

Barnes, T. G., DeMaso, S. J., and Bahm, M. A., 2013. The impact of 3 exotic, invasive grasses in the southeastern United States on wildlife. *Wildlife Society Bulletin*, 37(3):497–502. doi:10.1002/wsb.316.

Barrett, R. L., Peterson, P. M., and Romaschenko, K., 2020. A molecular phylogeny of *Eragrostis* (Poaceae: Chloridoideae: Eragrostideae): making lovegrass monophyletic in Australia. *Australian Systematic Botany*, 33(5):458–476. doi:10.1071/SB19034.

Batten, K. M., Scow, K. M., and Espeland, E. K., 2008. Soil microbial community associated with an invasive grass differentially impacts native plant performance. *Microbial Ecology*, 55(2):220–228. doi:10.1007/s00248-007-9269-3.

Beaulieu, J. M., Tank, D. C., and Donoghue, M. J., 2013. A Southern Hemisphere origin for campanulid angiosperms, with traces of the break-up of Gondwana. *BMC Evolutionary Biology*, 13(1):1–17. doi:10.1186/1471-2148-13-80.

Beerling, D. J. and Osborne, C. P., 2006. The origin of the savanna biome. *Global Change Biology*, 12(11):2023–2031. doi:10.1111/j.1365-2486.2006.01239.x.

Begg, G. S., Cook, S. M., Dye, R., Ferrante, M., Franck, P., Lavigne, C., Lövei, G. L., Mansion-Vaquie, A., Pell, J. K., Petit, S., *et al.*, 2017. A functional overview of conservation biological control. *Crop Protection*, 97:145–158. doi:10.1016/j.cropro.2016.11.008.

Bell, G. P., 1997. Ecology and management of *Arundo donax*, and approaches to riparian habitat restoration in southern California. In *Plant Invasion: Studies from North America and Europe*, pages 104—114, 1997.

Bell, K. L., Heard, T. A., Manion, G., Ferrier, S., and van Klinken, R. D., 2013. The role of geography and environment in species turnover: phytophagous arthropods on a Neotropical legume. *Journal of Biogeography*, 40(9):1755–1766. doi:10.1111/jbi.12102.

Bell, M. A. and Lloyd, G. T., 2015. strap: an R package for plotting phylogenies against stratigraphy and assessing their stratigraphic congruence. Technical report, Wiley Online Library.

Bergsten, J., Bilton, D. T., Fujisawa, T., Elliott, M., Monaghan, M. T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, G. N., *et al.*, 2012. The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology*, 61(5):851–869. doi:10.1093/sysbio/sys037.

Bernays, E., 1988. Host specificity in phytophagous insects: selection pressure from generalist predators. *Entomologia experimentalis et applicata*, 49(1-2):131–140. doi:10.1111/j.1570-7458.1988.tb02484.x.

Bernays, E. and Graham, M., 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology*, 69(4):886–892. doi:10.2307/1941237.

Berry, T. R. and van Noort, S., 2016. Review of Afrotropical *Cryptopimpla* Taschenberg (Hymenoptera, Ichneumonidae, Banchinae), with description of nine new species. *ZooKeys*, (640):103. doi:10.3897/zookeys.640.10334.

Bertone, M. A., Courtney, G. W., and Wiegmann, B. M., 2008. Phylogenetics and temporal diversification of the earliest true flies (insecta: Diptera) based on multiple nuclear genes. *Systematic Entomology*, 33(4):668–687. doi:10.1111/j.1365-3113.2008.00437.x.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K., Meier, R., Winker, K., Ingram, K. K., and Das, I., 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, 22(3):148–155. doi:10.1016/j.tree.2006.11.004.

Bilderbeek, R. beautier: BEAUti 2 from R. https://github.com/richelbilderbeek/beastier, 2018a.

Bilderbeek, R. tracerer: Tracer from R. https://github.com/richelbilderbeek/tracerer, 2018b.

Bin, F., Roversi, P. F., and Van Lenteren, J. C., 2012. Erroneous host identification frustrates systematics and delays implementation of biological control. *Redia*, 95:83–88.

Blair, C. and Bryson Jr, R. W., 2017. Cryptic diversity and discordance in single-locus species delimitation methods within horned lizards (Phrynosomatidae: *Phrynosoma*). *Molecular Ecology Resources*, 17(6):1168–1182. doi:10.1111/1755-0998.12658.

Blossey, B., Häfliger, P., Tewksbury, L., Dávalos, A., and Casagrande, R., 2018. Host specificity and risk assessment of *Archanara geminipuncta* and *Archanara neurica*, two potential biocontrol agents for invasive *Phragmites australis* in North America. *Biological Control*, 125:98–112. doi:10.1016/j.biocontrol.2018.05.019.

Blossey, B., Endriss, S. B., Casagrande, R., Häfliger, P., Hinz, H., Dávalos, A., Brown-Lima, C., Tewksbury, L., and Bourchier, R. S., 2020. When misconceptions impede best practices: evidence supports biological control of invasive *Phragmites*. *Biological Invasions*, 22(3): 873–883. doi:10.1007/s10530-019-02166-8.

Boland, J. M., 2008. The roles of floods and bulldozers in the break-up and dispersal of *Arundo donax* (Giant reed). *Madrono*, 55(3):216–222. doi:10.3120/0024-9637-55.3.216.

BOLD. BarCode of Life (BOLD), 2023. URL `https://www.ncbi.nlm.nih.gov/genbank/barcode/`. Accessed 2-01-2023.

Bordenstein, S. R., O'Hara, F. P., and Werren, J. H., 2001. Wolbachia-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*, 409(6821):707–710. doi:10.1038/35055543.

Bouchenak-Khelladi, Y., Maurin, O., Hurter, J., and Van der Bank, M., 2010. The evolutionary history and biogeography of Mimosoideae (Leguminosae): an emphasis on African acacias. *Molecular Phylogenetics and Evolution*, 57(2):495–508. doi:10.1016/j.ympev.2010.07.019.

Bouckaert, R., Vaughan, T. G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., Heled, J., Jones, G., Kühnert, D., De Maio, N., *et al.*, 2019. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *Public Library of Science Computational Biology*, 15(4):e1006650. doi:10.1371/journal.pcbi.1006650.

Brede, E. G., Adis, J., and Schneider, P., 2008. Genetic diversity, population structure and gene flow in native populations of a proposed biocontrol agent (*Cornops aquaticum*). *Biological Journal of the Linnean Society*, 95(4):666–676. doi:10.1111/j.1095-8312.2008.00993.x.

Brewer, S., 2008. Declines in plant species richness and endemic plant species in longleaf pine savannas invaded by *Imperata cylindrica*. *Biological Invasions*, 10(8):1257–1264. doi:10.1007/s10530-007-9200-3.

Bridge, P. D., Roberts, P. J., Spooner, B. M., and Panchal, G., 2003. On the unreliability of published DNA sequences. *New Phytologist*, 160(1):43–48. doi:10.1046/j.1469-8137.2003.00861.x.

Briese, D., 2003. The centrifugal phylogenetic method used to select plants for host-specificity testing of weed biological control agents: Can and should it be modernised? *CSIRO and CRC for Australian Weed Management*, pages 25–27.

Briese, D., 2005. Translating host-specificity test results into the real world: the need to harmonize the yin and yang of current testing procedures. *Biological Control*, 35(3):208–214. doi:10.1016/j.biocontrol.2005.02.001.

Briese, D. and Walker, A., 2008. Choosing the right plants to test: the host-specificity of *Longitarsus* sp.(Coleoptera: Chrysomelidae) a potential biological control agent of *Heliotropium amplexicaule*. *Biological Control*, 44(3):271–285. doi:10.1016/j.biocontrol.2007.05.001.

Britton, T., Anderson, C. L., Jacquet, D., Lundqvist, S., and Bremer, K., 2007. Estimating divergence times in large phylogenetic trees. *Systematic Biology*, 56(5):741–752. doi:10.1080/10635150701613783.

Brown, M. S., Blubaugh, C. K., and Chong, J. H., 2021. Biology and management of eriophyid mites in turfgrass. *Journal of Integrated Pest Management*, 12(1):25. doi:10.1093/jipm/pmab020.

Buckley, T. R., Attanayake, D., Nylander, J. A., and Bradler, S., 2010. The phylogenetic placement and biogeographical origins of the New Zealand stick insects (Phasmatodea). *Systematic Entomology*, 35(2):207–225. doi:10.1111/j.1365-3113.2009.00505.x.

Buhay, J. E., 2009. "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. *Journal of Crustacean Biology*, 29(1):96–110. doi:10.1651/08-3020.1.

Burgess, T. L., Bowers, J. E., and Turner, R. M., 1991. Exotic plants at the desert laboratory, Tucson, Arizona. *Madroño*, pages 96–114.

Búrquez-Montijo, A., Miller, M., Martínez-Yrízar, A., *et al.*, 2002. Mexican grasslands, thornscrub, and the transformation of the Sonoran Desert by invasive exotic buffelgrass (*Pennisetum ciliare*). *Invasive exotic species in the Sonoran region*.

Butler, B. D. W. and Fairfax, R. J., 2003. Buffel grass and fire in a gidgee and brigalow woodland: a case study from central Queensland. *Ecological Management & Restoration*, 4(2):120–125. doi:10.1046/j.1442-8903.2003.00146.x.

Byrne, D., Scheben, A., Scott, J. K., Webber, B. L., Batchelor, K. L., Severn-Ellis, A. A., Gooden, B., and Bell, K. L., 2022. Genomics reveals the history of a complex plant invasion and improves the management of a biological invasion from the South African–Australian biotic exchange. *Ecology and Evolution*, 12(8):e9179. doi:10.1002/ece3.9179.

Cabrera Walsh, G., Magalí Dalto, Y., Mattioli, F. M., Carruthers, R. I., and Anderson, L. W., 2013. Biology and ecology of Brazilian elodea (*Egeria densa*) and its specific herbivore, *Hydrellia* sp., in Argentina. *Biocontrol*, 58(1):133–147. doi:10.1007/s10526-012-9475-x.

Campbell, B., Heraty, J., Rasplus, J., Chan, K., Steffen-Campbell, J., Babcock, C., Austin, A., and Dowton, M., 2000. The Hymenoptera: evolution, biodiversity and biological control.

Campbell, B., Steffen-Campbell, J., and Werren, J., 1994. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology*, 2(4):225–237. doi:10.1111/j.1365-2583.1994.tb00142.x.

Canavan, K., Paterson, I., Hill, M., and Dudley, T., 2019a. Testing the Enemy Release Hypothesis on tall-statured grasses in South Africa, using *Arundo donax*, *Phragmites australis*, and *Phragmites mauritianus* as models. *Bulletin of Entomological Research*, 109 (3):287–299. doi:10.1017/S0007485318000627.

Canavan, K., Canavan, S., Harms, N. E., Lambertini, C., Paterson, I. D., and Thum, R., 2020. The potential for biological control on cryptic plant invasions. *Biological Control*, 144:104243. doi:10.1016/j.biocontrol.2020.104243.

Canavan, S., Meyerson, L. A., Packer, J. G., Pyšek, P., Maurel, N., Lozano, V., Richardson, D. M., Brundu, G., Canavan, K., Cicatelli, A., *et al.*, 2019b. Tall-statured grasses: a useful functional group for invasion science. *Biological Invasions*, 21(1):37–58. doi:10.1007/s10530-018-1815-z.

Cao, Y. and Dietrich, C. H., 2022. Phylogenomics of flavobacterial insect nutritional endosymbionts with implications for Auchenorrhyncha phylogeny. *Cladistics*, 38(1):38–58. doi:10.1111/cla.12474.

Carmean, D., Kimsey, L. S., and Berbee, M. L., 1992. 18S rDNA sequences and the holometabolous insects. *Molecular Phylogenetics and Evolution*, 1(4):270–278. doi:10.1016/1055-7903(92)90002-X.

Carpenter, D. J., Mathiassen, S. K., Boutin, C., Strandberg, B., Casey, C. S., and Damgaard, C., 2020. Effects of herbicides on flowering. *Environmental Toxicology and Chemistry*, 39(6):1244–1256. doi:10.1002/etc.4712.

Carson, W. P., Hovick, S. M., Baumert, A. J., Bunker, D. E., and Pendergast, T. H., 2008. Evaluating the post-release efficacy of invasive plant biocontrol by insects: a comprehensive approach. *Arthropod-Plant Interactions*, 2(2):77–86. doi:10.1007/s11829-008-9036-5.

Carstens, B. C., Pelletier, T. A., Reid, N. M., and Satler, J. D., 2013. How to fail at species delimitation. *Molecular ecology*, 22(17):4369–4383. doi:10.1111/mec.12413.

Casagrande, R. A., Häfliger, P., Hinz, H. L., Tewksbury, L., and Blossey, B., 2018. Grasses as appropriate targets in weed biocontrol: is the common reed, *Phragmites australis*, an anomaly? *BioControl*, 63(3):391–403. doi:10.1007/s10526-018-9871-y.

Casiraghi, M., Labra, M., Ferri, E., Galimberti, A., and De Mattia, F., 2010. DNA barcoding: a six-question tour to improve users' awareness about the method. *Briefings in bioinformatics*, 11(4):440–453. doi:10.1093/bib/bbq003.

Caswell, H., Reed, F., Stephenson, S., and Werner, P. A., 1973. Photosynthetic pathways and selective herbivory: a hypothesis. *The American Naturalist*, 107(956):465–480. doi:10.1086/282851.

Ceccarelli, F. S., Sharkey, M. J., and Zaldívar-Riverón, A., 2012. Species identification in the taxonomically neglected, highly diverse, neotropical parasitoid wasp genus *Notiospathius* (Braconidae: Doryctinae) based on an integrative molecular and morphological approach. *Molecular Phylogenetics and Evolution*, 62(1):485–495. doi:10.1016/j.ympev.2011.10.018.

Chambers, E. A. and Hillis, D. M., 2020. The multispecies coalescent over-splits species in the case of geographically widespread taxa. *Systematic Biology*, 69(1):184–193. doi:10.1093/sysbio/syz042.

Charleston, M. and Libeskind-Hadas, R., 2014. Event-based cophylogenetic comparative analysis. In *Modern phylogenetic comparative methods and their application in evolutionary biology*, pages 465–480. Springer. doi:10.1007/978-3-662-43550-2_23.

Charleston, M. A. and Perkins, S. L., 2006. Traversing the tangle: algorithms and applications for cophylogenetic studies. *Journal of biomedical informatics*, 39(1):62–71.

Chejara, K. V., Kriticos, D., Kristiansen, P., Sindel, B. M., Whalley, R., and Nadolny, C., 2010. The current and future potential geographical distribution of *Hyparrhenia hirta*. *Weed Research*, 50(2):174–184. doi:10.1111/j.1365-3180.2010.00765.x.

Chejara, V. K., Nadolny, C., Kristiansen, P., Whalley, R., Sindel, B. M., *et al.*, 2006. Impacts of *Hyparrhenia hirta* (L.) Stapf (Coolatai grass) on native vegetation in a travelling stock route in northern New South Wales. In *Papers and Proceedings. 15th Australian Weeds Conference'.(Eds C. Preston, JH Watts and ND Crossman.) pp*, pages 207–210, 2006.

Chen, Y., Xiao, H., Fu, J., and Huang, D.-W., 2004. A molecular phylogeny of eurytomid wasps inferred from DNA sequence data of 28S, 18S, 16S, and COI genes. *Molecular Phylogenetics and Evolution*, 31(1):300–307. doi:10.1016/S1055-7903(03)00282-3.

Chernomor, O., Von Haeseler, A., and Minh, B. Q., 2016. Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology*, 65(6):997–1008. doi:10.1093/sysbio/syw037.

Cibils-Stewart, X., Mace, W. J., Popay, A. J., Lattanzi, F. A., Hartley, S. S. E., Hall, C. R., Powell, J. R., and Johnson, S. N., 2022. Interactions between silicon and alkaloid defences in endophyte-infected grasses and the consequences for a folivore. *Functional Ecology*, 36(1):249–261. doi:10.1111/1365-2435.13916.

Cidade, F. W., Vigna, B. B., De Souza, F. H., Valls, J. F. M., Dall'Agnol, M., Zucchi, M. I., de Souza-Chies, T. T., and Souza, A. P., 2013. Genetic variation in polyploid forage grass: assessing the molecular genetic variability in the *Paspalum* genus. *BMC genetics*, 14:1–19. doi:10.1186/1471-2156-14-50.

Claridge, M., 1961. A contribution to the biology and taxonomy of some Palaearctic species of *Tetramesa* Walker (= *Isosoma* Walk.;= *Harmolita* Motsch.)(Hymenoptera: Eurytomidae), with particular reference to the British fauna. *Transactions of the Royal Entomological Society of London*, 113(9):175–217. doi:10.1111/j.1365-2311.1961.tb00807.x.

Clayton, W. D., Vorontsova, M. S., Harman, K. T., and Williamson, H. Grassbase - the online world grass flora, 2021.

Clement, M., Snell, Q., Walker, P., Posada, D., and Crandall, K., 2002. TCS: Estimating gene genealogies. *Parallel and Distributed Processing Symposium, International Proceedings*, 2:184.

Coetzee, J., Hill, M., Byrne, M., and Bownes, A., 2011. A review of the biological control programmes on *Eichhornia crassipes* (C. Mart.) Solms (Pontederiaceae), *Salvinia molesta* DS Mitch.(Salviniaceae), *Pistia stratiotes* L.(Araceae), *Myriophyllum aquaticum* (Vell.) Verdc.(Haloragaceae) and *Azolla filiculoides* Lam.(Azollaceae) in South Africa. *African Entomology*, 19(1):451–468. doi:10520/EJC32900.

Coetzee, J., Bownes, A., Martin, G., Miller, B., Smith, R., Weyl, P., and Hill, M., 2021. A review of the biocontrol programmes against aquatic weeds in South Africa. *African Entomology*, 29(3):935–964. doi:10520/ejc-ento_v29_n3_a18.

Coetzee, J. A., Miller, B. E., Kinsler, D., Sebola, K., and Hill, M. P., 2022. It's a numbers game: inundative biological control of water hyacinth (*Pontederia crassipes*), using *Megamelus scutellaris* (Hemiptera: Delphacidae) yields success at a high elevation, hypertrophic reservoir in South Africa. *Biocontrol Science and Technology*, pages 1–10. doi:10.1080/09583157.2022.2109594.

Coffman, G. C., 2020. Giant Reed (*Arundo donax*): Streams and Water Resources. In *Managing Water Resources and Hydrological Systems*, pages 327–335. CRC Press.

Collier, T. and Van Steenwyk, R., 2004. A critical evaluation of augmentative biological control. *Biological Control*, 31(2):245–256. doi:10.1016/j.biocontrol.2004.05.001.

Collin, R., Venera-Pontón, D. E., Driskell, A. C., Macdonald, K. S., Geyer, L. B., Lessios, H. A., and Boyle, M. J., 2020. NA barcoding of *Echinopluteus* larvae uncovers cryptic diversity in neotropical echinoids. *Invertebrate Biology*, 139(2):e12292. doi:10.1111/ivb.12292.

Collins, R. and Cruickshank, R., 2013. The seven deadly sins of DNA barcoding. *Molecular Ecology Resources*, 13(6):969–975. doi:10.1111/1755-0998.12046.

Conow, C., Fielder, D., Ovadia, Y., and Libeskind-Hadas, R., 2010. Jane: a new tool for the cophylogeny reconstruction problem. *Algorithms for Molecular Biology*, 5(1):1–10. doi:10.1186/1748-7188-5-16.

Cook, B. D., Page, T. J., and Hughes, J. M., 2008. Importance of cryptic species for identifying 'representative' units of biodiversity for freshwater conservation. *Biological Conservation*, 141(11):2821–2831. doi:10.1016/j.biocon.2008.08.018.

Cook, G. D. and Dias, L., 2006. It was no accident: deliberate plant introductions by Australian government agencies during the 20th century. *Australian Journal of Botany*, 54(7):601–625. doi:10.1071/BT05157.

Cornell, H., 1989. Endophage-ectophage ratios and plant defense. *Evolutionary Ecology*, 3(1):64–76. doi:10.1007/BF02147932.

Cristofaro, M., Sale, F., Campobasso, G., Knutson, L., and Sbordoni, V., 1998. Biology and host preference of *Nephopteryx divisella* (Lepidoptera: Pyralidae): Candidate agent for biological control of leafy spurge complex in North America. *Environmental Entomology*, 27(3):731–735.

Cruaud, A. and Rasplus, J.-Y., 2016. Testing cospeciation through large-scale cophylogenetic studies. *Current Opinion in Insect Science*, 18:53–59. doi:10.1016/j.cois.2016.10.004.

Cullen, J., 1995. Predicting effectiveness: fact and fantasy. In Delfosse, E. and Scott, R., editors, *Proceedings of the VIII International Symposium on Biological Control of Weeds*, pages 103–109. Agriculture Canada, 1995.

Culliney, T. W., 2005. Benefits of Classical Biological Control for Managing Invasive Plants. *Critical Reviews in Plant Sciences*, 24(2):131–150. doi:10.1080/07352680590961649.

Daehler, C. C. and Carino, D. A., 1998. Recent replacement of native pili grass (*Heteropogon contortus*) by invasive African grasses in the Hawaiian Islands. *Pacific Science*, 52(3):220–227.

Daehler, C. C. and Goergen, E. M., 2005. Experimental restoration of an indigenous Hawaiian grassland after invasion by buffel grass (*Cenchrus ciliaris*). *Restoration Ecology*, 13(2): 380–389. doi:10.1111/j.1526-100X.2005.00047.x.

Damasceno, G., Souza, L., Pivello, V. R., Gorgone-Barbosa, E., Giroldo, P. Z., and Fidelis, A., 2018. Impact of invasive grasses on cerrado under natural regeneration. *Biological Invasions*, 20(12):3621–3629. doi:10.1007/s10530-018-1800-6.

Daneshgar, P., Jose, S., Collins, A., and Ramsey, C., 2008. Cogongrass (*Imperata cylindrica*), an alien invasive grass, reduces survival and productivity of an establishing pine forest. *Forest Science*, 54(6):579–587. doi:10.1093/forestscience/54.6.579.

D'Antonio, C. M. and Vitousek, P. M., 1992. Biological invasions by exotic grasses, the grass/fire cycle, and global change. *Annual review of ecology and systematics*, 23(1):63–87. doi:10.1146/annurev.es.23.110192.000431.

Dawah, H., 1987. Biological species problems in some *Tetramesa* (Hymenoptera, Eurytomidae). *Biological journal of the Linnean Society*, 32(3):237–245.

De Biase, A., Colonnelli, E., Belvedere, S., La Marca, A., Cristofaro, M., and Smith, L., 2016. Genetic and morphological studies of *Trichosirocalus* species introduced to North America, Australia and New Zealand for the biological control of thistles. *Bulletin of Entomological Research*, 106(1):99–113. doi:10.1017/S000748531500084X.

De Biase, A., Smith, L., Brunetti, M., Belvedere, S., Primerano, S., Antonini, G., La Marca, A., Audisio, P., Biondi, M., and Cristofaro, M., 2019. Three prospective agents instead of one? Cryptic diversity of the biological control agent *Psylliodes chalcomera*. *Biological Control*, 136:103998. doi:10.1016/j.biocontrol.2019.103998.

De Queiroz, K., 1998. The general lineage concept of species, species criteria, and the process of speciation. *Endless forms: species and speciation*.

De Queiroz, K., 2005. Different species problems and their resolution. *BioEssays*, 27(12): 1263–1269. doi:10.1002/bies.20325.

Delić, T., Trontelj, P., Rendoš, M., and Fišer, C., 2017. The importance of naming cryptic species and the conservation of endemic subterranean amphipods. *Scientific Reports*, 7 (1):1–12. doi:10.1038/s41598-017-02938-z.

Dellicour, S. and Flot, J.-F., 2015. Delimiting species-poor data sets using single molecular markers: a study of barcode gaps, haplowebs and GMYC. *Systematic Biology*, 64(6): 900–908. doi:10.1093/ sysbio/syul3.

Dellicour, S. and Flot, J.-F., 2018. The hitchhiker's guide to single-locus species delimitation. *Molecular Ecology Resources*, 18(6):1234–1246. doi:10.1111/1755-0998.12908.

Délye, C., Jasieniuk, M., and Le Corre, V., 2013. Deciphering the evolution of herbicide resistance in weeds. *Trends in Genetics*, 29(11):649–658. doi:10.1016/j.tig.2013.06.001.

Demastes, J. W., Spradling, T. A., Hafner, M. S., Spies, G. R., Hafner, D. J., and Light, J. E., 2012. Cophylogeny on a fine scale: *Geomydoecus* chewing lice and their pocket gopher hosts, *Pappogeomys bulleri*. *Journal of Parasitology*, 98(2):262–270. doi:10.1645/GE-2904.1.

Deng, J., Yu, F., Li, H.-B., Gebiola, M., Desdevises, Y., Wu, S.-A., and Zhang, Y.-Z., 2013. Cophylogenetic relationships between *Anicetus* parasitoids (Hymenoptera: Encyrtidae) and their scale insect hosts (Hemiptera: Coccidae). *BMC Evolutionary Biology*, 13(1): 1–11. doi:10.1186/1471-2148-13-275.

Derkarabetian, S., Castillo, S., Koo, P. K., Ovchinnikov, S., and Hedin, M., 2019. A demonstration of unsupervised machine learning in species delimitation. *Molecular Phylogenetics and Evolution*, 139:106562. doi:10.1016/j.ympev.2019.106562.

Derr, J. F., 2008. Common reed (*Phragmites australis*) response to mowing and herbicide application. *Invasive Plant Science and Management*, 1(1):12–16. doi:10.1614/IPSM-07-001.1.

DeSalle, R., Egan, M. G., and Siddall, M., 2005. The unholy trinity: taxonomy, species delimitation and dna barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 360(1462):1905–1916. doi:10.1098/rstb.2005.1722.

Desneux, N., Blahnik, R., Delebecque, C. J., and Heimpel, G. E., 2012. Host phylogeny and specialisation in parasitoids. *Ecology Letters*, 15(5):453–460. doi:10.1111/j.1461-0248.2012.01754.x.

Després, L. One, two or more species? Mitonuclear discordance and species delimitation, 2019.

Dhileepan, K., Senaratne, K. W., and Raghu, S., 2006. A systematic approach to biological control agent exploration and prioritisation for prickly acacia (*Acacia nilotica* ssp. *indica*). *Australian Journal of Entomology*, 45(4):303–307.

Diaz, R., Overholt, W. A., Cuda, J. P., Pratt, P. D., and Fox, A., 2009. Host specificity of *Ischnodemus variegatus*, an herbivore of west indian marsh grass (*Hymenachne amplexicaulis*). *BioControl*, 54(2):307–321. doi:10.1007/s10526-008-9188-3.

DiGaulio, 1998. Eurytomidae. In Gibson, G., Huber, J., and Woolley, J., editors, *Annotated Keys to the Genera of Nearctic Chalcidoidea (Hymenoptera)*, page 477–495. National Research Council (NRC) of Canada, Ottawa.

Diniz-Filho, J. A. F., Soares, T. N., Lima, J. S., Dobrovolski, R., Landeiro, V. L., Telles, M. P. d. C., Rangel, T. F., and Bini, L. M., 2013. Mantel test in population genetics. *Genetics and molecular biology*, 36:475–485. doi:10.1590/S1415-47572013000400002.

Dismukes, W., Braga, M. P., Hembry, D. H., Heath, T. A., and Landis, M. J., 2022. Cophylogenetic methods to untangle the evolutionary history of ecological interactions. *Annual Review of Ecology, Evolution, and Systematics*, 53. doi:10.1146/annurev-ecolsys-102320-112823.

DiTomaso, J. M., 2000. Invasive weeds in rangelands: species, impacts, and management. *Weed science*, 48(2):255–265. doi:10.1614/0043-1745(2000)048[0255:IWIRSI]2.0.CO;2.

Ditomaso, J. M., Brooks, M. L., Allen, E. B., Minnich, R., Rice, P. M., and Kyser, G. B., 2006. Control of invasive weeds with prescribed burning. *Weed technology*, 20(2):535–548.

Doña, J., Sweet, A. D., Johnson, K. P., Serrano, D., Mironov, S., and Jovani, R., 2017. Cophylogenetic analyses reveal extensive host-shift speciation in a highly specialized and host-specific symbiont system. *Molecular Phylogenetics and Evolution*, 115:190–196. doi:10.1016/j.ympev.2017.08.005.

Dos Reis, M., Donoghue, P. C., and Yang, Z., 2016. Bayesian molecular clock dating of species divergences in the genomics era. *Nature Reviews Genetics*, 17(2):71. doi:10.1038/nrg.2015.8.

Dowling, T. E. and Secor, C. L., 1997. The role of hybridization and introgression in the diversification of animals. *Annual review of Ecology and Systematics*, 28(1):593–619. doi:10.1146/annurev.ecolsys.28.1.593.

Dresseno, A. L., Guido, A., Balogianni, V., and Overbeck, G. E., 2018. Negative effects of an invasive grass, but not of native grasses, on plant species richness along a cover gradient. *Austral Ecology*, 43(8):949–954. doi:10.1111/aec.12644.

Dudgeon, S., Kübler, J. E., West, J. A., Kamiya, M., and Krueger-Hadfield, S. A., 2017. Asexuality and the cryptic species problem. *Perspectives in Phycology*, 4:47–59. doi:10.1127/pip/2017/0070.

D'Urso, V., Lisi, O., Sabella, G., *et al.*, 2017. The alien leafhopper *Balclutha brevis* Lindberg, 1954 (Hemiptera Cicadellidae) and its host plant, the invasive Poaceae *Pennisetum setaceum* (Försskal) Chiov.: a real risk in the scenario of Mediterranean land biodiversity. *Biodiversity Journal*, 8(1):145–150.

Ebach, M. C., Valdecasas, A. G., and Wheeler, Q. D., 2011. Impediments to taxonomy and users of taxonomy: accessibility and impact evaluation. *Cladistics*, 27(5):550–557. doi:10.1111/j.1096-0031.2011.00348.x.

Egli, D., Olckers, T., Willows-Munro, S., and Harvey, K., 2020. DNA barcoding of endophagous immature stages elucidates the host-plant affinities of insects associated with the invasive *Senecio madagascariensis* in its native range in South Africa. *Biological Control*, 145:104245. doi:10.1016/j.biocontrol.2020.104245.

Ehlers, G., Caradus, J. R., and Fowler, S. V., 2020. The regulatory process and costs to seek approval for the development and release of new biological control agents in New Zealand. *BioControl*, 65(1):1–12. doi:10.1007/s10526-019-09975-9.

Ehlers, R.-U., Niemann, I., Hollmer, S., Strauch, O., Jende, D., Shanmugasundaram, M., Mehta, U., Easwaramoorthy, S., and Burnell, A., 2000. Mass production potential of the bacto-helminthic biocontrol complex *Heterorhabditis indica-Photorhabdus luminescens*. *Biocontrol Science and Technology*, 10(5):607–616. doi:10.1080/095831500750016406.

Ehrlich, P. R. and Raven, P. H., 1964. Butterflies and plants: a study in coevolution. *Evolution*, pages 586–608. doi:10.2307/2406212.

Elias-Costa, A. J., Confalonieri, V. A., Lanteri, A. A., and Rodriguero, M. S., 2019. Game of clones: Is *Wolbachia* inducing speciation in a weevil with a mixed reproductive mode? *Molecular Phylogenetics and Evolution*, 133:42–53. doi:10.1016/j.ympev.2018.12.027.

Ellstrand, N. C. and Schierenbeck, K. A., 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences*, 97(13): 7043–7050. doi:10.1073/pnas.97.13.7043.

Ellsworth, L. M., Litton, C. M., Dale, A. P., and Miura, T., 2014. Invasive grasses change landscape structure and fire behaviour in Hawaii. *Applied Vegetation Science*, 17(4): 680–689. doi:10.1111/avsc.12110.

Endara, M.-J., Nicholls, J. A., Coley, P. D., Forrister, D. L., Younkin, G. C., Dexter, K. G., Kidner, C. A., Pennington, R. T., Stone, G. N., and Kursar, T. A., 2018. Tracking of host defenses and phylogeny during the radiation of neotropical Inga-feeding sawflies (Hymenoptera; Argidae). *Frontiers in plant science*, 9:1237. doi:10.3389/fpls.2018.01237.

Erb, M., Robert, C. A., Hibbard, B. E., and Turlings, T. C., 2011. Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology*, 99(1): 7–15. doi:10.1111/j.1365-2745.2010.01757.x.

Esselstyn, J. A., Evans, B. J., Sedlock, J. L., Anwarali Khan, F. A., and Heaney, L. R., 2012. Single-locus species delimitation: a test of the mixed Yule–coalescent model, with an empirical application to Philippine round-leaf bats. *Proceedings of the Royal Society B: Biological Sciences*, 279(1743):3678–3686. doi:10.1098/rspb.2012.0705.

Estrada, J. A. and Flory, S. L., 2015. Cogongrass (*Imperata cylindrica*) invasions in the US: mechanisms, impacts, and threats to biodiversity. *Global Ecology and Conservation*, 3: 1–10. doi:10.1016/j.gecco.2014.10.014.

Everitt, J. H., Yang, C., and Deloach, C., 2005. Remote sensing of giant reed with quickbird satellite imagery. *Journal of Aquatic Plant Management*, 43:81–85.

Excoffier, L. and Lischer, H. E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3):564–567. doi:10.1111/j.1755-0998.2010.02847.x.

Excoffier, L., Smouse, P. E., and Quattro, J., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131(2):479–491. doi:10.1093/genetics/131.2.479.

Eyer, P., Seltzer, R., Reiner-Brodetzki, T., and Hefetz, A., 2017. An integrative approach to untangling species delimitation in the *Cataglyphis bicolor* desert ant complex in Israel. *Molecular Phylogenetics and Evolution*, 115:128–139. doi:10.1016/j.ympev.2017.07.024.

Ezard, T., Fujisawa, T., and Barraclough, T., 2009. Splits: species' limits by threshold statistics. *R package version 1.0.*

Fagan-Jeffries, E. P., Cooper, S. J., Bertozzi, T., Bradford, T. M., and Austin, A. D., 2018. DNA barcoding of microgastrine parasitoid wasps (Hymenoptera: Braconidae) using high-throughput methods more than doubles the number of species known for Australia. *Molecular Ecology Resources*, 18(5):1132–1143. doi:10.1111/1755-0998.12904.

Ferrari, R. R., Onuferko, T. M., Monckton, S. K., and Packer, L., 2020. The evolutionary history of the cellophane bee genus *Colletes* Latreille (Hymenoptera: Colletidae): molecular phylogeny, biogeography and implications for a global infrageneric classification. *Molecular Phylogenetics and Evolution*, 146:106750. doi:10.1016/j.ympev.2020.106750.

Firn, J. *et al.*, 2009. African lovegrass in Australia: a valuable pasture species or embarrassing invader? *TG: Tropical Grasslands*, 43(2):86.

Fischbein, D., Lantschner, M. V., and Corley, J. C., 2019. Modelling the distribution of forest pest natural enemies across invaded areas: Towards understanding the influence of climate on parasitoid establishment success. *Biological Control*, 132:177–188.

Fisher, J. L., Veneklaas, E. J., Lambers, H., and Loneragan, W. A., 2006. Enhanced soil and leaf nutrient status of a Western Australian Banksia woodland community invaded by *Ehrharta calycina* and *Pelargonium capitatum*. *Plant and Soil*, 284(1-2):253–264. doi:10.1007/s11104-006-0042-z.

Flanders, A. A., Kuvlesky Jr, W. P., Ruthven III, D. C., Zaiglin, R. E., Bingham, R. L., Fulbright, T. E., Hernández, F., and Brennan, L. A., 2006. Effects of invasive exotic grasses on south Texas rangeland breeding birds. *The Auk*, 123(1):171–182. doi:10.1093/auk/123.1.171.

Florencia, F. M., Carolina, T., Enzo, B., and Leonardo, G., 2017. Effects of the herbicide glyphosate on non-target plant native species from Chaco forest (Argentina). *Ecotoxicology and Environmental Safety*, 144:360–368. doi:10.1016/j.ecoenv.2017.06.049.

Flory, S. L. and Clay, K., 2010. Non-native grass invasion suppresses forest succession. *Oecologia*, 164(4):1029–1038. doi:10.1007/s00442-010-1697-y.

Flory, S. L. and Lewis, J., 2009. Nonchemical methods for managing Japanese stiltgrass (*Microstegium vimineum*). *Invasive Plant Science and Management*, 2(4):301–308. doi:10.1614/IPSM-09-026.1.

Flot, J.-F., Couloux, A., and Tillier, S., 2010. Haplowebs as a graphical tool for delimiting species: a revival of Doyle's "field for recombination" approach and its application to the coral genus *Pocillopora* in Clipperton. *BMC Evolutionary Biology*, 10(1):1–14. doi:10.1186/1471-2148-10-372.

Fonseca, E. M., Duckett, D. J., and Carstens, B. C., 2021. P2C2M.GMYC: An R package for assessing the utility of the Generalized Mixed Yule Coalescent model. *Methods in Ecology and Evolution*, 12(3):487–493. doi:10.1111/2041-210X.13541.

Fontaneto, D., Herniou, E. A., Boschetti, C., Caprioli, M., Melone, G., Ricci, C., and Barraclough, T. G., 2007. Independently evolving species in asexual bdelloid rotifers. *Public Library of Science Biology*, 5(4):e87. doi:10.1371/journal.pbio.0050087.

Fontaneto, D., Flot, J.-F., and Tang, C. Q., 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. *Marine Biodiversity*, 45(3):433–451. doi:10.1007/s12526-015-0319-7.

Foster, B. J., Hereward, J. P., Uelese, A., Walter, G. H., and Furlong, M. J., 2021. *Trichogramma* species in Samoa and their potential as biological control agents against the large cabbage moth, *Crocidolomia pavonana*. *Biological Control*, 164:104781. doi:10.1016/j.biocontrol.2021.104781.

Foxcroft, L. C., Richardson, D. M., Rejmánek, M., and Pyšek, P., 2010. Alien plant invasions in tropical and sub-tropical savannas: patterns, processes and prospects. *Biological Invasions*, 12(12):3913–3933. doi:10.1007/s10530-010-9823-7.

Françoso, E., Gomes, F., and Arias, M. C., 2016. A protocol for isolating insect mitochondrial genomes: a case study of NUMT in *Melipona flavolineata* (Hymenoptera: Apidae). *Mitochondrial DNA Part A*, 27(4):2401–2404. doi:10.3109/19401736.2015.1028049.

Francoso, E., Zuntini, A. R., Ricardo, P. C., Silva, J. P. N., Brito, R., Oldroyd, B. P., and Arias, M. C., 2019. Conserved numts mask a highly divergent mitochondrial-COI gene in a species complex of Australian stingless bees *Tetragonula* (Hymenoptera: Apidae). *Mitochondrial DNA Part A*, 30(7):806–817. doi:10.1080/24701394.2019.1665036.

Franklin, K. and Molina-Freaner, F., 2010. Consequences of buffelgrass pasture development for primary productivity, perennial plant richness, and vegetation structure in the drylands of Sonora, Mexico. *Conservation Biology*, 24(6):1664–1673. doi:10.1111/j.1523-1739.2010.01540.x.

Freifelder, R. R., Vitousek, P. M., and D'Antonio, C. M., 1998. Microclimate change and effect on fire following forest-grass conversion in seasonally dry tropical woodland 1. *Biotropica*, 30(2):286–297. doi:10.1111/j.1744-7429.1998.tb00062.x.

Freudenstein, J. V., Broe, M. B., Folk, R. A., and Sinn, B. T., 2017. Biodiversity and the species concept—lineages are not enough. *Systematic Biology*, 66(4):644–656. doi:10.1093/sysbio/syw098.

Frost, R. A. and Launchbaugh, K. L., 2003. Prescription grazing for rangeland weed management. *Rangelands Archives*, 25(6):43–47.

Frost, S., 1954. The numerical relationships between phytophagous insects and their hosts. *The Scientific Monthly*, 79(1):10–12.

Fujisawa, T. and Barraclough, T. G., 2013. Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic biology*, 62(5):707–724. doi:10.1093/sysbio/syt033.

Fusco, E. J., Finn, J. T., Balch, J. K., Nagy, R. C., and Bradley, B. A., 2019. Invasive grasses increase fire occurrence and frequency across US ecoregions. *Proceedings of the National Academy of Sciences*, 116(47):23594–23599. doi:10.1073/pnas.1908253116.

Futuyma, D. J., 2000. Some current approaches to the evolution of plant–herbivore interactions. *Plant Species Biology*, 15(1):1–9. doi:10.1046/j.1442-1984.2000.00029.x.

Futuyma, D. J. and Moreno, G., 1988. The evolution of ecological specialization. *Annual review of Ecology and Systematics*, 19(1):207–233. doi:10.1146/annurev.es.19.110188.001231.

Gaertner, M., Biggs, R., Te Beest, M., Hui, C., Molofsky, J., and Richardson, D. M., 2014. Invasive plants as drivers of regime shifts: identifying high-priority invaders that alter feedback relationships. *Diversity and Distributions*, 20(7):733–744. doi:10.1111/ddi.12182.

Gahan, A., 1924. The Systematic Position of the Genus *Harmolita* (Motschulsky), with Additional Note (Hymenoptera). *Proc. Ent. Soc. Washington,*, 26:224–231.

Gahan, A. B., 1922. *Descriptions of miscellaneous new reared parasitic Hymenoptera*, volume 61. US Government Printing Office.

Galtier, N., 2019. Delineating species in the speciation continuum: A proposal. *Evolutionary Applications*, 12(4):657–663. doi:10.1111/eva.12748.

Garrick, R. C., Sabree, Z. L., Jahnes, B. C., and Oliver, J. C., 2017. Strong spatial-genetic congruence between a wood-feeding cockroach and its bacterial endosymbiont, across a topographically complex landscape. *Journal of Biogeography*, 44(7):1500–1511. doi:10.1111/jbi.12992.

Gaskin, J. F. and Schaal, B. A., 2003. Molecular phylogenetic investigation of US invasive *Tamarix*. *Systematic Botany*, 28(1):86–95. doi:10.1043/0363-6445-28.1.86.

Gaskin, J. F., Bon, M.-C., Cock, M. J., Cristofaro, M., De Biase, A., De Clerck-Floate, R., Ellison, C. A., Hinz, H. L., Hufbauer, R. A., Julien, M. H., *et al.*, 2011. Applying molecular-based approaches to classical biological control of weeds. *Biological control*, 58 (1):1–21. doi:10.1016/j.biocontrol.2011.03.015.

Gaskin, J. F., Schwarzländer, M., Kinter, C. L., Smith, J. F., and Novak, S. J., 2013. Propagule pressure, genetic structure, and geographic origins of *Chondrilla juncea* (Asteraceae): an apomictic invader on three continents. *American Journal of Botany*, 100(9):1871–1882. doi:10.3732/ajb.1200621.

Gaskin, J. F., Goolsby, J. A., Bon, M.-C., Cristofaro, M., and Calatayud, P.-A., 2022. Identifying the geographic origins of invasive *Megathyrsus maximus* in the United States using molecular data. *Invasive Plant Science and Management*, 15(2):67–71. doi:10.1017/inp.2022.7.

Gates, M. W., 2008. *Species revision and generic systematics of world Rileyinae (Hymenoptera: Eurytomidae)*, volume 127. Univ of California Press.

Gauld, I. D. and Dubois, J., 2006. Phylogeny of the *Polysphincta* group of genera (Hymenoptera: Ichneumonidae; Pimplinae): a taxonomic revision of spider ectoparasitoids. *Systematic Entomology*, 31(3):529–564. doi:10.1111/j.1365-3113.2006.00334.x.

Gaytán, Á., Bergsten, J., Canelo, T., Pérez-Izquierdo, C., Santoro, M., and Bonal, R., 2020. DNA Barcoding and geographical scale effect: The problems of undersampling genetic diversity hotspots. *Ecology and Evolution*, 10(19):10754–10772. doi:10.1002/ece3.6733.

Genner, M. J., Ngatunga, B. P., Mzighani, S., Smith, A., and Turner, G. F., 2015. Geographical ancestry of Lake Malawi's cichlid fish diversity. *Biology Letters*, 11(6): 20150232. doi:10.1098/rsbl.2015.0232.

Gershenzon, J. and Ullah, C., 2022. Plants protect themselves from herbivores by optimizing the distribution of chemical defenses. *Proceedings of the National Academy of Sciences*, 119(4):e2120277119. doi:10.1073/pnas.2120277119.

Gettys, L. A. and Sutton, D. L., 2004. Comparison of torpedograss and pickerelweed susceptibility to glyphosate.

Ghajarieh, H., Bruford, M., Dawah, H. A., Fernandes, C., Dodd, C. S., *et al.*, 2006. Mitochondrial phylogenetics of UK eurytomids. *Journal of Entomology*, 3(2):167–179.

Gibson, D. J., 2009. *Grasses and grassland ecology*. Oxford University Press.

Gillespie, J. J., Munro, J. B., Heraty, J. M., Yoder, M. J., Owen, A. K., and Carmichael, A. E., 2005. A secondary structural model of the 28S rRNA expansion segments D2 and D3 for chalcidoid wasps (Hymenoptera: Chalcidoidea). *Molecular Biology and Evolution*, 22(7):1593–1608. doi:10.1093/molbev/msi152.

Godfree, R., Firn, J., Johnson, S., Knerr, N., Stol, J., and Doerr, V., 2017. Why non-native grasses pose a critical emerging threat to biodiversity conservation, habitat connectivity and agricultural production in multifunctional rural landscapes. *Landscape Ecology*, 32 (6):1219–1242. doi:10.1007/s10980-017-0516-9.

Goeden, R., 1983. Critique and revision of Harris' scoring system for selection of insect agents in biological control of weeds. *Protection Ecology*, 5(4):287–301.

Goolsby, J. A. and Moran, P., 2009. Host range of *Tetramesa romana* Walker (Hymenoptera: Eurytomidae), a potential biological control of giant reed, *Arundo donax* L. in North America. *Biological Control*, 49(2):160–168. doi:10.1016/j.biocontrol.2009.01.019.

Goolsby, J. A., Wright, A. D., and Pemberton, R. W., 2003. Exploratory surveys in Australia and Asia for natural enemies of Old World climbing fern, *Lygodium microphyllum*: Lygodiaceae. *Biological Control*, 28(1):33–46. doi:10.1016/S1049-9644(03)00054-9.

Goolsby, J. A., De Barro, P. J., Makinson, J. R., Pemberton, R. W., Hartley, D. M., and Frohlich, D. R., 2006a. Matching the origin of an invasive weed for selection of a herbivore haplotype for a biological control programme. *Molecular Ecology*, 15(1):287–297.

Goolsby, J. A., Van Klinken, R. D., and Palmer, W. A., 2006b. Maximising the contribution of native-range studies towards the identification and prioritisation of weed biocontrol agents. *Australian Journal of Entomology*, 45(4):276–286. doi:10.1111/j.1440-6055.2006.00551.x.

Goolsby, J. A., Gaskin, J. F., Tarin, D. V., Pepper, A. E., Henne, D. C., Auclair, A., Racelis, A. E., Summy, K. R., Moran, P. J., Thomas, D. B., *et al.*, 2014. Establishment and Spread of a Single Parthenogenic Genotype of the Mediterranean Arundo Wasp, *Tetramesa romana*, in the Variable Climate of Texas. *Southwestern Entomologist*, 39(4):675–690. doi:10.3958/059.039.0414.

Goolsby, J. A., Moran, P. J., Racelis, A. E., Summy, K. R., Jimenez, M. M., Lacewell, R. D., Perez de Leon, A., and Kirk, A. A., 2016. Impact of the biological control agent *Tetramesa romana* (Hymenoptera: Eurytomidae) on *Arundo donax* (Poaceae: Arundinoideae) along the Rio Grande River in Texas. *Biocontrol Science and Technology*, 26(1):47–60. doi:10.1080/09583157.2015.1074980.

Gordh, G., 1977. Biosystematics of natural enemies. In *Biological control by augmentation of natural enemies: insect and mite control with parasites and predators*, pages 125–148, 1977.

Gowda, P. H., Goolsby, J. A., Yang, C., Basu, S., Racelis, A., and Howell, T. A., 2011. Estimating water use by giant reed along the Rio Grande using a large aperture scintillometer. *Subtrop. Plant Sci*, 63:1–6.

Gratton, P., Trucchi, E., Trasatti, A., Riccarducci, G., Marta, S., Allegrucci, G., Cesaroni, D., and Sbordoni, V., 2016. Testing classical species properties with contemporary data: How "bad species" in the brassy ringlets (*Erebia tyndarus* complex, Lepidoptera) turned good. *Systematic Biology*, 65(2):292–303. doi:10.1093/sysbio/syv087.

Grice, A., Vanderduys, E. P., Perry, J. J., and Cook, G. D., 2013. Patterns and processes of invasive grass impacts on wildlife in Australia. *Wildlife Society Bulletin*, 37(3):478–485. doi:10.1002/wsb.314.

Grice, A. C., Friedel, M. H., Marshall, N. A., and Van Klinken, R. D., 2012. Tackling contentious invasive plant species: a case study of buffel grass in Australia. *Environmental Management*, 49(2):285–294. doi:10.1007/s00267-011-9781-6.

Grimaldi, D., Engel, M. S., Engel, M. S., and Engel, M. S., 2005. *Evolution of the Insects*. Cambridge University Press.

Guido, A., Hoss, D., and Pillar, V. D., 2019. Competitive effects and responses of the invasive grass *Eragrostis plana* in Río de la Plata grasslands. *Austral Ecology*, 44(8):1478–1486. doi:10.1111/aec.12822.

Guido, A., Quiñones, A., Pereira, A. L., and Da Silva, E. R., 2020. Are the invasive grasses *Cynodon dactylon* and *Eragrostis plana* more phytotoxic than a co-occurring native? *Ecología Austral*, 30(2):295–303. doi:10.25260/EA.20.30.2.0.1090.

Gullan, P. J. and Cranston, P. S., 2014. *The insects: an outline of entomology*. John Wiley & Sons, West Sussex, UK, 5th edition.

Guo, Q., Huang, M., Liu, Y., Zhang, X., and Gu, Z., 2018. Morphological plasticity in *Myxobolus* Bütschli, 1882: a taxonomic dilemma case and renaming of a parasite species of the common carp. *Parasites & vectors*, 11(1):1–11. doi:10.1186/s13071-018-2943-0.

Gyan, N. M., Yaakov, B., Weinblum, N., Singh, A., Cna'ani, A., Ben-Zeev, S., Saranga, Y., and Tzin, V., 2020. Variation between three *Eragrostis tef* accessions in defense responses to *Rhopalosiphum padi* aphid infestation. *Frontiers in plant science*, 11:598483. doi:10.3389/fpls.2020.598483.

Haag-Liautard, C., Coffey, N., Houle, D., Lynch, M., Charlesworth, B., and Keightley, P. D., 2008. Direct Estimation of the Mitochondrial DNA Mutation Rate in *Drosophila melanogaster*. *Public Library of Science Biology*, 6(8). doi:10.1371/journal.pbio.0060204.

Hall, A. A., Steinbauer, M. J., Taylor, G. S., Johnson, S. N., Cook, J. M., and Riegler, M., 2017. Unravelling mummies: cryptic diversity, host specificity, trophic and coevolutionary interactions in psyllid–parasitoid food webs. *BMC Evolutionary Biology*, 17(1):1–15. doi:10.1186/s12862-017-0959-2.

Hamilton, C. A., Hendrixson, B. E., Brewer, M. S., and Bond, J. E., 2014. An evaluation of sampling effects on multiple DNA barcoding methods leads to an integrative approach for delimiting species: a case study of the North American tarantula genus *Aphonopelma* (Araneae, Mygalomorphae, Theraphosidae). *Molecular Phylogenetics and Evolution*, 71: 79–93. doi:10.1016/j.ympev.2013.11.007.

Han, S., 2012. Transmission of mulberry dwarf phytoplasma by a *Balclutha punctata*. *Journal of Korean Society of Forest Science*, 101(4):635–639.

Han, Y., Buckley, Y. M., and Firn, J., 2012. An invasive grass shows colonization advantages over native grasses under conditions of low resource availability. *Plant Ecology*, 213(7): 1117–1130. doi:10.1007/s11258-012-0070-0.

Hanski, I., 1998. Metapopulation dynamics. *Nature*, 396(6706):41–49. doi:10.1038/23876.

Hardion, L., Verlaque, R., Saltonstall, K., Leriche, A., and Vila, B., 2014. Origin of the invasive *Arundo donax* (Poaceae): a trans-Asian expedition in herbaria. *Annals of Botany*, 114(3):455–462. doi:10.1093/aob/mcu143.

Harms, N. E., Williams, D. A., and Purcell, M. F., 2021. The role of overseas genetic surveys to potentially accelerate biological control development for a new *Hydrilla verticillata* introduction in the USA. *BioControl*, 66(2):271–280. doi:10.1007/s10526-020-10050-x.

Harris, P., 1973. The selection of effective agents for the biological control of weeds. *The Canadian Entomologist*, 105(12):1495–1503.

Havill, N. P., Davis, G., Mausel, D. L., Klein, J., McDonald, R., Jones, C., Fischer, M., Salom, S., and Caccone, A., 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. *Biological Control*, 63(3): 359–369. doi:10.1016/j.biocontrol.2012.08.001.

Havill, N. P., Griffin, B. P., Andersen, J. C., Foottit, R. G., Justesen, M. J., Caccone, A., D'Amico, V., and Elkinton, J. S., 2021. Species delimitation and invasion history of the balsam woolly adelgid, *Adelges* (*Dreyfusia*) *piceae* (hemiptera: Aphidoidea: Adelgidae), species complex. *Systematic Entomology*, 46(1):186–204. doi:10.1111/syen.12456.

Haye, T., Kuhlmann, U., Goulet, H., and Mason, P., 2006. Controlling *Lygus* plant bugs (Heteroptera: Miridae) with European *Peristenus relictus* (Hymenoptera: Braconidae) in Canada–risky or not? *Bulletin of Entomological Research*, 96(2):187–196.

Heap, I., 2014. Global perspective of herbicide-resistant weeds. *Pest management science*, 70(9):1306–1315. doi:10.1002/ps.3696.

Heard, T. A., 2002. Host specificity testing of biocontrol agents of weeds. In *Proceedings of a Workshop on Biological Control of Invasive Plants in Native Hawaiian Ecosystems. Pacific Cooperative Studies Unit, University of Hawaii at Manoa. Edited by Smith, CW, Denslow, JE, and Hight, SD Technical Report*, volume 129, pages 21–29, 2002.

Hebert, P. D. N., Cywinska, A., Ball, S. L., and deWaard, J. R., 2003a. Biological Identifications through DNA Barcodes. *Proceedings of the Royal Society B: Biological Sciences*, 270(1512):313–321. doi:10.1098/rspb.2002.2218.

Hebert, P. D. N., Ratnasingham, S., and de Waard, J. R., 2003b. Barcoding Animal Life: Cytochrome c Oxidase Subunit 1 Divergences among Closely Related Species. *Proceedings of the Royal Society B: Biological Sciences*, 270(Suppl_1):S96–S99. doi:10.1098/rsbl.2003.0025.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., and Hallwachs, W., 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences*, 101(41):14812–14817. doi:10.1073/pnas.0406166101.

Heibl, C. PHYLOCH: R language tree plotting tools and interfaces to diverse phylogenetic software packages. http://www.christophheibl.de/rpackages.html, 2019.

Heimpel, G. E., Abram, P. K., and Brodeur, J., 2021. A phylogenetic perspective on parasitoid host ranges with implications for biological control. *Current Opinion in Insect Science*, 44:95–100. doi:10.1016/j.cois.2021.04.003.

Henneicke, K., Dawah, H., and Jervis, M., 1992. Taxonomy and biology of final-instar larvae of some Eurytomidae (Hymenoptera: Chalcidoidea) associated with grasses in the UK. *Journal of Natural History*, 26(5):1047–1087. doi:10.1080/00222939200770621.

Henry, T. J., 2017. Description of a cryptic new species of the plant bug genus *Eccritotarsus* (Heteroptera: Miridae: Bryocorinae) from Peru, a new biocontrol agent of water hyacinth, *Eichhornia crassipes* (Pontederiaceae). *Proceedings of the Entomological Society of Washington*, 119(3):398–407. doi:10.4289/0013-8797.119.3.398.

Heraty, J. M. and Darling, D. C., 2009. Fossil Eucharitidae and Perilampidae (Hymenoptera: Chalcidoidea) from Baltic amber. *Zootaxa*, 2306(1):1–16. doi:10.11646/zootaxa.2306.1.1.

Hernández-Vera, G., Mitrović, M., Jović, J., Toševski, I., Caldara, R., Gassmann, A., and Emerson, B. C., 2010. Host-associated genetic differentiation in a seed parasitic weevil *Rhinusa antirrhini* (Coleptera: Curculionidae) revealed by mitochondrial and nuclear sequence data. *Molecular Ecology*, 19(11):2286–2300. doi:10.1111/j.1365-294X.2010.04639.x.

Hey, J., 2006. On the failure of modern species concepts. *Trends in Ecology & Evolution*, 21(8):447–450. doi:10.1016/j.tree.2006.05.011.

Hey, J., Waples, R. S., Arnold, M. L., Butlin, R. K., and Harrison, R. G., 2003. Understanding and confronting species uncertainty in biology and conservation. *Trends in Ecology & Evolution*, 18(11):597–603. doi:10.1016/j.tree.2003.08.014.

Hill, G. E., 2017. The mitonuclear compatibility species concept. *The Auk: Ornithological Advances*, 134(2):393–409. doi:10.1642/AUK-16-201.1.

Hill, M., Conlong, D., Zachariades, C., Coetzee, J., Paterson, I., Miller, B., Foxcroft, L., and Van Der Westhuizen, L., 2021. The role of mass-rearing in weed biological control projects in South Africa. *African Entomology*, 29(3):1030–1044. doi:10.4001/003.029.1030.

Hillis, D. M. and Dixon, M. T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly review of biology*, 66(4):411–453. doi:10.1086/417338.

Hinz, H. L., Winston, R. L., and Schwarzländer, M., 2019. How safe is weed biological control? A global review of direct nontarget attack. *The Quarterly Review of Biology*, 94(1):1–27. doi:10.1086/702340.

Hinz, H., Schwarzländer, M., and Gaskin, J., 2008. Does phylogeny explain the host-choice behaviour of potential biological control agents for Brassicaceae weeds? In *Proceedings of the XII International Symposium on Biological Control of Weeds*, pages 410–417. International Wallingford UK, 2008.

Hippee, A. C., Beer, M. A., Bagley, R. K., Condon, M. A., Kitchen, A., Lisowski, E. A., Norrbom, A. L., and Forbes, A. A., 2021. Host shifting and host sharing in a genus of specialist flies diversifying alongside their sunflower hosts. *Journal of evolutionary biology*, 34(2):364–379. doi:10.1111/jeb.13740.

Hoelzer, G. A. and Meinick, D. J., 1994. Patterns of speciation and limits to phylogenetic resolution. *Trends in Ecology & Evolution*, 9(3):104–107. doi:10.1016/0169-5347(94)90207-0.

Hoffman, A. M., Perretta, H., Lemoine, N. P., and Smith, M. D., 2019. Blue grama grass genotype affects palatability and preference by semi-arid steppe grasshoppers. *Acta Oecologica*, 96:43–48. doi:10.1111/j.1439-0418.1991.tb00294.x.

Hoffmann, J., Moran, V., and Impson, F., 1998. Promising results from the first biological control programme against a solanaceous weed (*Solanum elaeagnifolium*). *Agriculture, Ecosystems & Environment*, 70(2-3):145–150. doi:10.1016/S0167-8809(98)00120-0.

Hoffmann, J., Impson, F., and Volchansky, C., 2002. Biological control of cactus weeds: implications of hybridization between control agent biotypes. *Journal of Applied Ecology*, 39(6):900–908. doi:10.1046/j.1365-2664.2002.00766.x.

Hoffmann, W. A. and Haridasan, M., 2008. The invasive grass, *Melinis minutiflora*, inhibits tree regeneration in a Neotropical savanna. *Austral Ecology*, 33(1):29–36. doi:10.1111/j.1442-9993.2007.01787.x.

Hofmann, E. P., Nicholson, K. E., Luque-Montes, I. R., Köhler, G., Cerrato-Mendoza, C. A., Medina-Flores, M., Wilson, L. D., and Townsend, J. H., 2019. Cryptic diversity, but to what extent? Discordance between single-locus species delimitation methods within mainland anoles (Squamata: Dactyloidae) of northern central America. *Frontiers in Genetics*, page 11. doi:10.3389/fgene.2019.00011.

Holl, K. D., Howard, E. A., Brown, T. M., Chan, R. G., de Silva, T. S., Mann, E. T., Russell, J. A., and Spangler, W. H., 2014. Efficacy of exotic control strategies for restoring coastal prairie grasses. *Invasive Plant Science and Management*, 7(4):590–598. doi:10.1614/IPSM-D-14-00031.1.

Holly, D. C., Ervin, G. N., Jackson, C. R., Diehl, S. V., and Kirker, G. T., 2009. Effect of an invasive grass on ambient rates of decomposition and microbial community structure: a search for causality. *Biological Invasions*, 11(8):1855–1868. doi:10.1007/s10530-008-9364-5.

Hong, D.-Y., 2020. Gen-morph species concept—a new and integrative species concept for outbreeding organisms. *Journal of Systematics and Evolution*, 58(5):725–742. doi:10.1111/jse.12660.

Hopkins, T., Roininen, H., van Noort, S., Broad, G. R., Kaunisto, K., and Sääksjärvi, I. E., 2019. Extensive sampling and thorough taxonomic assessment of Afrotropical Rhyssinae (Hymenoptera, Ichneumonidae) reveals two new species and demonstrates the limitations of previous sampling efforts. *ZooKeys*, 878:33. doi:10.3897/zookeys.878.37845.

Howarth, F. G., 1991. Environmental impacts of classical biological control. *Annual Review of Entomology*, 36(1):485–509. doi:10.1146/annurev.en.36.010191.002413.

Huang, J.-P., 2020. Is population subdivision different from speciation? From phylogeography to species delimitation. *Ecology and Evolution*, 10(14):6890–6896. doi:10.1002/ece3.6524.

Huelsenbeck, J. P. and Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17(8):754–755. doi:10.1093/bioinformatics/17.8.754.

Hui, C. and Richardson, D. M., 2017. *Invasion dynamics*. Oxford University Press.

Huitu, O., Forbes, K. M., Helander, M., Julkunen-Tiitto, R., Lambin, X., Saikkonen, K., Stuart, P., Sulkama, S., and Hartley, S., 2014. Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores. *Frontiers in plant science*, 5:478. doi:10.3389/fpls.2014.00478.

Hupalo, K., Copilaș-Ciocianu, D., Leese, F., and Weiss, M., 2022. COI is not always right: integrative taxonomy reveals striking overestimation of species diversity in a Mediterranean freshwater amphipod. *Research Square preprint*.

Hussain, F., Murad, A., and Durrani, M., 2004. Weed communities in the wheat fields of Mastuj, District Chitral, Pakistan. *Pak. J. Weed Sci. Res*, 10(3-4):101–108.

Hutchinson, M. C., Cagua, E. F., Balbuena, J. A., Stouffer, D. B., and Poisot, T., 2017. paco: implementing Procrustean Approach to Cophylogeny in R. *Methods in Ecology and Evolution*, 8(8):932–940. doi:10.1111/2041-210X.12736.

Hwang, U.-W. and Kim, W., 1999. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. *The Korean journal of parasitology*, 37(4):215. doi:10.3347/kjp.1999.37.4.215.

Iline, I. and Phillips, C., 2004. Allozyme markers to help define the South American origins of *Microctonus hyperodae* (Hymenoptera: Braconidae) established in New Zealand for biological control of Argentine stem weevil. *Bulletin of Entomological Research*, 94(3):229–234.

Impson, F. A. and Hoffmann, J. H., 2019. The efficacy of three seed-destroying *Melanterius* weevil species (Curculionidae) as biological control agents of invasive Australian *Acacia* trees (Fabaceae) in South Africa. *Biological Control*, 132:1–7. doi:10.1016/j.biocontrol.2019.01.007.

Isagi, Y. and Torii, A., 1997. Range expansion and its mechanisms in a naturalized bamboo species, *Phyllostachys pubescens*, in Japan. *Journal of Sustainable Forestry*, 6(1-2):127–141. doi:10.1300/J091v06n01_08.

Isbister, K. M., Lamb, E. G., and Stewart, K. J., 2017. Herbicide toxicity testing with non-target boreal plants: The sensitivity of *Achillea millefolium* L. and *Chamerion angustifolium* L. to triclopyr and imazapyr. *Environmental Management*, 60(1):136–156. doi:10.1007/s00267-017-0867-7.

Ivády, G., Madar, L., Dzsudzsák, E., Koczok, K., Kappelmayer, J., Krulisova, V., Macek, M., Horváth, A., and Balogh, I., 2018. Analytical parameters and validation of homopolymer detection in a pyrosequencing-based next generation sequencing system. *BMC genomics*, 19(1):1–8. doi:10.1186/s12864-018-4544-x.

Ivanov, V., Lee, K. M., and Mutanen, M., 2018. Mitonuclear discordance in wolf spiders: Genomic evidence for species integrity and introgression. *Molecular Ecology*, 27(7): 1681–1695. doi:10.1111/mec.14564.

Jacobs, B. F., 2004. Palaeobotanical studies from tropical Africa: relevance to the evolution of forest, woodland and savannah biomes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 359(1450):1573–1583. doi:10.1098/rstb.2004.1533.

Jaenike, J., 1990. Host specialization in phytophagous insects. *Annual Review of Ecology and Systematics*, 21(1):243–273. doi:10.1146/annurev.es.21.110190.001331.

Jain, S., Ale, S., Munster, C. L., Ansley, R. J., and Kiniry, J. R., 2015. Simulating the hydrologic impact of *Arundo donax* invasion on the headwaters of the Nueces River in Texas. *Hydrology*, 2(3):134–147. doi:10.3390/hydrology2030134.

Jiménez, M. M., Goolsby, J. A., Racelis, A. E., de Leon, A. P., and Arroyos, D. N., 2017. Introducción, Establecimiento, y Dispersión en México de la Avispa *Tetramesa romana* 1, Agente de Control Biológico del Carrizo Gigante. *Southwestern Entomologist*, 42(2): 427–434. doi:10.3958/059.042.0213.

Johnson, S. N., Lopaticki, G., and Hartley, S. E., 2014. Elevated atmospheric co2 triggers compensatory feeding by root herbivores on a c3 but not a c4 grass. *PLoS One*, 9(3): e90251. doi:10.1371/journal.pone.0090251.

Jones, E. T., Heyne, E., and Schmidt, J., 1952. A new source of wheat jointworm resistance in wheat. *Transactions of the Kansas Academy of Science (1903-)*, 55(4):427–430. doi:10.2307/3625992.

Jourdan, M., Thomann, T., Kriticos, D. J., Bon, M.-C., Sheppard, A., and Baker, G. H., 2019. Sourcing effective biological control agents of conical snails, *Cochlicella acuta*, in Europe and north Africa for release in southern Australia. *Biological Control*, 134:1–14.

Jousselin, E., Van Noort, S., Berry, V., Rasplus, J.-Y., Rønsted, N., Erasmus, J. C., and Greeff, J. M., 2008. One fig to bind them all: host conservatism in a fig wasp community unraveled by cospeciation analyses among pollinating and nonpollinating fig wasps. *Evolution: International Journal of Organic Evolution*, 62(7):1777–1797. doi:10.1111/j.1558-5646.2008.00406.x.

Jousselin, E., Desdevises, Y., and Coeur d'Acier, A., 2009. Fine-scale cospeciation between *Brachycaudus* and *Buchnera aphidicola*: bacterial genome helps define species and evolutionary relationships in aphids. *Proceedings of the Royal Society B: Biological Sciences*, 276(1654):187–196. doi:10.1098/rspb.2008.0679.

Kaiser-Bunbury, C. N., Mougal, J., Valentin, T., Gabriel, R., and Blüthgen, N., 2015. Herbicide application as a habitat restoration tool: impact on native island plant communities. *Applied Vegetation Science*, 18(4):650–660. doi:10.1111/avsc.12183.

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K., Von Haeseler, A., and Jermiin, L. S., 2017. Modelfinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14(6):587–589. doi:10.1038/nmeth.4285.

Kapli, P., Yang, Z., and Telford, M. J., 2020. Phylogenetic tree building in the genomic age. *Nature Reviews Genetics*, 21(7):428–444. doi:10.1038/s41576-020-0233-0.

Kariñho-Betancourt, E., 2020. Coevolution: Plant-herbivore interactions and secondary metabolites of plants. *Co-Evolution of Secondary Metabolites*, pages 47–76. doi:10.1007/978-3-319-76887-8_41-11.

Katan, J., 2015. Soil solarization: the idea, the research and its development. *Phytoparasitica*, 43(1):1–4. doi:10.1007/s12600-014-0419-0.

Katoh, K., Rozewicki, J., and Yamada, K. D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in bioinformatics*, 20 (4):1160–1166. doi:10.1093/bib/bbx108.

Keeley, P. E. and Thullen, R. J., 1989. Growth and interaction of johnsongrass (*Sorghum halepense*) with cotton (*Gossypium hirsutum*). *Weed Science*, 37(3):339–344. doi:10.1017/S0043174500072027.

Kelch, D. G., McClay, A., *et al.*, 2004. Putting the phylogeny into the centrifugal phylogenetic method. In *XI International Symposium on Biological Control of Weeds*, page 287, 2004.

Kergoat, G. J., Condamine, F. L., Toussaint, E. F., Capdevielle-Dulac, C., Clamens, A.-L., Barbut, J., Goldstein, P. Z., and Le Ru, B., 2018. Opposite macroevolutionary responses to environmental changes in grasses and insects during the Neogene grassland expansion. *Nature communications*, 9(1):1–9. doi:10.1038/s41598-018-23754-z.

Klein, P. and Smith, C. M., 2021. Invasive johnsongrass, a threat to native grasslands and agriculture. *Biologia*, 76(2):413–420. doi:10.2478/s11756-020-00625-5.

Kleinjan, C. and Hoffmann, J., 2013. Advances in clarifying the phylogenetic relationships of acacias: Relevance for biological control. *Acta oecologica*, 48:21–29. doi:10.1016/j.cois.2021.04.003.

Klopfstein, S., Kropf, C., and Baur, H., 2016. *Wolbachia* endosymbionts distort DNA barcoding in the parasitoid wasp genus *Diplazon* (Hymenoptera: Ichneumonidae). *Zoological Journal of the Linnean Society*, 177(3):541–557. doi:10.1111/zoj.12380.

Klopfstein, S., Langille, B., Spasojevic, T., Broad, G. R., Cooper, S. J., Austin, A. D., and Niehuis, O., 2019. Hybrid capture data unravel a rapid radiation of pimpliform parasitoid wasps (Hymenoptera: Ichneumonidae: Pimpliformes). *Systematic Entomology*, 44(2): 361–383. doi:10.1111/syen.12415.

Kolesik, P., Adair, R. J., and Eick, G., 2005. Nine new species of *Dasineura* (Diptera: Cecidomyiidae) from flowers of Australian *Acacia* (Mimosaceae). *Systematic Entomology*, 30(3):454–479. doi:10.1111/j.1365-3113.2005.00287.x.

Kriticos, D., 2003. The roles of ecological models in evaluating weed biological control agents and projects. In Spafford-Jacob, H. and Briese, D., editors, *Improving the Selection, Testing and Evaluation of Weed Biological Control Agents*, pages 69–74. Cooperative Research Centre for Australian Weed Management, Adelaide, Australia.

Krull, S. and Basedow, T., 2005. Evaluation of the biological control of the pink wax scale *Ceroplastes rubens* Maskell (Hom., Coccidae) with the introduced parasitoid *Anicetus beneficus* Ishii & Yasumatsu (Hym., Encyrtidae) in the Central province of Papua New Guinea. *Journal of Applied Entomology*, 129(6):323–329. doi:10.1111/j.1439-0418.2005.00975.x.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, 35(6):1547. doi:10.1093/molbev/msy096.

Kunin, V., Engelbrektson, A., Ochman, H., and Hugenholtz, P., 2010. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental microbiology*, 12(1):118–123. doi:10.1111/j.1462-2920.2009.02051.x.

Larsson, A., 2014. AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics*, 30(22):3276–3278. doi:10.1093/bioinformatics/btu531.

Lawson, D. M., Giessow, J. A., and Giessow, J. H., 2005. The Santa Margarita River *Arundo donax* control project: development of methods and plant community response. *In: Kus, Barbara E., and Beyers, Jan L., technical coordinators. Planning for Biodiversity: Bringing Research and Management Together. Gen. Tech. Rep. PSW-GTR-195. Albany, CA: Pacific Southwest Research Station, Forest Service, US Department of Agriculture: 229-244*, 195.

Lees, A. C. and Pimm, S. L., 2015. Species, extinct before we know them? *Current Biology*, 25(5):R177–R180. doi:10.1016/j.cub.2014.12.017.

Legendre, P., Desdevises, Y., and Bazin, E., 2002. A statistical test for host–parasite coevolution. *Systematic Biology*, 51(2):217–234. doi:10.1080/10635150252899734.

Leigh, J. W. and Bryant, D., 2015. POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9):1110–1116. doi:10.1111/2041-210X.12410.

Li, H.-T., Yi, T.-S., Gao, L.-M., Ma, P.-F., Zhang, T., Yang, J.-B., Gitzendanner, M. A., Fritsch, P. W., Cai, J., Luo, Y., *et al.*, 2019. Origin of angiosperms and the puzzle of the Jurassic gap. *Nature Plants*, 5(5):461–470. doi:10.1038/s41477-019-0421-0.

Li, T., Blande, J. D., Gundel, P. E., Helander, M., and Saikkonen, K., 2014. Epichloë endophytes alter inducible indirect defences in host grasses. *PLoS One*, 9(6):e101331. doi:10.1371/journal.pone.0101331.

Li, X., Sarma, S. J., Sumner, L. W., Jones, A. D., and Last, R. L., 2022. Switchgrass metabolomics reveals striking genotypic and developmental differences in specialized metabolic phenotypes. *Journal of Agricultural and Food Chemistry*, 70(26):8010–8023. doi:10.1021/acs.jafc.2c01306.

Li, Y., Zhou, X., Feng, G., Hu, H., Niu, L., Hebert, P. D., and Huang, D., 2010. COI and ITS2 sequences delimit species, reveal cryptic taxa and host specificity of fig-associated *Sycophila* (Hymenoptera, Eurytomidae). *Molecular Ecology Resources*, 10(1):31–40. doi:10.1111/j.1755-0998.2009.02671.x.

Light, J. E. and Hafner, M. S., 2007. Cophylogeny and disparate rates of evolution in sympatric lineages of chewing lice on pocket gophers. *Molecular Phylogenetics and Evolution*, 45(3):997–1013. doi:10.1016/j.ympev.2007.09.001.

Lim, G. S., Balke, M., and Meier, R., 2012. Determining species boundaries in a world full of rarity: singletons, species delimitation methods. *Systematic Biology*, 61(1):165–169. doi:10.1093/sysbio/syr030.

Lin, Y.-P., Edwards, R. D., Kondo, T., Semple, T. L., and Cook, L. G., 2017. Species delimitation in asexual insects of economic importance: the case of black scale (*Parasaissetia nigra*), a cosmopolitan parthenogenetic pest scale insect. *Public Library of Science One*, 12(5):e0175889. doi:10.1371/journal.pone.0175889.

Linder, H. P., Lehmann, C. E., Archibald, S., Osborne, C. P., and Richardson, D. M., 2018. Global grass (Poaceae) success underpinned by traits facilitating colonization, persistence and habitat transformation. *Biological Reviews*, 93(2):1125–1144. doi:10.1111/brv.12388.

Lockwood, J. L., Hoopes, M. F., and Marchetti, M. P., 2013. *Invasion Ecology*. John Wiley & Sons, West Sussex, 2nd edition. ISBN 1118570820.

Lohse, K., 2009. Can mtDNA barcodes be used to delimit species? A response to Pons *et al.* (2006). *Systematic Biology*, 58(4):439–442. doi:10.1093/sysbio/syp039.

Lopez, J. V., Yuhki, N., Masuda, R., Modi, W., and O'Brien, S. J., 1994. Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. *Journal of molecular evolution*, 39(2):174–190. doi:10.1007/BF00163806.

Lotfalizadeh, H., Rasplus, J.-Y., Cristofaro, M., and Marini, F., 2020. *Tetramesa amica* and its parasitoid *Eurytoma amicophaga* (Hymenoptera, Eurytomidae): two new species associated with medusahead, *Taeniatherum caput-medusae* (Poaceae). *ZooKeys*, 1005: 133. doi:10.3897/zookeys.1005.56353.

Lotfalizadeh, H., Delvare, G., and Rasplus, J.-Y., 2007a. *Eurytoma caninae* sp. n. (Hymenoptera, Eurytomidae), a common species previously overlooked with *E. rosae*. *Zootaxa*, 1640(1):55–68. doi:10.11646/zootaxa.1640.1.3.

Lotfalizadeh, H., Delvare, G., and Rasplus, J.-Y., 2007b. Phylogenetic analysis of Eurytominae (Chalcidoidea: Eurytomidae) based on morphological characters. *Zoological Journal of the Linnean Society*, 151(3):441–510. doi:10.1111/j.1096-3642.2007.00308.x.

Luo, A., Ling, C., Ho, S. Y., and Zhu, C.-D., 2018. Comparison of methods for molecular species delimitation across a range of speciation scenarios. *Systematic Biology*, 67(5): 830–846. doi:10.1093/sysbio/syy011.

Madeira, P., Tipping, P. W., Gandolfo, D. E., Center, T. D., Van, T. K., O'brien, C. W., *et al.*, 2006. Molecular and morphological examination of *Cyrtobagous* sp. collected from Argentina, Paraguay, Brazil, Australia, and Florida. *BioControl*, 51(5):679–701. doi:10.1007/s10526-005-5271-1.

Madjdzadeh, S. M. Morphological and genetic variation in the genus *Tetramesa* and its parasitoids (Hymenoptera: Chalcidoidea) and its application to Iranian biogeography, 2004. PhD thesis.

Magoga, G., Fontaneto, D., and Montagna, M., 2021. Factors affecting the efficiency of molecular species delimitation in a species-rich insect family. *Molecular Ecology Resources*, 21(5):1475–1489. doi:10.1111/1755-0998.13352.

Mal, T. K. and Narine, L., 2004. The biology of Canadian weeds. 129. *Phragmites australis* (Cav.) Trin. ex Steud. *Canadian Journal of Plant Science*, 84(1):365–396.

Malagón-Aldana, L. A., Jensen, A. R., Smith, D. R., Shinohara, A., and Vilhelmsen, L., 2022. Sawflies out of Gondwana: phylogenetics and biogeography of Argidae (Hymenoptera). *Systematic Entomology*, 47(1):231–258. doi:10.1111/syen.12527.

Maluleke, M., Fraser, G. C., and Hill, M. P., 2021. Economic evaluation of chemical and biological control of four aquatic weeds in South Africa. *Biocontrol Science and Technology*, 31(9):896–911. doi:10.1080/09583157.2021.1900783.

Manea, A., Sloane, D. R., and Leishman, M. R., 2016. Reductions in native grass biomass associated with drought facilitates the invasion of an exotic grass into a model grassland system. *Oecologia*, 181(1):175–183. doi:10.1007/s00442-016-3553-1.

Maneesakorn, P., An, R., Daneshvar, H., Taylor, K., Bai, X., Adams, B. J., Grewal, P. S., and Chandrapatya, A., 2011. Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (Heterorhabditis: Rhabditida) and their symbiotic bacteria (Photorhabdus: Enterobacteriaceae). *Molecular Phylogenetics and Evolution*, 59(2):271–280. doi:10.1016/j.ympev.2011.02.012.

Marks, M., Lapin, B., Randall, J., *et al.*, 1994. *Phragmites australis* (*P. communis*): threats, management and monitoring. *Natural Areas Journal*, 14(4):285–294.

Marshall, D. C., Hill, K. B., Moulds, M., Vanderpool, D., Cooley, J. R., Mohagan, A. B., and Simon, C., 2016. Inflation of molecular clock rates and dates: molecular phylogenetics, biogeography, and diversification of a global cicada radiation from Australasia (Hemiptera: Cicadidae: Cicadettini). *Systematic Biology*, 65(1):16–34. doi:10.1093/sysbio/syv069.

Marshall, M., Goolsby, J., Vacek, A., Moran, P., Kirk, A., Mendoza, E. C., Cristofaro, M., Bownes, A., Mastoras, A., Kashefi, J., *et al.*, 2018. Densities of the arundo wasp, *Tetramesa romana* (Hymenoptera: Eurytomidae) across its native range in Mediterranean Europe and introduced ranges in North America and Africa. *Biocontrol Science and Technology*, 28(8):772–785. doi:10.1080/09583157.2018.1493090.

Marshall, V., Lewis, M., and Ostendorf, B., 2012. Buffel grass (*Cenchrus ciliaris*) as an invader and threat to biodiversity in arid environments: a review. *Journal of Arid Environments*, 78:1–12. doi:10.1016/j.jaridenv.2011.11.005.

Marushia, R. G. and Allen, E. B., 2011. Control of exotic annual grasses to restore native forbs in abandoned agricultural land. *Restoration Ecology*, 19(1):45–54. doi:10.1111/j.1526-100X.2009.00540.x.

Masubelele, M. L., Hoffman, M., Bond, W., and Gambiza, J., 2014. A 50 year study shows grass cover has increased in shrublands of semi-arid South Africa. *Journal of Arid Environments*, 104:43–51. doi:10.1016/j.jaridenv.2014.01.011.

Mateo Leach, I., Hesseling, A., Huibers, W., Witsenboer, H., Beukeboom, L., and Van De Zande, L., 2009. Transcriptome and proteome analysis of ovaries of arrhenotokous and thelytokous *Venturia canescens*. *Insect Molecular Biology*, 18(4):477–482. doi:10.1111/j.1365-2583.2009.00890.x.

Mathenge, C. W., Riegler, M., Beattie, G. A. C., Spooner-Hart, R. N., and Holford, P., 2015. Genetic variation amongst biotypes of *Dactylopius tomentosus*. *Insect science*, 22 (3):360–374. doi:10.1111/1744-7917.12120.

Mau-Crimmins, T., 2007. Effects of removing *Cynodon dactylon* from a recently abandoned agricultural field. *Weed Research*, 47(3):212–221. doi:10.1111/j.1365-3180.2007.00556.x.

McArdle, S. L., Nadolny, C., Sindel, B. M., *et al.*, 2004. Invasion of native vegetation by Coolatai grass *Hyparrhenia hirta*: impacts on native vegetation and management implications. *Pacific Conservation Biology*, 10(1):49–56. doi:10.1071/PC040049.

McCulloch, G. A., Makinson, J. R., Purcell, M. F., Raghu, S., and Walter, G. H., 2022. A prospective and iterative, ecological and molecular approach to finding safe weed biological control agents. *Biological Control*, page 104887. doi:10.1016/j.biocontrol.2022.104887.

McDonald, C. J. and McPherson, G. R., 2013. Creating hotter fires in the Sonoran Desert: Buffelgrass produces copious fuels and high fire temperatures. *Fire Ecology*, 9(2):26–39. doi:10.4996/fireecology.0902026.

McEvoy, P. B., 1996. Host specificity and biological pest control. *BioScience*, 46(6):401–405. doi:10.2307/1312873.

McFadyen, R. E. C., 1998. Biological control of weeds. *Annual Review of Entomology*, 43 (1):369–393. doi:10.1146/annurev.ento.43.1.369.

McFadyen, R. and Marohasy, J., 1990. A leaf feeding moth, *Euclasta whalleyi* [Lep.: Pyralidae] for the biological control of *Cryptostegia grandiflora* [Asclepiadaceae] in Queensland, Australia. *Entomophaga*, 35(3):431–435.

McNaughton, S. and Tarrants, J., 1983. Grass leaf silicification: natural selection for an inducible defense against herbivores. *Proceedings of the National Academy of Sciences*, 80(3):790–791. doi:10.1073/pnas.80.3.790.

McNaughton, S., Tarrants, J., McNaughton, M., and Davis, R., 1985. Silica as a defense against herbivory and a growth promotor in African grasses. *Ecology*, 66(2):528–535. doi:10.2307/1940401.

Medina, R. F., Reyna, S. M., and Bernal, J. S., 2012. Population genetic structure of a specialist leafhopper on *Zea*: likely anthropogenic and ecological determinants of gene flow. *Entomologia Experimentalis et Applicata*, 142(3):223–235. doi:10.1016/j.ympev.2006.10.015.

Meierotto, S., Sharkey, M. J., Janzen, D. H., Hallwachs, W., Hebert, P. D., Chapman, E. G., and Smith, M. A., 2019. A revolutionary protocol to describe understudied hyperdiverse taxa and overcome the taxonomic impediment. *Deutsche Entomologische Zeitschrift*, 66: 119. doi:10.3897/dez.66.34683.

Meiklejohn, K. A., Damaso, N., and Robertson, J. M., 2019. Assessment of BOLD and GenBank–Their accuracy and reliability for the identification of biological materials. *Public Library of Science One*, 14(6):e0217084. doi:10.1371/journal.pone.0217084.

Melander, B. and Jørgensen, M., 2005. Soil steaming to reduce intrarow weed seedling emergence. *Weed Research*, 45(3):202–211. doi:10.1111/j.1365-3180.2005.00449.x.

Menezes, R. S., Lloyd, M. W., and Brady, S. G., 2020. Phylogenomics indicates Amazonia as the major source of Neotropical swarm-founding social wasp diversity. *Proceedings of the Royal Society B*, 287(1928):20200480. doi:10.1098/rspb.2020.0480.

Mercadier, G., Goolsby, J. A., Jones, W. A., and Tamesse, J. L., 2009. Results of a preliminary survey in Cameroon, Central Africa, for potential natural enemies of Guineagrass, *Panicum maximum*. *Subtropical Plant Science*, 61:31–36.

Meredith, R. W., Westerman, M., Case, J. A., and Springer, M. S., 2008. A phylogeny and timescale for marsupial evolution based on sequences for five nuclear genes. *Journal of Mammalian Evolution*, 15(1):1–36. doi:10.1007/s10914-007-9062-6.

Michereff, M. F., Nascimento, I. N., Santana, G. T., Sarria, A. L., Borges, M., Laumann, R. A., Withall, D. M., Caulfield, J. C., Birkett, M. A., and Blassioli-Moraes, M. C., 2022. Neotropical maize genotypes with different levels of benzoxazinoids affect fall armyworm development. *Physiological Entomology*, 47(4):232–241. doi:10.1111/phen.12392.

Milam, J., Johnson, D. E., Andersen, J. C., Fassler, A. B., Narango, D. L., and Elkinton, J. S., 2020. Validating morphometrics with DNA barcoding to reliably separate three cryptic species of *Bombus cresson* (Hymenoptera: Apidae). *Insects*, 11(10):669. doi:10.3390/insects11100669.

Miller, M. A., Pfeiffer, W., and Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 gateway computing environments workshop (GCE)*, pages 1–8. Ieee, 2010.

Mills, S., Alcántara-Rodríguez, J. A., Ciros-Pérez, J., Gómez, A., Hagiwara, A., Galindo, K. H., Jersabek, C. D., Malekzadeh-Viayeh, R., Leasi, F., Lee, J.-S., *et al.*, 2017. Fifteen species in one: deciphering the *Brachionus plicatilis* species complex (Rotifera, Monogononta) through DNA taxonomy. *Hydrobiologia*, 796(1):39–58. doi:10.1007/s10750-016-2725-7.

Minogue, P. J., Miller, J. H., and Lauer, D. K., 2012. Use of glyphosate and imazapyr for Cogongrass (*Imperata cylindrica*) management in southern pine forests. *Southern Journal of Applied Forestry*, 36(1):19–25. doi:10.5849/sjaf.10-025.

Minuti, G., Coetzee, J. A., Ngxande-Koza, S., Hill, M. P., and Stiers, I., 2021. Prospects for the biological control of *Iris pseudacorus* L. (Iridaceae). *Biocontrol Science and Technology*, 31(3):314–335. doi:10.1080/09583157.2020.1853050.

Mitskas, M. B., Tsolis, C. E., Eleftherohorinos, I. G., and Damalas, C. A., 2003. Interference between corn and johnsongrass (*Sorghum halepense*) from seed or rhizomes. *Weed Science*, 51(4):540–545. doi:10.1614/0043-1745(2003)051[0540:IBCAJS]2.0.CO;2.

Moffat, C. E., Lalonde, R. G., Ensing, D. J., De Clerck-Floate, R. A., Grosskopf-Lachat, G., and Pither, J., 2013. Frequency-dependent host species use by a candidate biological control insect within its native European range. *Biological Control*, 67(3):498–508. doi:10.1016/j.biocontrol.2013.10.007.

Monaghan, M. T., Wild, R., Elliot, M., Fujisawa, T., Balke, M., Inward, D. J., Lees, D. C., Ranaivosolo, R., Eggleton, P., Barraclough, T. G., *et al.*, 2009. Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology*, 58(3):298–311. doi:10.1093/sysbio/syp027.

Moncrieff, G. R., Scheiter, S., Slingsby, J. A., and Higgins, S. I., 2015. Understanding global change impacts on South African biomes using Dynamic Vegetation Models. *South African Journal of Botany*, 101:16–23. doi:10.1016/j.sajb.2015.02.004.

Moore, B. D. and Johnson, S. N., 2017. Get tough, get toxic, or get a bodyguard: identifying candidate traits conferring belowground resistance to herbivores in grasses. *Frontiers in plant science*, 7:1925. doi:10.3389/fpls.2016.01925.

Moore, G., Li, F., Kui, L., and West, J., 2016. Flood water legacy as a persistent source for riparian vegetation during prolonged drought: an isotopic study of *Arundo donax* on the Rio Grande. *Ecohydrology*, 9(6):909–917. doi:10.1002/eco.1698.

Moran, P. J. and Goolsby, J. A., 2009. Biology of the galling wasp *Tetramesa romana*, a biological control agent of giant reed. *Biological Control*, 49(2):169–179. doi:10.1016/j.biocontrol.2009.01.017.

Moran, P. J., Goolsby, J. A., Racelis, A. E., Cohen, A. C., Ciomperlik, M. A., Summy, K. R., Sands, D. P., and Kirk, A. A., 2014. Mass rearing of the stem-galling wasp *Tetramesa romana*, a biological control agent of the invasive weed *Arundo donax*. In *Mass production of beneficial organisms*, pages 163–201. Elsevier. doi:10.1016/B978-0-12-391453-8.00006-6.

Moran, P. J., Vacek, A. T., Racelis, A. E., Pratt, P. D., and Goolsby, J. A., 2017. Impact of the arundo wasp, *Tetramesa romana* (Hymenoptera: Eurytomidae), on biomass of the invasive weed, *Arundo donax* (Poaceae: Arundinoideae), and on revegetation of riparian habitat along the Rio Grande in Texas. *Biocontrol Science and Technology*, 27(1):96–114. doi:10.1080/09583157.2016.1258453.

Moran, V. C., Hoffmann, J. H., and Zimmermann, H. G., 2013. 100 years of biological control of invasive alien plants in South Africa: history, practice and achievements: news & views. *South African Journal of Science*, 109(9):1–6. doi:10.1590/sajs.2013/a0022.

Morin, L., Reid, A. M., Sims-Chilton, N., Buckley, Y., Dhileepan, K., Hastwell, G. T., Nordblom, T., and Raghu, S., 2009. Review of approaches to evaluate the effectiveness of weed biological control agents. *Biological control*, 51(1):1–15. doi:10.1016/j.biocontrol.2009.05.017.

Moritz, C. and Cicero, C., 2004. DNA Barcoding: Promise and Pitfalls. *Public Library of Science Biology*, 2(10):e354. doi:10.1371/journal.pbio.0020354.

Mulvey, L. P., Warnock, R. C., and De Baets, K., 2022. Where traditional extinction estimates fall flat: using novel cophylogenetic methods to estimate extinction risk in platyhelminths. *Proceedings of the Royal Society B*, 289(1981):20220432. doi:10.1098/rspb.2022.0432.

Munro, J. B., Heraty, J. M., Burks, R. A., Hawks, D., Mottern, J., Cruaud, A., Rasplus, J.-Y., and Jansta, P., 2011. A molecular phylogeny of the Chalcidoidea (Hymenoptera). *Public Library of Science One*, 6(11):e27023. doi:10.1371/journal.pone.0027023.

Muranaka, T. and Washitani, I., 2004. Aggressive invasion of *Eragrostis curvla* in gravelly floodplains of Japanese rivers: current status, ecological effects and countermeasures. *Global Environmental Research*, 8(2):155–162.

Murphy, D. J., 2008. A review of the classification of *Acacia* (Leguminosae, Mimosoideae). *Muelleria*, 26(1):10–26.

Murphy, N. P., Carey, D., Castro, L. R., Dowton, M., and Austin, A. D., 2007. Phylogeny of the platygastroid wasps (Hymenoptera) based on sequences from the 18S rRNA, 28S rRNA and cytochrome oxidase I genes: implications for the evolution of the ovipositor system and host relationships. *Biological Journal of the Linnean Society*, 91(4):653–669. doi:10.1111/j.1095-8312.2007.00825.x.

Nartshuk, E., 2014. Grass-fly larvae (Diptera, Chloropidae): diversity, habitats, and feeding specializations. *Entomological Review*, 94(4):514–525. doi:10.1134/S001387381404006X.

Natural History Museum. Universal Chalcidoidea Database: Species in genus *Tetramesa*. https://www.nhm.ac.uk/our-science/data/chalcidoids/database/, 2021. Accessed 11-01-2021.

Nawaz, M., McCulloch, G. A., Brookes, D. R., Zonneveld, R., and Walter, G. H., 2021. Native range surveys for host-specific *Acacia auriculiformis* biocontrol agents–A role for DNA barcoding. *Biological Control*, 158:104594. doi:10.1016/j.biocontrol.2021.104594.

Neser, O., 2008. *Eurytoma bryophylli* sp. n. (Hymenoptera: Eurytomidae), a leaf-borer of *Bryophyllum delagoense* (Crassulaceae) from Madagascar and a candidate for the biocontrol of the plant in Australia. *African Entomology*, 16(1):60–67. doi:10.4001/1021-3589-16.1.60.

Nguyen, H.-T. T., Dang, B. T., Glenner, H., and Geffen, A. J., 2020. Cophylogenetic analysis of the relationship between anemonefish *Amphiprion* (Perciformes: Pomacentridae) and their symbiotic host anemones (Anthozoa: Actiniaria). *Marine Biology Research*, 16(2): 117–133. doi:10.1080/17451000.2020.1711952.

Nguyen, L.-T., Schmidt, H. A., Von Haeseler, A., and Minh, B. Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, 32(1):268–274. doi:10.1093/molbev/msu300.

Noort, S. v. and Compton, S., 1996. Convergent evolution of agaonine and sycoecine (Agaonidae, Chalcidoidea) head shape in response to the constraints of host fig morphology. *Journal of Biogeography*, 23(4):415–424. doi:10.1111/j.1365-2699.1996.tb00003.x.

Noyes, J. Universal Chalcidoidea Database. http://www.nhm.ac.uk/chalcidoids, 2012. Accessed 13-01-2021.

Nyman, T., Farrell, B. D., Zinovjev, A. G., and Vikberg, V., 2006. Larval habits, host-plant associations, and speciation in nematine sawflies (Hymenoptera: Tenthredinidae). *Evolution*, 60(8):1622–1637. doi:10.1111/j.0014-3820.2006.tb00507.x.

Oberprieler, S. K., Andersen, A. N., and Moritz, C. C., 2018. Ants in Australia's monsoonal tropics: CO1 barcoding reveals extensive unrecognised diversity. *Diversity*, 10(2):36. doi:10.3390/d10020036.

Obeysekara, P. T., Legrand, A., and Lavigne, G., 2014. Use of herbivore-induced plant volatiles as search cues by *Tiphia vernalis* and *Tiphia popilliavora* to locate their below-ground scarabaeid hosts. *Entomologia Experimentalis et Applicata*, 150(1):74–85. doi:10.1111/eea.12138.

Okamoto, K. W., Amarasekare, P., Post, D. M., Vasseur, D. A., and Turner, P. E., 2019. The interplay between host community structure and pathogen life-history constraints in driving the evolution of host-range shifts. *Functional Ecology*, 33(12):2338–2353. doi:10.1111/1365-2435.13467.

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H. B. A., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M. O., Lahti, L., McGlinn, D., Ouellette, M.-H., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C. J., and Weedon, J. *vegan: Community Ecology Package*, 2022. URL `https://CRAN.R-project.org/package=vegan`. R package version 2.6-2.

Olckers, T., Zimmermann, H., and Hoffmann, J., 1995. Interpreting ambiguous results of host-specificity tests in biological control of weeds: assessment of two *Leptinotarsa* species (Chrysomelidae) for the control of *Solanum elaeagnifolium* (Solanaceae) in South Africa. *Biological Control*, 5(3):336–344.

Olmi, M., van Noort, S., and Guglielmino, A., 2016. Contribution to the knowledge of Afrotropical Dryinidae, Embolemidae and Sclerogibbidae (Hymenoptera), with description of new species from Central African Republic and Uganda. *ZooKeys*, (578): 45. doi:10.3897/zookeys.578.7820.

Olofsson, H., Ripa, J., and Jonzén, N., 2009. Bet-hedging as an evolutionary game: the trade-off between egg size and number. *Proceedings of the Royal Society B: Biological Sciences*, 276(1669):2963–2969. doi:10.1098/rspb.2009.0500.

O'Meara, B. C., 2010. New heuristic methods for joint species delimitation and species tree inference. *Systematic Biology*, 59(1):59–73. doi:10.1093/sysbio/syp077.

Osborne, C. P., 2008. Atmosphere, ecology and evolution: what drove the Miocene expansion of C4 grasslands? *Journal of Ecology*, 96(1):35–45. doi:10.1111/j.1365-2745.2007.01323.x.

O'Brien, P. A., Andreakis, N., Tan, S., Miller, D. J., Webster, N. S., Zhang, G., and Bourne, D. G., 2021. Testing cophylogeny between coral reef invertebrates and their bacterial and archaeal symbionts. *Molecular Ecology*, 30(15):3768–3782. doi:10.1111/mec.16006.

Padial, J. M., Miralles, A., De la Riva, I., and Vences, M., 2010. The integrative future of taxonomy. *Frontiers in Zoology*, 7(1):1–14. doi:10.1186/1742-9994-7-16.

Page, R. D., 2003. *Tangled trees: phylogeny, cospeciation, and coevolution*. University of Chicago Press.

Palmer, W. A., Yobo, K. S., Witt, A. B., *et al.*, 2008. Prospects for the biological control of the weedy *Sporobolus* grasses in Australia. In *Proceedings of the 16th Australian Weeds Conference, Cairns Convention Centre, North Queensland, Australia*, pages 18–22, 2008.

Papadopoulou, A., Monaghan, M. T., Barraclough, T. G., and Vogler, A. P., 2009. Sampling error does not invalidate the Yule-coalescent model for species delimitation. A response to Lohse (2009). *Systematic Biology*, 58(4):442–444. doi:10.1093/sysbio/syp038.

Papadopoulou, A., Anastasiou, I., and Vogler, A. P., 2010. Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. *Molecular Biology and Evolution*, 27(7):1659–1672. doi:10.1093/molbev/msq051.

Paradis, E., Claude, J., and Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, 20(2):289–290. doi:10.1093/bioinformatics/btg412.

Parslow, B. A., Schwarz, M. P., and Stevens, M. I., 2021. Molecular diversity and species delimitation in the family Gasteruptiidae (Hymenoptera: Evanioidea). *Genome*, 64(3): 253–264. doi:10.1139/gen-2019-0186.

Paterson, I. D., Downie, D. A., and Hill, M. P., 2009. Using molecular methods to determine the origin of weed populations of *Pereskia aculeata* in South Africa and its relevance to biological control. *Biological Control*, 48(1):84–91. doi:10.1016/j.biocontrol.2008.09.012.

Paterson, I. D., Mangan, R., Downie, D. A., Coetzee, J. A., Hill, M. P., Burke, A. M., Downey, P. O., Henry, T. J., and Compton, S. G., 2016. Two in one: cryptic species discovered in biological control agent populations using molecular data and crossbreeding experiments. *Ecology and Evolution*, 6(17):6139–6150. doi:10.1002/ece3.2297.

Paterson, I. D., Coetzee, J. A., Weyl, P., Griffith, T. C., Voogt, N., and Hill, M. P., 2019. Cryptic species of a water hyacinth biological control agent revealed in South Africa: host specificity, impact, and thermal tolerance. *Entomologia Experimentalis et Applicata*, 167 (7):682–691. doi:10.1111/eea.12812.

Paterson, I. D., Hill, M. P., Canavan, K., and Downey, P. O., 2021a. Prioritisation of targets for weed biological control II: the South African Biological Control Target Selection system. *Biocontrol Science and Technology*, 31(6):566–583. doi:10.1080/09583157.2021.1918637.

Paterson, I., Vitorino, M., de Cristo, S., Martin, G., and Hill, M., 2014. Prioritisation of potential agents for the biological control of the invasive alien weed, *Pereskia aculeata* (Cactaceae), in South Africa. *Biocontrol Science and Technology*, 24(4):407–425.

Paterson, I., Klein, H., Muskett, P., Griffith, T., Mayonde, S., Mofokeng, K., Mnqeta, Z., and Venter, N., 2021b. Biological control of Cactaceae in South Africa. *African Entomology*, 29(3):713–734. doi:10.4001/003.029.0713.

Paynter, Q., Gourlay, A., Oboyski, P., Fowler, S., Hill, R., Withers, T., Parish, H., and Hona, S., 2008. Why did specificity testing fail to predict the field host-range of the gorse pod moth in New Zealand? *Biological Control*, 46(3):453–462. doi:10.1016/j.biocontrol.2008.05.004.

Paynter, Q., Paterson, I. D., and Kwong, R. M., 2020. Predicting non-target impacts. *Current Opinion in Insect Science*. doi:10.1016/j.cois.2020.02.002.

Pearson, D. L., Hamilton, A. L., and Erwin, T. L., 2011. Recovery plan for the endangered taxonomy profession. *BioScience*, 61(1):58–63. doi:10.1525/bio.2011.61.1.11.

Pearson, D. E., Ortega, Y. K., Runyon, J. B., and Butler, J. L., 2016. Secondary invasion: the bane of weed management. *Biological Conservation*, 197:8–17. doi:10.1016/j.biocon.2016.02.029.

Pejchar, L. and Mooney, H. A., 2009. Invasive Species, Ecosystem Services and Human Well-Being. *Trends in Ecology & Evolution*, 24(9):497–504. doi:10.1016/j.tree.2009.03.016.

Pemberton, R., 2002. Selection of appropriate future target weeds for biological control. In *Biological control of invasive plants in the eastern United States*, pages 375 – 386. US Department of Agriculture Forest Service, Morgantown, WV, 2002.

Perrard, A., Pickett, K., Villemant, C., Kojima, J.-i., and Carpenter, J., 2013. Phylogeny of hornets: a total evidence approach (Hymenoptera, Vespidae, Vespinae, Vespa). *Journal of Hymenoptera Research*, 32:1–15. doi:10.3897/jhr.32.4685.

Perrichot, V., Boudinot, B. E., Engel, M. S., Xu, C., Bojarski, B., and Szwedo, J., 2022. Ants (Hymenoptera: Formicidae) from Miocene Ethiopian amber: filling gaps in the geological record of African terrestrial biota. *Zoological Journal of the Linnean Society*. doi:10.1093/zoolinnean/zlac053.

Peters, M. Genetic and Morphological Variation in *Taeniatherum caput-medusae* (Medusahead): Taxonomic Diversity, Geographic Origins, Multiple Introductions and Founder Effects, 2013. Masters thesis dissertation.

Peters, R. S., Niehuis, O., Gunkel, S., Bläser, M., Mayer, C., Podsiadlowski, L., Kozlov, A., Donath, A., van Noort, S., Liu, S., *et al.*, 2018. Transcriptome sequence-based phylogeny of chalcidoid wasps (Hymenoptera: Chalcidoidea) reveals a history of rapid radiations, convergence, and evolutionary success. *Molecular Phylogenetics and Evolution*, 120:286–296. doi:10.1016/j.ympev.2017.12.005.

Peterson, M. A., Collavo, A., Ovejero, R., Shivrain, V., and Walsh, M. J., 2018. The challenge of herbicide resistance around the world: a current summary. *Pest Management Science*, 74(10):2246–2259. doi:10.1002/ps.4821.

Peterson, P. M., Romaschenko, K., and Johnson, G., 2010. A classification of the Chloridoideae (Poaceae) based on multi-gene phylogenetic trees. *Molecular Phylogenetics and Evolution*, 55(2):580–598. doi:10.1016/j.ympev.2010.01.018.

Peterson, P. M., Romaschenko, K., Arrieta, Y. H., and Saarela, J. M., 2014. A molecular phylogeny and new subgeneric classification of Sporobolus (Poaceae: Chloridoideae: Sporobolinae). *Taxon*, 63(6):1212–1243. doi:10.12705/636.19.

Phillips, M. L., McNellis, B. E., Allen, M. F., and Allen, E. B., 2019. Differences in root phenology and water depletion by an invasive grass explains persistence in a Mediterranean ecosystem. *American Journal of Botany*, 106(9):1210–1218. doi:10.1002/ajb2.1344.

Phillips, W. J., 1936. A second revision of the chalcid flies of the genus *Harmolita* (*Isosoma*) of America north of Mexico, with descriptions of 20 new species. *United States Department of Agriculture*.

Phillips, W. J. and Emery, W. T., 1919. A revision of the chalcid-flies of the genus *Harmolita* of America north of Mexico. *Proceedings of the U.S. National Museum*.

Piganeau, G., Gardner, M., and Eyre-Walker, A., 2004. A Broad Survey of Recombination in Animal Mitochondria. *Molecular Biology and Evolution*, 21(12):2319–2325. doi:10.1093/molbev/msh244.

Pikatek, M., Lutz, M., Jacobs, A., Villablanca, F., and Wood, A. R., 2015. Epitypification of *Tilletia ehrhartae*, a smut fungus with potential for nature conservation, biosecurity and biocontrol. *European Journal of Plant Pathology*, 143(1):151–158. doi:10.1007/s10658-015-0672-1.

Pinto, J. and Stouthamer, R., 1994. Systematics of the Trichogrammatidae with emphasis on *Trichogramma*. In *Biological control with egg parasitoids*, pages 1–36.

Poelman, E. H., Broekgaarden, C., Van Loon, J. J., and Dicke, M., 2008. Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology*, 17(14):3352–3365. doi:10.1111/j.1365-294X.2008.03838.x.

Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., Kamoun, S., Sumlin, W. D., and Vogler, A. P., 2006. Sequence-based species delimitation for the dna taxonomy of undescribed insects. *Systematic biology*, 55(4):595–609. doi:10.1080/10635150600852011.

Powell, C., Caleca, V., Sinno, M., van Staden, M., van Noort, S., Rhode, C., Allsopp, E., and van Asch, B., 2019. Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wild and cultivated olives in the Western Cape of South Africa. *Genome*, 62(3):183–199. doi:10.1139/gen-2018-0068.

Prasad, V., Strömberg, C. A., Alimohammadian, H., and Sahni, A., 2005. Dinosaur coprolites and the early evolution of grasses and grazers. *Science*, 310(5751):1177–1180. doi:10.1126/science.1118806.

Prentis, P., White, E., Radford, I. J., Lowe, A. J., and Clarke, A., 2007. Can hybridization cause local extinction: a case for demographic swamping of the Australian native *Senecio pinnatifolius* by the invasive *Senecio madagascariensis?* *New Phytologist*, 176(4):902–912. doi:10.1111/j.1469-8137.2007.02217.x.

Puillandre, N., Lambert, A., Brouillet, S., and ACHAZ, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular ecology*, 21(8):1864–1877. doi:10.1111/j.1365-294X.2011.05239.x.

Puillandre, N., Brouillet, S., and Achaz, G., 2021. ASAP: assemble species by automatic partitioning. *Molecular Ecology Resources*, 21(2):609–620. doi:10.1111/1755-0998.13281.

Pyšek, P., Pergl, J., Essl, F., Lenzner, B., Dawson, W., Kreft, H., Weigelt, P., Winter, M., Kartesz, J., Nishino, M., *et al.*, 2017. Naturalized alien flora of the world. *Preslia.*, 89 (3):203–274. doi:10.23855/preslia.2017.203.

Pyšek, P., Pergl, J., van Kleunen, M., Dawson, W., Essl, F., Kreft, H., Weigelt, P., Wilson, J. R., Winter, M., and Richardson, D. M., 2020. South Africa as a donor of naturalised and invasive plants to other parts of the world. In *Biological Invasions in South Africa*, pages 759–785. Springer. doi:10.1007/978-3-030-32394-3_26.

Quirion, B., Simek, Z., Dávalos, A., and Blossey, B., 2018. Management of invasive *Phragmites australis* in the Adirondacks: a cautionary tale about prospects of eradication. *Biological Invasions*, 20(1):59–73. doi:10.1007/s10530-017-1513-2.

R Core Team. R: A language and environment for statistical computing, 2013. URL `http://www.R-project.org/`.

Racelis, A., Davey, R., Goolsby, J., De León, A. P., Varner, K., and Duhaime, R., 2012. Facilitative ecological interactions between invasive species: *Arundo donax* stands as favorable habitat for cattle ticks (Acari: Ixodidae) along the US–Mexico border. *Journal of medical entomology*, 49(2):410–417. doi:10.1603/ME11104.

Racelis, A., Wagle, P., Escamilla, J., Goolsby, J., and Gowda, P., 2021. Interannual variability and seasonal dynamics of evapotranspiration of *Arundo donax* l. and populations of its biological control agent (*Tetramesa romana*). *Ecohydrology & Hydrobiology*. doi:10.1016/j.ecohyd.2021.07.002.

Racelis, A. E., Goolsby, J., Penk, R., Jones, W., and Roland, T., 2010. Development of an Inundative, Aerial Release Technique for the Arundo Wasp, Biological Control Agent of the Invasive *Arundo donax* L. *Southwestern Entomologist*, 35(4):495–501. doi:https://doi.org/10.3958/059.035.0401.

Rafter, M. A., McKay, F., Parisi, M., Sosa, A., Heard, T. A., White, A., Fichera, G., Brookes, D., Nagalingam, K., Kaye, L., *et al.*, 2022. Biology, host specificity and DNA barcoding of cryptic *Eueupithecia* species (Lepidoptera: Geometridae), and implications for biological control of *Parkinsonia aculeata* (Fabaceae) in Australia. *Austral Entomology*, 61(1):124–132. doi:10.1111/aen.12586.

Ramakrishnan, A. P., Meyer, S. E., Waters, J., Stevens, M. R., Coleman, C. E., and Fairbanks, D. J., 2004. Correlation between molecular markers and adaptively significant genetic variation in *Bromus tectorum* (Poaceae), an inbreedingannual grass. *American Journal of Botany*, 91(6):797–803. doi:10.3732/ajb.91.6.797.

Ramanand, H., Bhikraj, K., Willows-Munro, S., Bownes, A., and Olckers, T. Molecular studies on *Arundo donax* and an adventive population of a stem-galling wasp, *Tetramesa romana*, in South Africa , 2018.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., and Suchard, M. A., 2018a. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic biology*, 67(5):901. doi:10.1093/sysbio/syy032.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., and Suchard, M. A., 2018b. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic biology*, 67(5):901. doi:10.1093/sysbio/syy032.

Ranasinghe, U. G. S. L., Eberle, J., Thormann, J., Bohacz, C., Benjamin, S. P., and Ahrens, D., 2022. Multiple species delimitation approaches with COI barcodes poorly fit each other and morphospecies–An integrative taxonomy case of Sri Lankan *Sericini* chafers (Coleoptera: Scarabaeidae). *Ecology and Evolution*, 12(5):e8942. doi:10.1002/ece3.8942.

Randall, R. P. *et al.* A global compendium of weeds., 2017.

Rannala, B., 2015. The art and science of species delimitation. *Current Zoology*, 61(5): 846–853. doi:10.1093/czoolo/61.5.846.

Ratnasingham, S. and Hebert, P. D., 2013. A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. *Public Library of Science One*, 8(7):e66213. doi:10.1371/journal.pone.0066213.

Rauth, S. J., Hinz, H. L., Gerber, E., and Hufbauer, R. A., 2011. The benefits of pre-release population genetics: a case study using *Ceutorhynchus scrobicollis*, a candidate agent of garlic mustard, *Alliaria petiolata*. *Biological Control*, 56(1):67–75. doi:10.1016/j.biocontrol.2010.09.015.

Ray, C. A., Sherman, J. J., Godinho, A. L., Hanson, N., and Parker, I. M., 2018. Impacts and best management practices for Erect Veldtgrass (*Ehrharta erecta*). *Invasive Plant Science and Management*, 11(1):40–48. doi:10.1017/inp.2018.2.

Rector, B. G., De Biase, A., Cristofaro, M., Primerano, S., Belvedere, S., Antonini, G., and Sobhian, R., 2010. DNA fingerprinting to improve data collection efficiency and yield in an open-field host-specificity test of a weed biological control candidate. *Invasive Plant Science and Management*, 3(4):429–439. doi:10.1614/IPSM-D-10-00019.1.

Redak, R. A., 1987. Forage quality: secondary chemistry of grasses. *Integrated Pest Management on Rangeland*, pages 38–55.

Reed, H. E., Seastedt, T. R., and Blair, J. M., 2005. Ecological consequences of C4 grass invasion of a C4 grassland: a dilemma for management. *Ecological applications*, 15(5): 1560–1569. doi:10.1890/04-0407.

Reeg, J., Schad, T., Preuss, T. G., Solga, A., Körner, K., Mihan, C., and Jeltsch, F., 2017. Modelling direct and indirect effects of herbicides on non-target grassland communities. *Ecological Modelling*, 348:44–55. doi:10.1016/j.ecolmodel.2017.01.010.

Reid, M. K., Naidu, P., Paterson, I. D., Mangan, R., and Coetzee, J. A., 2021. Population genetics of invasive and native *Nymphaea mexicana* Zuccarini: Taking the first steps to initiate a biological control programme in South Africa. *Aquatic Botany*, 171:103372. doi:10.1016/j.aquabot.2021.103372.

Reid, N. M. and Carstens, B. C., 2012. Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology*, 12(1):1–11. doi:10.1186/1471-2148-12-196.

Reidenbach, K. R., Cook, S., Bertone, M. A., Harbach, R. E., Wiegmann, B. M., and Besansky, N. J., 2009. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. *BMC Evolutionary Biology*, 9(1):1–14. doi:10.1186/1471-2148-9-298.

Reinert, J. A., Taliaferro, C. M., and McAfee, J. A., 2007. Susceptibility of Bermudagrass (*Cynodon*) varieties to bermduagrass mite (*Eriophyes cynodoniensis*). In *II International Conference on Turfgrass Science and Management for Sports Fields 783*, pages 519–528, 2007.

Relyea, R. A., 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological applications*, 15(2):618–627. doi:10.1890/03-5342.

Revell, L. J., 2012. phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, 3:217–223. doi:10.1111/j.2041-210X.2011.00169.x.

Rhodes, A. C., Plowes, R. M., Goolsby, J. A., Gaskin, J. F., Musyoka, B., Calatayud, P.-A., Cristofaro, M., Grahmann, E. D., Martins, D. J., and Gilbert, L. E., 2021a. The dilemma of guinea grass (*Megathyrsus maximus*): a valued pasture grass and a highly invasive species. *Biological Invasions*, 23(12):3653–3669. doi:10.1007/s10530-021-02607-3.

Rhodes, A. C., Rutledge, J., DuPont, B., Plowes, R. M., and Gilbert, L. E., 2021b. Targeted grazing of an invasive grass improves outcomes for native plant communities and wildlife habitat. *Rangeland Ecology & Management*, 75:41–50. doi:10.1016/j.rama.2020.11.007.

Richardson, D. M., Pyšek, P., Rejmánek, M., Barbour, M. G., Panetta, F. D., and West, C. J., 2000. Naturalization and invasion of alien plants: concepts and definitions. *Diversity and distributions*, 6(2):93–107. doi:10.1046/j.1472-4642.2000.00083.x.

Riek, E. F., 1955. Fossil insects from the Triassic beds at Mt. Crosby, Queensland. *Australian Journal of Zoology*, 3(4):654–691. doi:10.1071/ZO9550654.

Rinella, M. J., Haferkamp, M. R., Masters, R. A., Muscha, J. M., Bellows, S. E., and Vermeire, L. T., 2010. Growth regulator herbicides prevent invasive annual grass seed production. *Invasive Plant Science and Management*, 3(1):12–16. doi:10.1614/IPSM-D-09-00007.1.

Robertson, M. P., Kriticos, D. J., and Zachariades, C., 2008. Climate matching techniques to narrow the search for biological control agents. *Biological Control*, 46(3):442–452.

Roehrdanz, R., Olson, D., Bourchier, R., Sears, S., Cortilet, A., and Fauske, G., 2006. Mitochondrial DNA diversity and *Wolbachia* infection in the flea beetle *Aphthona nigriscutis* (Coleoptera: Chrysomelidae): an introduced biocontrol agent for leafy spurge. *Biological Control*, 37(1):1–8. doi:10.1016/j.biocontrol.2005.12.004.

Rogers, G. F., 1985. Mortality of burned *Cereus giganteus*. *Ecology*, 66(2):630–632.

Rojas-Sandoval, J. and Meléndez-Ackerman, E., 2012. Effects of an invasive grass on the demography of the Caribbean cactus *Harrisia portoricensis*: Implications for cacti conservation. *Acta Oecologica*, 41:30–38. doi:10.1016/j.actao.2012.04.004.

Rosenblueth, M., Sayavedra, L., Sámano-Sánchez, H., Roth, A., and Martínez-Romero, E., 2012. Evolutionary relationships of flavobacterial and enterobacterial endosymbionts with their scale insect hosts (Hemiptera: Coccoidea). *Journal of Evolutionary Biology*, 25(11): 2357–2368. doi:10.1111/j.1420-9101.2012.02611.x.

Rossi, R. D., Martins, C. R., Viana, P. L., Rodrigues, E. L., and Figueira, J. E. C., 2014. Impact of invasion by molasses grass (*Melinis minutiflora* P. Beauv.) on native species and on fires in areas of campo-cerrado in Brazil. *Acta Botanica Brasilica*, 28(4):631–637. doi:10.1590/0102-33062014abb3390.

Rossiter, N. A., Setterfield, S. A., Douglas, M. M., and Hutley, L. B., 2003. Testing the grass-fire cycle: alien grass invasion in the tropical savannas of northern Australia. *Diversity and distributions*, 9(3):169–176. doi:10.1046/j.1472-4642.2003.00020.x.

Rossiter-Rachor, N., Setterfield, S., Hutley, L., McMaster, D., Schmidt, S., and Douglas, M., 2017. Invasive *Andropogon gayanus* (Gamba grass) alters litter decomposition and nitrogen fluxes in an Australian tropical savanna. *Scientific Reports*, 7(1):1–10. doi:10.1038/s41598-017-08893-z.

Rossiter-Rachor, N. A., Setterfield, S. A., Douglas, M. M., Hutley, L. B., Cook, G. D., and Schmidt, S., 2009. Invasive *Andropogon gayanus* (gamba grass) is an ecosystem transformer of nitrogen relations in Australian savanna. *Ecological Applications*, 19(6):1546–1560. doi:10.1890/08-0265.1.

Rougerie, R., Smith, M. A., Fernandez-Triana, J., Lopez-Vaamonde, C., Ratnasingham, S., and Hebert, P. D., 2011. Molecular analysis of parasitoid linkages (MAPL): gut contents of adult parasitoid wasps reveal larval host. *Molecular Ecology*, 20(1):179–186. doi:10.1111/j.1365-294X.2010.04918.x.

Rubinoff, D. and Holland, B. S., 2005. Between Two Extremes: Mitochondrial DNA Is Neither the Panacea nor the Nemesis of Phylogenetic and Taxonomic Inference. *Systematic Biology*, 54(6):952–961. doi:10.1080/10635150500234674.

Rubinoff, D., Cameron, S., and Will, K., 2006. A Genomic Perspective on the Shortcomings of Mitochondrial DNA for “Barcoding” Identification. *Journal of Heredity*, 97(6):581–594. doi:10.1093/jhered/esl036.

Rubinoff, D., Reil, J. B., Osborne, K. H., Gregory, C. J., Geib, S. M., and Dupuis, J. R., 2020. Phylogenomics reveals conservation challenges and opportunities for cryptic endangered species in a rapidly disappearing desert ecosystem. *Biodiversity and Conservation*, pages 1–16. doi:10.1007/s10531-020-01968-w.

Rutman, S., Dickson, L., and Tellman, B., 2002. Management of buffelgrass on organ pipe cactus national monument, Arizona. *Invasive exotic species in the Sonoran region. University of Arizona Press, Tucson*, pages 311–318.

Saikkonen, K., Gundel, P. E., and Helander, M., 2013. Chemical ecology mediated by fungal endophytes in grasses. *Journal of chemical ecology*, 39:962–968. doi:10.1007/s10886-013-0310-3.

Samacá-Sáenz, E., Santos, B. F., Martínez, J. J., Egan, S. P., Shaw, S. R., Hanson, P. E., and Zaldívar-Riverón, A., 2022. Ultraconserved elements-based systematics reveals evolutionary patterns of host-plant family shifts and phytophagy within the predominantly parasitoid braconid wasp subfamily Doryctinae. *Molecular Phylogenetics and Evolution*, 166:107319. doi:10.1016/j.ympev.2021.107319.

Sanderson, M. J., 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. *Bioinformatics*, 19(2):301–302. doi:10.1093/bioinformatics/19.2.301.

Santichaivekin, S., Yang, Q., Liu, J., Mawhorter, R., Jiang, J., Wesley, T., Wu, Y.-C., and Libeskind-Hadas, R., 2020. eMPRess: a systematic cophylogeny reconciliation tool. *Bioinformatics*. doi:10.1093/bioinformatics/btaa978.

Santos, B. F., Payne, A., Pickett, K. M., and Carpenter, J. M., 2015. Phylogeny and historical biogeography of the paper wasp genus *Polistes* (Hymenoptera: Vespidae): implications for the overwintering hypothesis of social evolution. *Cladistics*, 31(5):535–549. doi:10.1111/cla.12103.

Sato, J. J., Ohdachi, S. D., Echenique-Diaz, L. M., Borroto-Páez, R., Begué-Quiala, G., Delgado-Labañino, J. L., Gámez-Díez, J., Alvarez-Lemus, J., Nguyen, S. T., Yamaguchi, N., *et al.*, 2016. Molecular phylogenetic analysis of nuclear genes suggests a Cenozoic over-water dispersal origin for the Cuban solenodon. *Scientific reports*, 6(1):1–8. doi:10.1038/srep31173.

Schaffner, U., 2001. Host Range Testing of Insects for Biological Weed Control: How Can It Be Better Interpreted? Data on the host range of biocontrol candidates are particularly relevant in assessing potential detrimental effects to nontarget organisms. *BioScience*, 51(11):951–959.

Schaffner, U., Smith, L., and Cristofaro, M., 2018. A review of open-field host range testing to evaluate non-target use by herbivorous biological control candidates. *BioControl*, 63 (3):405–416. doi:10.1007/s10526-018-9875-7.

Schardl, C. L., Leuchtmann, A., and Spiering, M. J., 2004. Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.*, 55:315–340. doi:10.1146/annurev.arplant.55.031903.141735.

Scherz, M. D., Masonick, P., Meyer, A., and Hulsey, C. D., 2022. Between a Rock and a Hard Polytomy: Phylogenomics of the Rock-Dwelling Mbuna Cichlids of Lake Malaŵi. *Systematic Biology*, 71(3):741–757. doi:10.1093/sysbio/syac006.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E., and Crozier, R. H., 2010. Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, 55:421–438. doi:10.1146/annurev-ento-112408-085432.

Schmidt, A. R., Perrichot, V., Svojtka, M., Anderson, K. B., Belete, K. H., Bussert, R., Dörfelt, H., Jancke, S., Mohr, B., Mohrmann, E., *et al.*, 2010. Cretaceous African life captured in amber. *Proceedings of the National Academy of Sciences*, 107(16):7329–7334. doi:10.1073/pnas.1000948107.

Schmidt, S., Driver, F., and De Barro, P., 2006. The phylogenetic characteristics of three different 28S rRNA gene regions in *Encarsia* (Insecta, Hymenoptera, Aphelinidae). *Organisms Diversity & Evolution*, 6(2):127–139. doi:10.1016/j.ode.2005.07.002.

Schmitz, P., Cibois, A., and Landry, B., 2007. Molecular phylogeny and dating of an insular endemic moth radiation inferred from mitochondrial and nuclear genes: The genus *Galagete* (Lepidoptera: Autostichidae) of the Galapagos Islands. *Molecular Phylogenetics and Evolution*, 45(1):180–192. doi:10.1016/j.ympev.2007.05.010.

Schwander, T. and Oldroyd, B. P., 2016. Androgenesis: where males hijack eggs to clone themselves. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 371 (1706):20150534. doi:10.1098/rstb.2015.0534.

Schwarzfeld, M. D. and Sperling, F. A., 2015. Comparison of five methods for delimitating species in *Ophion* Fabricius, a diverse genus of parasitoid wasps (Hymenoptera, Ichneumonidae). *Molecular Phylogenetics and Evolution*, 93:234–248. doi:10.1016/j.ympev.2015.08.003.

Schwarzländer, M., Hinz, H. L., Winston, R., and Day, M., 2018. Biological control of weeds: an analysis of introductions, rates of establishment and estimates of success, worldwide. *BioControl*, 63(3):319–331. doi:10.1007/s10526-018-9890-8.

Seah, Y. G., Ariffin, A. F., and Jaafar, T. N. A. M., 2017. Levels of COI divergence in Family Leiognathidae using sequences available in GenBank and BOLD Systems: A review on the accuracy of public databases. *Aquaculture, Aquarium, Conservation & Legislation*, 10(2):391–401.

Seastedt, T. R., 2015. Biological control of invasive plant species: a reassessment for the Anthropocene. *New phytologist*, 205(2):490–502. doi:10.1111/nph.13065.

Seawright, E. K., Rister, M. E., Lacewell, R. D., McCorkle, D. A., Sturdivant, A. W., Yang, C., and Goolsby, J. A., 2009. Economic implications for the biological control of *Arundo donax*: Rio Grande Basin. *Southwestern Entomologist*, 34(4):377–394. doi:10.3958/059.034.0403.

Sebastian, D. J., Fleming, M. B., Patterson, E. L., Sebastian, J. R., and Nissen, S. J., 2017. Indaziflam: a new cellulose-biosynthesis-inhibiting herbicide provides long-term control of invasive winter annual grasses. *Pest management science*, 73(10):2149–2162. doi:10.1002/ps.4594.

Secord, D. and Kareiva, P., 1996. Perils and pitfalls in the host specificity paradigm. *BioScience*, 46(6):448–453.

Seifert, B., 2020. The gene and gene expression (gage) species concept: an universal approach for all eukaryotic organisms. *Systematic biology*, 69(5):1033–1038. doi:10.1093/sysbio/syaa032.

Setterfield, S. A., Rossiter-Rachor, N. A., Hutley, L. B., Douglas, M. M., and Williams, R. J., 2010. Biodiversity research: turning up the heat: the impacts of *Andropogon gayanus* (gamba grass) invasion on fire behaviour in northern Australian savannas. *Diversity and distributions*, 16(5):854–861. doi:10.1111/j.1472-4642.2010.00688.x.

Sha, Z.-L., Zhu, C.-D., Murphy, R., and Huang, D.-W., 2007. *Diglyphus isaea* (Hymenoptera: Eulophidae): a probable complex of cryptic species that forms an important biological control agent of agromyzid leaf miners. *Journal of Zoological Systematics and Evolutionary Research*, 45(2):128–135. doi:10.1111/j.1439-0469.2006.00375.x.

Shafer, A. B., Williams, G. R., Shutler, D., Rogers, R. E., and Stewart, D. T., 2009. Cophylogeny of *Nosema* (Microsporidia: Nosematidae) and bees (Hymenoptera: Apidae) suggests both cospeciation and a host-switch. *Journal of Parasitology*, 95(1):198–203. doi:10.1645/GE-1724.1.

Shaffer, H. B. and Thomson, R. C., 2007. Delimiting species in recent radiations. *Systematic biology*, 56(6):896–906. doi:10.1080/10635150701772563.

Shanker, K., Vijayakumar, S., and Ganeshaiah, K., 2017. Unpacking the species conundrum: philosophy, practice and a way forward. *Journal of genetics*, 96(3):413–430. doi:10.1007/s12041-017-0800-0.

Sharkey, M. J., Baker, A., McCluskey, K., Smith, A., Naik, S., Ratnasingham, S., Manjunath, R., Perez, K., Sones, J., D'Souza, M., *et al.*, 2021a. Addendum to a minimalist revision of Costa Rican Braconidae: 28 new species and 23 host records. *ZooKeys*, 1075:77. doi:10.3897/zookeys.1075.72197.

Sharkey, M. J., Janzen, D. H., Hallwachs, W., Chapman, E. G., Smith, M. A., Dapkey, T., Brown, A., Ratnasingham, S., Naik, S., Manjunath, R., *et al.*, 2021b. Minimalist revision and description of 403 new species in 11 subfamilies of Costa Rican braconid parasitoid wasps, including host records for 219 species. *ZooKeys*, 4:1–665. doi:10.3897/zookeys.1013.55600.

Shaw, J., Lickey, E. B., Schilling, E. E., and Small, R. L., 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare iii. *American journal of botany*, 94(3):275–288. doi:10.3732/ajb.94.3.275.

Sheikh, S. I., Ward, A. K., Zhang, Y. M., Davis, C. K., Zhang, L., Egan, S. P., and Forbes, A. A., 2022. *Ormyrus labotus* (Hymenoptera: Ormyridae): Another Generalist That Should not be a Generalist is not a Generalist. *Insect Systematics and Diversity*, 6(1): 8. doi:10.1093/isd/ixac001.

Shen, Y.-Y., Chen, X., and Murphy, R. W., 2013. Assessing DNA barcoding as a tool for species identification and data quality control. *Public Library of Science One*, 8(2):e57125. doi:10.1371/journal.pone.0057125.

Shields, M. W., Johnson, A. C., Pandey, S., Cullen, R., González-Chang, M., Wratten, S. D., and Gurr, G. M., 2019. History, current situation and challenges for conservation biological control. *Biological Control*, 131:25–35. doi:10.1016/j.biocontrol.2018.12.010.

Shrestha, S., Adkins, S. W., Graham, G. C., and Loch, D. S., 2003. Phylogeny of the *Sporobolus indicus* complex, based on internal transcribed spacer (ITS) sequences. *Australian Systematic Botany*, 16(2):165–176. doi:10.1071/SB02009.

Shrestha, S., Adkins, S. W., Graham, G. C., and Loch, D. S., 2005. An identification tool for the Australian weedy *Sporobolus* species based on random amplified polymorphic DNA (RAPD) profiles. *Australian Journal of Agricultural Research*, 56(2):157–167. doi:10.1071/AR04180.

Simmonds, F., 1972. Approaches to biological control problems. *Entomophaga*, 17(3):251–264.

Skendzic, E. M., Columbus, J. T., and Cerros-Tlatilpa, R., 2007. Phylogenetics of Andropogoneae (Poaceae: Panicoideae) based on nuclear ribosomal internal transcribed spacer and chloroplast trnL–F sequences. *Aliso: A Journal of Systematic and Floristic Botany*, 23(1):530–544.

Smith, L., De Lillo, E., and Amrine, J., 2010. Effectiveness of eriophyid mites for biological control of weedy plants and challenges for future research. *Experimental and Applied Acarology*, 51(1):115–149. doi:10.1007/s10493-009-9299-2.

Smith, L., Cristofaro, M., Bon, M.-C., De Biase, A., Petanović, R., and Vidović, B., 2018. The importance of cryptic species and subspecific populations in classic biological control of weeds: a North American perspective. *BioControl*, 63(3):417–425. doi:10.1007/s10526-017-9859-z.

Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W., and Hebert, P. D., 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of the National Academy of Sciences*, 103(10):3657–3662. doi:10.1073/pnas.0511318103.

Smith, M. A., Rodriguez, J. J., Whitfield, J. B., Deans, A. R., Janzen, D. H., Hallwachs, W., and Hebert, P. D., 2008. Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *Proceedings of the National Academy of Sciences*, 105(34):12359–12364. doi:10.1073/pnas.0805319105.

Smyth, A., Friedel, M., and O'Malley, C., 2009. The influence of buffel grass (*Cenchrus ciliaris*) on biodiversity in an arid Australian landscape. *The Rangeland Journal*, 31(3): 307–320. doi:10.1071/RJ08026.

Sokal, R. R., 1979. Testing statistical significance of geographic variation patterns. *Systematic zoology*, 28(2):227–232. doi:10.2307/2412528.

Soucy, S. M., Huang, J., and Gogarten, J. P., 2015. Horizontal gene transfer: building the web of life. *Nature Reviews Genetics*, 16(8):472–482. doi:10.1038/nrg3962.

Southwood, T., 1961. The number of species of insect associated with various trees. *The Journal of Animal Ecology*, pages 1–8.

Spears, B. M. and Barr, W. F., 1985. Effect of jointworms on the growth and reproduction of four native range grasses of Idaho. *Rangeland Ecology & Management/Journal of Range Management Archives*, 38(1):44–46.

Stanbury, K. E., Stevens, J. C., and Ritchie, A. L., 2018. Legacy issues in post-pine (*Pinus pinaster*) restoration environments: Weeds compromise seedling growth and function more than edaphic factors. *Land Degradation & Development*, 29(6):1694–1704. doi:10.1002/ldr.2945.

Stanford, A. M., Harden, R., and Parks, C. R., 2000. Phylogeny and biogeography of *Juglans* (Juglandaceae) based on matK and ITS sequence data. *American Journal of Botany*, 87 (6):872–882. doi:10.2307/2656895.

Stanton, D. W., Frandsen, P., Waples, R. K., Heller, R., Russo, I.-R. M., Orozco-terWengel, P. A., Pedersen, C.-E. T., Siegismund, H. R., and Bruford, M. W., 2019. More grist for the mill? Species delimitation in the genomic era and its implications for conservation. *Conservation Genetics*, 20(1):101–113. doi:10.1007/s10592-019-01149-5.

Staver, A. C., Archibald, S., and Levin, S. A., 2011. The global extent and determinants of savanna and forest as alternative biome states. *Science*, 334(6053):230–232. doi:10.1126/science.1210465.

Steppan, S. J., Adkins, R., Spinks, P., and Hale, C., 2005. Multigene phylogeny of the Old World mice, Murinae, reveals distinct geographic lineages and the declining utility of mitochondrial genes compared to nuclear genes. *Molecular Phylogenetics and Evolution*, 37(2):370–388. doi:10.1016/j.ympev.2005.04.016.

Stevens, J. M. and Fehmi, J. S., 2009. Competitive effect of two nonnative grasses on a native grass in southern Arizona. *Invasive Plant Science and Management*, 2(4):379–385. doi:10.1614/IPSM-09-022.1.

Still, C. J., Berry, J. A., Collatz, G. J., and DeFries, R. S., 2003. Global distribution of C3 and C4 vegetation: carbon cycle implications. *Global Biogeochemical Cycles*, 17(1):6–1. doi:10.1029/2001GB001807.

Stinca, A. and Mei, G., 2019. *Ehrharta erecta* Lam.(Poaceae, Ehrhartoideae): distribution in Italy and taxonomy of one of the most invasive plant species in the world. *BioInvasions Record*, 8(4). doi:10.3391/bir.2019.8.4.02.

StiremanIII, J. O., Nason, J. D., and Heard, S. B., 2005. Host-associated genetic differentiation in phytophagous insects: general phenomenon or isolated exceptions? Evidence from a goldenrod-insect community. *Evolution*, 59(12):2573–2587. doi:10.1111/j.0014-3820.2005.tb00970.x.

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., and Rambaut, A., 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus evolution*, 4(1). doi:10.1093/ve/vey016.

Suckling, D. M. and Sforza, R. F. H., 2014. What magnitude are observed non-target impacts from weed biocontrol? *Public Library of Science One*, 9(1):e84847. doi:10.1371/journal.pone.0084847.

Sukumaran, J. and Knowles, L. L., 2017. Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences*, 114(7):1607–1612. doi:10.1073/pnas.1607921114.

Sutton, G., Bownes, A., Visser, V., Mapaura, A., and Canavan, K., 2021a. Progress and prospects for the biological control of invasive alien grasses (Poaceae) in South Africa. *African Entomology*, 29(3):837–858. doi:10.4001/003.029.0837.

Sutton, G., 2019. Searching for a needle in a haystack: Where to survey for climatically-matched biological control agents for two grasses (*Sporobolus* spp.) invading Australia. *Biological Control*, 129:37–44. doi:10.1016/j.biocontrol.2018.11.012.

Sutton, G., Canavan, K., Day, M., Den Breeyen, A., Goolsby, J., Cristofaro, M., McConnachie, A., and Paterson, I., 2019. Grasses as suitable targets for classical weed biological control. *BioControl*, pages 1–18. doi:10.1007/s10526-019-09968-8.

Sutton, G. F., Canavan, K., Day, M. D., and Paterson, I. D., 2021b. Field-based ecological studies to assess prospective biological control agents for invasive alien plants: An example from giant rat's tail grass. *Journal of Applied Ecology*, 58(5):1043–1054. doi:10.1111/1365-2664.13834.

Sword, G., Joern, A., and Senior, L., 2005. Host plant-associated genetic differentiation in the snakeweed grasshopper, *Hesperotettix viridis* (Orthoptera: Acrididae). *Molecular Ecology*, 14(7):2197–2205. doi:10.1111/j.1365-294X.2005.02546.x.

Szűcs, M., Schwarzländer, M., and Gaskin, J. F., 2011. Reevaluating establishment and potential hybridization of different biotypes of the biological control agent *Longitarsus jacobaeae* using molecular tools. *Biological Control*, 58(1):44–52. doi:10.1016/j.biocontrol.2011.03.010.

Talavera, G., Dincă, V., and Vila, R., 2013. Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. *Methods in Ecology and Evolution*, 4 (12):1101–1110. doi:10.1111/2041-210X.12107.

Tang, C. Q., Humphreys, A. M., Fontaneto, D., and Barraclough, T. G., 2014. Effects of phylogenetic reconstruction method on the robustness of species delimitation using single-locus data. *Methods in Ecology and Evolution*, 5(10):1086–1094. doi:10.1111/2041-210X.12246.

Tariq, S., Hakim, A., Siddiqi, A. A., and Owais, M., 2022. An Image Dataset of Fruitfly Species (*Bactrocera zonata* and *Bactrocera dorsalis*) and Automated Species Classification through Object Detection. *Data in Brief*, page 108366. doi:10.1016/j.dib.2022.108366.

Taylor, D. B. and Dhileepan, K., 2019. Implications of the changing phylogenetic relationships of *Acacia* sl on the biological control of *Vachellia nilotica* ssp. indica in Australia. *Annals of Applied Biology*, 174(2):238–247. doi:10.1111/aab.12499.

Taylor, S., Downie, D., and Paterson, I., 2011. Genetic diversity of introduced populations of the water hyacinth biological control agent *Eccritotarsus catarinensis* (Hemiptera: Miridae). *Biological Control*, 58(3):330–336. doi:10.1016/j.biocontrol.2011.05.008.

Tedesco, P., Bigorne, R., Bogan, A., Giam, X., Jézéquel, C., and Hugueny, B., 2014. Estimating how many undescribed species have gone extinct. *Conservation Biology*, 28 (5):1360–1370. doi:10.1111/cobi.12285.

Thomas, P., 2006. Mortality over 16 years of cacti in a burnt desert grassland. *Plant Ecology*, 183(1):9–17. doi:10.1007/s11258-005-9001-7.

Toews, D. P. and Brelsford, A., 2012. The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*, 21(16):3907–3930. doi:10.1111/j.1365-294X.2012.05664.x.

Tooker, J. F. and Giron, D., 2020. The evolution of endophagy in herbivorous insects. *Frontiers in Plant Science*, 11:581816. doi:10.3389/fpls.2020.581816.

Toševski, I., Caldara, R., Jović, J., Hernźndez-vera, G., Baviera, C., Gassmann, A., and Emerson, B. C., 2011. Morphological, molecular and biological evidence reveal two cryptic species in *Mecinus janthinus* Germar (Coleoptera, Curculionidae), a successful biological control agent of Dalmatian toadflax, *Linaria dalmatica* (Lamiales, Plantaginaceae). *Systematic Entomology*, 36(4):741–753. doi:10.1111/j.1365-3113.2011.00593.x.

Toševski, I., Jović, J., Krstić, O., and Gassmann, A., 2013. PCR-RFLP-based method for reliable discrimination of cryptic species within *Mecinus janthinus* species complex (Mecinini, Curculionidae) introduced in North America for biological control of invasive toadflaxes. *Biocontrol*, 58(4):563–573. doi:10.1007/s10526-013-9506-2.

Toussaint, E. F., Condamine, F. L., Kergoat, G. J., Capdevielle-Dulac, C., Barbut, J., Silvain, J.-F., and Le Ru, B. P., 2012. Palaeoenvironmental shifts drove the adaptive radiation of a noctuid stemborer tribe (Lepidoptera, Noctuidae, Apameini) in the Miocene. doi:10.1371/journal.pone.0041377.

Trewick, S. A. and Morgan-Richards, M., 2005. After the deluge: mitochondrial DNA indicates Miocene radiation and Pliocene adaptation of tree and giant weta (Orthoptera: Anostostomatidae). *Journal of Biogeography*, 32(2):295–309. doi:10.1111/j.1365-2699.2004.01179.x.

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., and Minh, B. Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic acids research*, 44(W1):W232–W235. doi:10.1093/nar/gkw256.

Trontelj, P. and Fišer, C., 2009. Perspectives: Cryptic species diversity should not be trivialised. *Systematics and Biodiversity*, 7(1):1–3. doi:10.1017/S1477200008002909.

Tscharntke, T., 1993. Connections of insect population dynamics with community structure in *Phragmites* habitats. In *Dynamics of Populations*, pages 37–44.

Tscharntke, T. and Greiler, H.-J., 1995. Insect communities, grasses, and grasslands. *Annual Review of Entomology*, 40(1):535–558. doi:10.1146/annurev.en.40.010195.002535.

Uno, K. T., Polissar, P. J., Jackson, K. E., and deMenocal, P. B., 2016. Neogene biomarker record of vegetation change in eastern Africa. *Proceedings of the National Academy of Sciences*, 113(23):6355–6363. doi:10.1073/pnas.152126711.

Unruh, T. R., Miliczky, E. R., Horton, D. R., Thomsen-Archer, K., Rehfield-Ray, L., and Jones, V. P., 2016. Gut content analysis of arthropod predators of codling moth in Washington apple orchards. *Biological Control*, 102:85–92. doi:10.1016/j.biocontrol.2016.05.014.

Vacek, A. T., Goolsby, J. A., Calatayud, P.-A., Le Ru, B., Musyoka, B., and Kariyat, R. R., 2021. Importation and Preliminary Evaluation of the Stem Boring Moth *Buakea kaeuae* as a Potential Biological Control Agent of Invasive Guineagrass, *Megathyrsus maximus*. *Southwestern Entomologist*, 46(1):257–260. doi:10.3958/059.046.0125.

Valan, M., Makonyi, K., Maki, A., Vondráček, D., and Ronquist, F., 2019. Automated taxonomic identification of insects with expert-level accuracy using effective feature transfer from convolutional networks. *Systematic Biology*, 68(6):876–895. doi:10.1093/sysbio/syz014.

Valan, M., Vondracek, D., and Ronquist, F., 2021. Awakening taxonomist's third eye: exploring the utility of computer vision and deep learning in insect systematics. *Systematic Entomology*. doi:10.1111/syen.12492.

van der Kooi, C. J. and Ollerton, J., 2020. The origins of flowering plants and pollinators. *Science*, 368(6497):1306–1308. doi:10.1126/science.aay366.

van Der Linden, C. F., WallisDeVries, M. F., and Simon, S., 2021. Great chemistry between us: The link between plant chemical defenses and butterfly evolution. *Ecology and Evolution*, 11(13):8595–8613. doi:10.1002/ece3.7673.

van Driesche, R., Hoddle, M., and Center, T., 2009. *Control of Pests and Weeds by Natural Enemies: An Introduction to Biological Control*. John Wiley & Sons.

van Klinken, R. D. and Friedel, M. H., 2018. Unassisted invasions: understanding and responding to australia's high-impact environmental grass weeds. *Australian Journal of Botany*, 65(8):678–690. doi:10.1071/BT17152.

Van Klinken, R. D. and Raghu, S., 2006. A scientific approach to agent selection. *Australian Journal of Entomology*, 45(4):253–258.

van Klinken, R. D., 1999. Host specificity testing: why do we do it and how we can do it better. In *Proceedings, host specificity testing of exotic arthropod biological control agents: the biological basis for improvement in safety, X international symposium on biological control of weeds*, pages 54–68, 1999.

van Noort, S. WaspWeb: Hymenoptera of the Afrotropical region. www.waspweb.org, 2020. Accessed 11-01-2021.

van Noort, S., Buffington, M. L., and Forshage, M., 2015. Afrotropical Cynipoidea (Hymenoptera). *ZooKeys*, (493):1. doi:10.3897/zookeys.493.6353.

van Steenderen, C. J. M. and Sutton, G. F., 2022. SPEDE-sampler: an R Shiny application to assess how methodological choices and taxon-sampling can affect Generalised Mixed Yule Coalescent (GMYC) output and interpretation. *Molecular Ecology Resources*, 00: 1–16. doi:10.1111/1755-0998.13591.

van Wilgen, B. W. and Lange, W. J. D., 2011. The Costs and Benefits of Biological Control of Invasive Alien Plants in South Africa. *African Entomology*, 19(2):504–514. doi:10.4001/003.019.0228.

Van Wyk, E. and Van Wilgen, B., 2002. The cost of water hyacinth control in South Africa: a case study of three options. *African Journal of Aquatic Science*, 27(2):141–149. doi:10.2989/16085914.2002.9626585.

Vea, I. M. and Grimaldi, D. A., 2016. Putting scales into evolutionary time: the divergence of major scale insect lineages (Hemiptera) predates the radiation of modern angiosperm hosts. *Scientific Reports*, 6(1):1–11. doi:10.1038/srep23487.

Vélez-Gavilán, J. *et al.* *Digitaria eriantha* (pangola grass)., 2018.

Vilà, M. and Hulme, P. E., 2017. Non-Native Species, Ecosystem Services, and Human Well-Being. In *Impact of Biological Invasions on Ecosystem Services*, Invading Nature - Springer Series in Invasion Ecology, pages 1–14. Springer, Cham. ISBN 978-3-319-45119-0 978-3-319-45121-3.

Vilà, M., Espinar, J. L., Hejda, M., Hulme, P. E., Jarošík, V., Maron, J. L., Pergl, J., Schaffner, U., Sun, Y., and Pyšek, P., 2011. Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. *Ecology Letters*, 14(7):702–708. doi:10.1111/j.1461-0248.2011.01628.x.

Vilgalys, R., 2003. Taxonomic misidentification in public DNA databases. *New Phytologist*, 160(1):4–5. doi:10.1046/j.1469-8137.2003.00894.x.

Visser, V., Wilson, J. R., Fish, L., Brown, C., Cook, G. D., and Richardson, D. M., 2016. Much more give than take: South africa as a major donor but infrequent recipient of invasive non-native grasses. *Global Ecology and Biogeography*, 25(6):679–692. doi:10.1111/geb.12445.

Vuataz, L., Rutschmann, S., Monaghan, M. T., and Sartori, M., 2016. Molecular phylogeny and timing of diversification in Alpine *Rhithrogena* (Ephemeroptera: Heptageniidae). *BMC Evolutionary Biology*, 16(1):1–16. doi:10.1186/s12862-016-0758-1.

Waage, J. and Greathead, D., 1988. Biological control: challenges and opportunities. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 318(1189): 111–128. doi:10.1098/rstb.1988.0001.

Wagner, V. and Nelson, C. R., 2014. Herbicides can negatively affect seed performance in native plants. *Restoration Ecology*, 22(3):288–291. doi:10.1111/rec.12089.

Waichert, C., Wilson, J. S., Pitts, J. P., and von Dohlen, C. D., 2020. Phylogenetic species delimitation for the widespread spider wasp *Ageniella accepta* (Hymenoptera: Pompilidae), with new synonyms. *Insect Systematics & Evolution*, 51(3):532–549. doi:10.1163/1876312X-00002207.

Walker, F., 1832. *Monographia chalciditum*. *Entomological Magazine*, 1:115–142.

Walsh, H., Kidd, M., Moum, T., and Friesen, V., 1999. Polytomies and the power of phylogenetic inference. *Evolution*, 53(3):932–937. doi:10.1111/j.1558-5646.1999.tb05386.x.

Wang, J.-X., Liu, J., Miao, Y.-H., Huang, D.-W., and Xiao, J.-H., 2020a. Tracking the distribution and burst of nuclear mitochondrial DNA sequences (NUMTs) in Fig Wasp Genomes. *Insects*, 11(10):680. doi:10.3390/insects11100680.

Wang, L.-G., Lam, T. T.-Y., Xu, S., Dai, Z., Zhou, L., Feng, T., Guo, P., Dunn, C. W., Jones, B. R., Bradley, T., *et al.*, 2020b. treeio: an R package for phylogenetic tree input and output with richly annotated and associated data. *Molecular Biology and Evolution*, 37(2):599–603. doi:10.1093/molbev/msz240.

Wang, X., He, Z., Shi, S., and Wu, C.-I., 2020c. Genes and speciation: is it time to abandon the biological species concept? *National Science Review*, 7(8):1387–1397. doi:10.1093/nsr/nwz220.

Wapshere, A., 1974. A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology*, 77(2):201–211.

Wapshere, A., 1990. Biological control of grass weeds in Australia: an appraisal. *Plant Protection Quarterly*, 5(2):62–75.

War, A. R., Taggar, G. K., Hussain, B., Taggar, M. S., Nair, R. M., and Sharma, H. C., 2018. Plant defence against herbivory and insect adaptations. *AoB Plants*, 10(4):ply037. doi:10.1093/aobpla/ply037.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., and Sharma, H. C., 2012. Mechanisms of plant defense against insect herbivores. *Plant signaling & behavior*, 7(10):1306–1320. doi:10.4161/psb.21663.

Wardill, T. J., Graham, G. C., Zalucki, M., Palmer, W. A., Playford, J., and Scott, K. D., 2005. The importance of species identity in the biocontrol process: identifying the subspecies of *Acacia nilotica* (Leguminosae: Mimosoideae) by genetic distance and the implications for biological control. *Journal of Biogeography*, 32(12):2145–2159. doi:10.1111/j.1365-2699.2005.01348.x.

Watts, D. A. and Moore, G. W., 2011. Water-use dynamics of an invasive reed, *Arundo donax*, from leaf to stand. *Wetlands*, 31(4):725–734. doi:10.1007/s13157-011-0188-1.

Waugh, J., 2007. DNA Barcoding in Animal Species: Progress, Potential and Pitfalls. *BioEssays*, 29(2):188–197. doi:10.1002/bies.20529.

Wheeler, G. and Madeira, P., 2017. Phylogeny within the Anacardiaceae predicts host range of potential biological control agents of Brazilian peppertree. *Biological Control*, 108:22–29. doi:10.1016/j.biocontrol.2017.01.017.

Wheeler, Q. D., 2005. Losing the plot: DNA “barcodes” and taxonomy. *Cladistics*, 21(4): 405–407. doi:10.1111/j.1096-0031.2005.00075.x.

White, T. J., Bruns, T., Lee, S., Taylor, J., *et al.*, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1):315–322.

Wiegmann, B. M., Mitter, C., Regier, J. C., Friedlander, T. P., Wagner, D. M., and Nielsen, E. S., 2000. Nuclear genes resolve Mesozoic-aged divergences in the insect order Lepidoptera. *Molecular Phylogenetics and Evolution*, 15(2):242–259. doi:10.1006/mpev.1999.0746.

Wiens, J. J., 2007. Species delimitation: new approaches for discovering diversity. *Systematic biology*, 56(6):875–878. doi:10.1080/10635150701748506.

Wilder, B. T., Jarnevich, C. S., Baldwin, E., Black, J. S., Franklin, K. A., Grissom, P., Hovanes, K. A., Olsson, A., Malusa, J., Kibria, A. S., *et al.*, 2021. Grassification and fast-evolving fire connectivity and risk in the Sonoran Desert, United States. *Frontiers in Ecology and Evolution*, 9:655561. doi:10.3389/fevo.2021.655561.

Will, K. W. and Rubinoff, D., 2004. Myth of the Molecule: DNA Barcodes for Species Cannot Replace Morphology for Identification and Classification. *Cladistics*, 20(1):47–55. doi:10.1111/j.1096-0031.2003.00008.x.

Will, K. W., Mishler, B. D., and Wheeler, Q. D., 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic biology*, 54(5):844–851. doi:10.1080/10635150500354878.

Williams, C. and Hayes, R. M., 1984. Johnsongrass (*Sorghum halepense*) competition in soybeans (*Glycine max*). *Weed Science*, 32(4):498–501.

Williams, D. G. and Baruch, Z., 2000. African grass invasion in the Americas: ecosystem consequences and the role of ecophysiology. *Biological Invasions*, 2(2):123–140. doi:10.1023/A:1010040524588.

Williams, J. L., Zhang, Y. M., LaPolla, J. S., Schultz, T. R., and Lucky, A., 2022. Phylogenomic Delimitation of Morphologically Cryptic Species in Globetrotting *Nylanderia* (Hymenoptera: Formicidae) Species Complexes. *Insect Systematics and Diversity*, 6(1). doi:10.1093/isd/ixab027.

Williams, W. I., Friedman, J. M., Gaskin, J. F., and Norton, A. P., 2014. Hybridization of an invasive shrub affects tolerance and resistance to defoliation by a biological control agent. *Evolutionary Applications*, 7(3):381–393. doi:10.1111/eva.12134.

Williamson, M. and Fitter, A., 1996. The varying success of invaders. *Ecology*, 77(6): 1661–1666. doi:10.2307/2265769.

Winkler, I., Scheffer, S. J., Lewis, M. L., Ottens, K. J., Rasmussen, A. P., Gomes-Costa, G. A., Santillan, L. M. H., Condon, M. A., and Forbes, A. A., 2018. Anatomy of a neotropical insect radiation. *BMC Evolutionary Biology*, 18(1):1–13. doi:10.1186/s12862-018-1146-9.

Winkler, I. S., Mitter, C., and Scheffer, S. J., 2009. Repeated climate-linked host shifts have promoted diversification in a temperate clade of leaf-mining flies. *Proceedings of the National Academy of Sciences*, 106(43):18103–18108. doi:10.1073/pnas.0904852106.

Winston, R., Schwarzländer, M., Hinz, H. L., Day, M. D., Cock, M. J., Julien, M., *et al.* Biological control of weeds: A world catalogue of agents and their target weeds., 2021. URL `https://www.ibiocontrol.org/catalog/`.

Winter, S., Fennessy, J., and Janke, A., 2018. Limited introgression supports division of giraffe into four species. *Ecology and Evolution*, 8(20):10156–10165. doi:10.1002/ece3.4490.

Witt, A. and McConnachie, A., 2004. The potential for classical biological control of invasive grass species with special reference to invasive *Sporobolus* spp.(Poaceae) in Australia. In *XI International Symposium on Biological Control of Weeds*, page 198, 2004.

Yan, F., Lü, J., Zhang, B., Yuan, Z., Zhao, H., Huang, S., Wei, G., Mi, X., Zou, D., Xu, W., *et al.*, 2018. The Chinese giant salamander exemplifies the hidden extinction of cryptic species. *Current Biology*, 28(10):R590–R592. doi:10.1016/j.cub.2018.04.004.

Yang, L., Kong, H., Huang, J.-P., and Kang, M., 2019. Different species or genetically divergent populations? Integrative species delimitation of the *Primulina hochiensis* complex from isolated karst habitats. *Molecular Phylogenetics and Evolution*, 132:219–231. doi:10.1016/j.ympev.2018.12.011.

Yang, X.-H., Zhu, D.-H., Liu, Z., Zhao, L., and Su, C.-Y., 2013. High levels of multiple infections, recombination and horizontal transmission of *Wolbachia* in the *Andricus mukaigawae* (Hymenoptera; Cynipidae) communities. *Public Library of Science One*, 8 (11):e78970. doi:10.1371/journal.pone.0078970.

Yeates, D. K., Seago, A., Nelson, L., Cameron, S. L., Joseph, L., and Trueman, J. W., 2011. Integrative taxonomy, or iterative taxonomy? *Systematic Entomology*, 36(2):209–217. doi:10.1111/j.1365-3113.2010.00558.x.

Yoshimoto, C. M., 1975. Cretaceous chalcidoid fossils from Canadian amber. *The Canadian Entomologist*, 107(5):499–527. doi:10.4039/Ent107499-5.

Yu, G., Smith, D. K., Zhu, H., Guan, Y., and Lam, T. T.-Y., 2017. ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods in Ecology and Evolution*, 8(1):28–36.

Zachariades, C., Paterson, I. D., Strathie, L. W., Hill, M. P., and van Wilgen, B. W., 2017. Assessing the status of biological control as a management tool for suppression of invasive alien plants in South Africa. *Bothalia-African Biodiversity & Conservation*, 47(2):1–19. doi:10.4102/abc.v47i2.2142.

Zachos, F. E., 2016. *Species concepts in biology*, volume 801. Springer.

Zachos, F. E., 2018. (New) Species concepts, species delimitation and the inherent limitations of taxonomy. *Journal of Genetics*, 97(4):811–815. doi:10.1007/s12041-018-0965-1.

Zachos, J. C., Dickens, G. R., and Zeebe, R. E., 2008. An early Cenozoic perspective on greenhouse warming and carbon-cycle dynamics. *Nature*, 451(7176):279–283. doi:10.1038/nature06588.

Zalucki, M., Day, M., and Playford, J., 2007. Will biological control of *Lantana camara* ever succeed? Patterns, processes & prospects. *Biological Control*, 42(3):251–261. doi:10.1016/j.biocontrol.2007.06.002.

Zamani, A., Vahtera, V., Sääksjärvi, I. E., and Scherz, M. D., 2020. The omission of critical data in the pursuit of 'revolutionary' methods to accelerate the description of species. *Systematic Entomology*. doi:10.1111/syen.12444.

Zamani, A., Fric, Z. F., Gante, H. F., Hopkins, T., Orfinger, A. B., Scherz, M. D., Bartoňová, A. S., and Pos, D. D., 2022. DNA barcodes on their own are not enough to describe a species. *Systematic Entomology*, pages 1–5. doi:10.1111/syen.12538.

Zenni, R. D., Sampaio, A. B., Lima, Y. P., Pessoa-Filho, M., Lins, T. C., Pivello, V. R., and Daehler, C., 2019. Invasive *Melinis minutiflora* outperforms native species, but the magnitude of the effect is context-dependent. *Biological Invasions*, 21(2):657–667. doi:10.1007/s10530-018-1854-5.

Zerova, M., 1992. Problems of phylogeny and developmental trends in the family Eurytomidae. In *Chalcid Forum*, volume 15, pages 2–6, 1992.

Zhang, A., He, L., Crozier, R., Muster, C., and Zhu, C.-D., 2010. Estimating sample sizes for DNA barcoding. *Molecular Phylogenetics and Evolution*, 54(3):1035–1039. doi:10.1016/j.ympev.2009.09.014.

Zhang, D.-X., Nagabhyru, P., and Schardl, C. L., 2009. Regulation of a chemical defense against herbivory produced by symbiotic fungi in grass plants. *Plant Physiology*, 150(2): 1072–1082. doi:10.1104/pp.109.138222.

Zhang, J., Kapli, P., Pavlidis, P., and Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, 29(22):2869–2876.

Zhang, L., Hood, G. R., Ott, J. R., and Egan, S. P., 2019a. Temporal isolation between sympatric host plants cascades across multiple trophic levels of host-associated insects. *Biology letters*, 15(12):20190572. doi:10.1098/rsbl.2019.0572.

Zhang, Y. M., Gates, M. W., and Shorthouse, J. D., 2014. Testing species limits of Eurytomidae (Hymenoptera) associated with galls induced by *Diplolepis* (Hymenoptera: Cynipidae) in Canada using an integrative approach. *The Canadian Entomologist*, 146 (3):321–334. doi:10.4039/tce.2013.70.

Zhang, Y. M., Lászlo, Z., Looney, C., Dénes, A.-L., Hanner, R. H., and Shorthouse, J. D., 2019b. DNA barcodes reveal inconsistent species boundaries in *Diplolepis* rose gall wasps and their *Periclistus* inquilines (Hymenoptera: Cynipidae). *The Canadian Entomologist*, 151(6):717–727. doi:10.4039/tce.2019.59.

Zhang, Y.-Z., Si, S.-l., Zheng, J.-T., Li, H.-L., Fang, Y., Zhu, C.-D., and Vogler, A. P., 2011. DNA barcoding of endoparasitoid wasps in the genus *Anicetus* reveals high levels of host specificity (Hymenoptera: Encyrtidae). *Biological Control*, 58(3):182–191. doi:10.1016/j.biocontrol.2011.05.006.

Zhang, Y. M., Sheikh, S., Ward, A. K. G., Forbes, A. A., Prior, K., Stone, G. N., Gates, M., Egan, S., Zhang, L. N., Davis, C., Weinersmith, K., Melika, G., and Lucky, A., 2022. Delimiting the cryptic diversity and host preferences of *Sycophila* parasitoid wasps associated with oak galls using phylogenomic data. *bioRxiv*. doi:10.1101/2022.01.21.477213.

Zhao, Y., Zhang, W.-Y., Wang, R.-L., and Niu, D.-L., 2020. Divergent domains of 28S ribosomal RNA gene: DNA barcodes for molecular classification and identification of mites. *Parasites & Vectors*, 13(1):1–12. doi:10.1186/s13071-020-04124-z.

Zvereva, E. L., Kruglova, O. Y., and Kozlov, M. V., 2010. Drivers of host plant shifts in the leaf beetle *Chrysomela lapponica*: natural enemies or competition? *Ecological Entomology*, 35(5):611–622. doi:10.1111/j.1365-2311.2010.01220.x.

6

Supplementary Section

Table S1: Sample information for all the wasp sequences (28S and COI) included in this work. Unshaded cells in the COI and 28S columns denote missing data. GenBank codes beginning with AY317 were sourced from (Chen *et al.*, 2004). PNS = pronotal spot, NPNS = non-pronotal spot, NA = unknown, SA = South Africa, EC = Eastern Cape, FS = Free State, KZN = KwaZulu-Natal, MP = Mpumalanga, GHT = Grahamstown, PE = Port Elizabeth, EL = East London, f = female, m = male.

Sequence ID	Morphospecies assignment	COI	28S	Morphotype	Country	Province	Latitude	Longitude	Host	Collection_date	Sex
CVS_0002	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_2019_00019	Tetramesa sp. 4	■	■	PNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2019_00030	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2019	
GFS_2019_00035	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2019	
GFS_2019_00039	Tetramesa sp. 4	■	■	PNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2019_00041	Tetramesa sp. 4	■	■	PNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2019_00043	Tetramesa sp. 5	■	■	NPNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2019	
GFS_2019_00044	Tetramesa sp. 4		■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	Eragrostis curvula	2019	
GFS_2019_00063	Tetramesa sp. 5	■	■	NPNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2019_00069	Tetramesa sp. 4	■	■	PNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2019_00079	Tetramesa sp. 4	■	■	PNS	SA, Burnt Kraal, GHT	EC	-33.2817	26.4864	*Eragrostis curvula*	2019	
GFS_2020_00015	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_0003	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00043	Tetramesa sp. 1	■	■	PNS	SA, Valhalla, EL	EC	-32.8719	28.0838	*Sporobolus pyramidalis*	2020	
GFS_2020_00053	Tetramesa sp. 1	■	■	PNS	SA, Valhalla, EL	EC	-32.8719	28.0838	*Sporobolus pyramidalis*	2020	
GFS_2020_00054	Eurytomidae sp.?	■	■	NA	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00055	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00062	Bruchophagus sp 1	■		NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00079	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00083	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00085	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	m
GFS_2020_00086	Tetramesa sp. 4		■	PNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2020	f
GFS_2020_00087	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00091	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	f
GFS_2020_00102	Tetramesa sp. 4	■	■	PNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2020	
GFS_2020_00111	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	f
GFS_2020_00116	Tetramesa sp. 1	■	■	PNS	SA, Valhalla, EL	EC	-32.8719	28.0838	*Sporobolus pyramidalis*	2020	f
GFS_2020_00120	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	f
GFS_2020_00130	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	f
GFS_2020_00132	Tetramesa sp. 5	■	■	NPNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00152	Tetramesa sp. 1	■	■	PNS	SA, Valhalla, EL	EC	-32.8719	28.0838	*Sporobolus pyramidalis*	2020	m
GFS_2020_00155	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00184	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00196	Tetramesa sp. 5	■	■	NPNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2020	
GFS_2020_00233	Tetramesa sp. 4	■	■	PNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2020	
GFS_2020_00492	Tetramesa sp. 4	■	■	PNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	f
GFS_2020_00528	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00554	Tetramesa sp. 4	■	■	PNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	m
GFS_2020_00555	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00562	Tetramesa sp. 5	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	
GFS_2020_00563	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00574	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00576	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00595	Tetramesa sp. 5	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	m
GFS_2020_00606	Tetramesa sp. 5	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	
GFS_2020_00614	Tetramesa sp. 4	■	■	PNS	SA, Baakens River, Targetkloof, PE	EC	-33.9686	25.5923	*Eragrostis curvula*	2020	
GFS_2020_00626	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00638	Tetramesa sp. 5	■	■	NPNS	SA, Glen Muir, EL	EC	-32.8768	28.0950	*Eragrostis curvula*	2020	
GFS_2020_00645	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00667	Tetramesa sp. 4		■	PNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	
GFS_2020_00670	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00680	Tetramesa sp. 4	■	■	PNS	SA, Mondplaas	EC	-33.9630	24.9545	*Eragrostis curvula*	2020	f
GFS_2020_00702	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2020_00720	Tetramesa sp. 7	■	■	NPNS	SA, Mondplaas	EC	-33.9630	24.9545	*Hyparrhenia hirta*	2020	
GFS_2021_00245	Tetramesa sp. 4 (pre-pupa)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00254_F	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_2021_00255	Tetramesa sp. 5 (larva)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00267	Unknown	■		NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00291_M	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_2021_00302	Tetramesa sp. 5			NPNS	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	m
GFS_2021_00307	Tetramesa sp. 5 (larva)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	

Sequence ID	Morphospecies assignment	COI	28S	Morphotype	Country	Province	Latitude	Longitude	Host	Collection_date	Sex
GFS_2021_00329	Tetramesa sp. 1	■	■	PNS	SA, Drummond, Durban	KZN	-29.7622	30.6919	*Sporobolus pyramidalis*	Apr-2021	m
GFS_2021_00334	Tetramesa sp. 5 (larva)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00354	Tetramesa sp. 4	■	■	PNS	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Apr-2021	m
GFS_2021_00373	Tetramesa sp. 4 (larva)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00374	Tetramesa sp. 5	■	■	NPNS	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	f
GFS_2021_00411	Tetramesa sp. 5 (larva)	■	■	NA	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*	Feb-2021	
GFS_2021_00726	Tetramesa sp. 11	■	■	NPNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00730	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00741	Tetramesa sp. 9	■		NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	f
GFS_2021_00745	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00746	Tetramesa sp. 14	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00749	Tetramesa sp. 11	■	■	NPNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	m
GFS_2021_00750	Unknown	■	■	NA	SA, Mkuze Bus stop, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00751	Tetramesa sp. 9 (?)		■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	f
GFS_2021_00762	Tetramesa sp. 8	■	■	PNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	m
GFS_2021_00770	Tetramesa sp. 11	■	■	NPNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00773	Tetramesa sp. 10	■	■	PNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis trichophora*	May-2021	f
GFS_2021_00786	Tetramesa sp. 14	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00803	Tetramesa sp. 14	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00805	Tetramesa sp. 8	■	■	PNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	m
GFS_2021_00806	Tetramesa sp. 11	■	■	NPNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	m
GFS_2021_00810	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00826	Tetramesa sp. 14	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00846	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_2021_00853	Tetramesa sp. 14	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00883	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	m
GFS_2021_00886	Tetramesa sp. 9 (?)	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	f
GFS_2021_00887	Tetramesa sp. 13	■	■	PNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Eragrostis superba*	May-2021	f
GFS_2021_00894	Tetramesa sp. 9	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Cynodon dactylon*	May-2021	m
GFS_2021_00901	Tetramesa sp. 12	■	■	PNS	SA, Drummond, Durban	KZN	-29.7622	30.6919	*Eustachys paspaloides*	May-2021	f
GFS_2021_00902	Tetramesa sp. 10	■	■	PNS	SA, Mkuze GR turn-off, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	m
GFS_2021_00918	Tetramesa sp. 10	■	■	PNS	SA, Mkuze Bus stop, Mkuze	KZN	-27.6221	32.0247	*Eragrostis trichophora*	May-2021	f
GFS_PHI_1007	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1027_1	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_PHI_1027_2	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_PHI_1028F	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1034	Tetramesa sp. 22	■	■	?	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Panicum maximum*	Feb-2022	
GFS_PHI_1036	Tetramesa sp. 21	■	■	NPNS	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Panicum maximum*	Feb-2022	
GFS_PHI_1040_F	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_PHI_1052F	Unknown	■	■	NA	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1078F	Unknown	■	■	NA	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1080F	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1096_F	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_PHI_1096M	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	m
GFS_PHI_1101F	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_PHI_1101M	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	m
GFS_PHI_1132	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1132M	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		m
GFS_PHI_1149_M1	Tetramesa sp. 1	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	m
GFS_PHI_1164F	Unknown	■	■	NA	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1186	Tetramesa sp. 22	■	■	SB	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Panicum maximum*	Feb-2022	
GFS_PHI_1189F	Tetramesa sp. 18		■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis gummiflua*		f
GFS_PHI_1189M	Tetramesa sp. 18	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis gummiflua*		m
GFS_PHI_1190F	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_PHI_1196F	Tetramesa sp. 10	■	■	PNS	SA, Hluhluwe Mine	KZN	-28.0333	32.2833	*Eragrostis trichophora*		f
GFS_PHI_1196M	Tetramesa sp. 11	■	■	PNS	SA, Hluhluwe Mine	KZN	-28.0333	32.2833	*Eragrostis trichophora*		m
GFS_PHI_1200_F	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	f
GFS_PHI_1201F	Unknown	■	■	NA	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1205F	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1232F	Tetramesa sp. 26	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f

Sequence ID	Morphospecies assignment	COI	28S	Morphotype	Country	Province	Latitude	Longitude	Host	Collection_date	Sex
GFS_PHI_1238F	Tetramesa sp. 5	■		NPNS	SA, Coombs Valley, GHT	EC	-33.2804	26.7988	*Eragrostis curvula*		f
GFS_PHI_1238M	Tetramesa sp. 11	■	■	PNS	SA, Hluhluwe Mine	KZN	-28.0333	32.2833	*Eragrostis trichophora*		m
GFS_PHI_1416F	Tetramesa sp. 26	■		PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis rigidior*		f
GFS_PHI_1419F	Tetramesa sp. 18	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis gummiflua*		f
GFS_PHI_1436_1	Tetramesa sp. 2	■		NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_PHI_1436_2	Tetramesa sp. 2	■		NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_PHI_1436_3	Tetramesa sp. 2	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Sporobolus pyramidalis*	Sep-2021	
GFS_PHI_1447	Tetramesa sp. 18	■	■	PNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis gummiflua*		m
GFS_PHI_1447M	Tetramesa sp. 18	■	■	NPNS	SA, Phinda	KZN	-27.8909	32.2097	*Eragrostis gummiflua*		m
GFS-PHI-1022	Tetramesa sp. 22	■	■	SB	SA, Khuleni Powerlines, Hluhluwe	KZN	-28.0333	32.2833	*Panicum maximum*	Feb-2022	
IP_2021_0001	Eurytoma	■	■	NA	SA, Phinda	KZN	-27.8909	32.2097	*Panicum maximum*	Sep-2021	
KCP_2011_00169	Tetramesa romana	■	■	PNS	SA, Bethlehem area	FS	-28.4315	28.0129	*Arundo donax*	Sep-11	
KCP_2011_00173	Tetramesa romana	■	■	PNS	SA, Bethlehem area	FS	-28.4005	27.2529	*Arundo donax*	Sep-11	
KCP_2012_00170	Tetramesa romana	■	■	PNS	SA, Bethlehem area	FS	-28.5110	27.3018	*Arundo donax*	Nov-12	
KCP_2012_00172	Tetramesa romana	■	■	PNS	SA, Bethlehem area	FS	-28.4211	27.2640	*Arundo donax*	Nov-12	
KCP_2020_00012	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	22/11/2020	f
KCP_2020_00019	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.3951	27.2617	*Eragrostis curvula*	24/11/2020	f
KCP_2020_00032	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.3951	27.2617	*Eragrostis curvula*	25/11/2020	m
KCP_2020_00033	Tetramesa sp. 5	■	■	NPNS	SA, Rietspruit area	FS	-28.3951	27.2617	*Eragrostis curvula*	25/11/2020	f
KCP_2020_00036	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4524	27.3318	*Eragrostis curvula*	26/11/2020	f
KCP_2020_00037	Tetramesa sp 5	■	■	NPNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	27/11/2020	m
KCP_2020_00039	Tetramesa sp. 5	■	■	NPNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	27/11/2020	m
KCP_2020_00040	Tetramesa sp. 20	■	■	NPNS	SA, Rietspruit area	FS	-28.4514	27.3352	*Eragrostis plana*	27/11/2020	m
KCP_2020_00041	Tetramesa sp. 5	■	■	NPNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	27/11/2020	m
KCP_2020_00044	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4524	27.3318	*Eragrostis curvula*	27/11/2020	f
KCP_2020_00045	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4524	27.3318	*Eragrostis curvula*	28/11/2020	m
KCP_2020_00047	Tetramesa sp. 20	■	■	NPNS	SA, Rietspruit area	FS	-28.4514	27.3352	*Eragrostis plana*	27/11/2020	m
KCP_2020_00048	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	27/11/2020	m
KCP_2020_00052	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4524	27.3318	*Eragrostis curvula*	27/11/2020	f
KCP_2020_00054	Tetramesa sp. 20	■	■	NPNS	SA, Rietspruit area	FS	-28.4514	27.3352	*Eragrostis plana*	30/11/2020	f
KCP_2020_00055	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.3951	27.2617	*Eragrostis curvula*	30/11/2020	f
KCP_2020_00056	Tetramesa sp. 20		■	NPNS	SA, Rietspruit area	FS	-28.4514	27.3352	*Eragrostis plana*	30/11/2020	m
KCP_2020_00061	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.3951	27.2617	*Eragrostis curvula*	30/11/2020	f
KCP_2020_00062	Tetramesa sp. 4	■	■	PNS	SA, Rietspruit area	FS	-28.4520	27.3330	*Eragrostis curvula*	30/11/2020	f
KCP_2020_00063	Tetramesa sp. 20	■	■	NPNS	SA, Rietspruit area	FS	-28.4514	27.3352	*Eragrostis plana*	30/11/2020	m
KCP_2020_00075	Tetramesa sp. 16	■	■	NPNS	SA, Rietspruit area	FS	-28.4340	27.3537	*Eragrostis tef*	16/3/2021	f
KCP_2020_00076	Tetramesa sp. 16	■	■	NPNS	SA, Rietspruit area	FS	-28.4340	27.3537	*Eragrostis tef*	16/3/2021	f
KCP_2020_00077	Tetramesa sp. 16	■	■	NPNS	SA, Rietspruit area	FS	-28.4340	27.3537	*Eragrostis tef*	16/3/2021	m
KCP_2020_00079	Tetramesa sp. 16	■	■	NPNS	SA, Rietspruit area	FS	-28.4340	27.3537	*Eragrostis tef*	16/3/2021	f
KCP_2020_00085	Tetramesa sp. 18	■	■	NPNS	SA, Rietspruit area	FS	-28.4417	27.3617	*Eragrostis gummiflua*	16/3/2021	m
KCP_2020_00089	Tetramesa sp. 18	■	■	NPNS	SA, Rietspruit area	FS	-28.4417	27.3617	*Eragrostis gummiflua*	11/3/2021	f
KCP_2020_00090	Tetramesa sp. 18	■	■	NPNS	SA, Rietspruit area	FS	-28.4417	27.3617	*Eragrostis gummiflua*	16/3/2021	f
KCP_2020_00092	Tetramesa sp. 17	■	■	PNS	SA, Rietspruit area	FS	-28.4417	27.3617	*Eragrostis gummiflua*	16/3/2021	f
KCP_2020_00096	Tetramesa sp. 19	■	■	NPNS	SA, Rietspruit area	FS	-28.4415	27.3616	*Eragrostis lehmanniana*	11/3/2021	f
KCP_2020_00140	Tetramesa sp. 18	■	■	NPNS	SA, Rietspruit area	FS	-28.4417	27.3617	*Eragrostis gummiflua*	18/3/2021	m
KCP_2020_00175	Tetramesa sp. 20	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis plana*	6-Feb-22	m
KCP_2020_00177	Tetramesa sp. 23	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis biflora*	7-Feb-22	m
KCP_2020_00178	Tetramesa sp. 23	■		PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis biflora*	7-Feb-22	f
KCP_2020_00184	Tetramesa sp. 18	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis gummiflua*		f
KCP_2020_00185	Tetramesa sp. 18	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis gummiflua*	6-Feb-22	f
KCP_2020_00186	Tetramesa sp. 18		■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis gummiflua*	6-Feb-22	f
KCP_2020_00194	Tetramesa sp. 24	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis capensis*	11-Feb	f
KCP_2020_00199	Tetramesa sp. 25	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis plana*	14-Feb-22	f
KCP_2020_00201	Tetramesa sp. 25	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis plana*	15-Feb-22	f
KCP_2020_00207	Tetramesa sp. 25	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis plana*		f
KCP_2020_00213	Tetramesa sp. 20	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis plana*	11-Feb-22	f
KCP_2020_00217	Tetramesa sp. 23	■	■	PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis biflora*	12-Feb-22	m
KCP_2020_00219	Tetramesa sp. 17	■		PNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis gummiflua*	10-Feb-22	m
KCP_2020_00224	Tetramesa sp. 20	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7689	27.5267	*Eragrostis plana*	17-Feb-22	f
KCP_2020_00225	Tetramesa sp. 20	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis plana*		f
KCP_2020_00233	Tetramesa sp. 20	■	■	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis plana*	18-Feb-22	m

Sequence ID	Morphospecies assignment	COI	28S	Morphotype	Country	Province	Latitude	Longitude	Host	Collection_date	Sex
KCP_2020_00235	Tetramesa sp. 18	🟦		NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis gummiflua*	18-Feb-22	m
KCP_2020_00238	Tetramesa sp. 4	🟦	🟦	PNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis curvula*	15-Feb-22	m
KCP_2020_00239	Tetramesa sp. 24	🟦	🟦	PNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis capensis*	24-Feb-22	f
KCP_2020_00241	Tetramesa sp. 18	🟦	🟦	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis gummiflua*	6-Mar-22	m
KCP_2020_00242	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis curvula*	28-Feb-22	f
KCP_2020_00243	Tetramesa sp. 18	🟦	🟦	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis gummiflua*	6-Mar-22	f
KCP_2020_00244	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis curvula*	3-Mar-22	f
KCP_2020_00245	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Kombuisland farm, Marquard	FS	-28.7556	27.5583	*Eragrostis curvula*	29 February 2022	f
KCP_2020_00247	Unknown	🟦	🟦	NPNS	SA	KZN			*Phragmites mauritianus*	24-Jan-15	f
LMY_2021_00110	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	m
LMY_2021_00117	Tetramesa sp. 4	🟦	🟦	PNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	
LMY_2021_00125	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	f
LMY_2021_00197	Tetramesa sp. 4	🟦	🟦	PNS	SA	NC	-30.3470	24.5632	*Eragrostis curvula*		
LMY_2021_00220	Tetramesa sp. 4	🟦	🟦	PNS	SA	NC	-30.3470	24.5632	*Eragrostis curvula*		
LMY_2021_00231	Tetramesa sp. 5	🟦	🟦	NPNS	SA	NC	-30.3470	24.5632	*Eragrostis curvula*		
LMY_2021_00251	Tetramesa sp. 11	🟦	🟦	NPNS	SA, Mpumalanga	MP	-25.0461	31.0991	*Eragrostis trichophora*	Feb-2022	m
LMY_2021_00282	Tetramesa sp. 5	🟦	🟦	NPNS	SA	NC	-30.3470	24.5632	*Eragrostis curvula*		
LMY_2021_00287	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	f
LMY_2021_00289	Tetramesa sp. 5	🟦	🟦	NPNS	SA	NC	-30.3470	24.5632	*Eragrostis curvula*		
LMY_2021_00300	Tetramesa sp. 5	🟦	🟦	NPNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	f
LMY_2021_00308	Tetramesa sp. 4	🟦	🟦	PNS	SA, Mpumalanga	MP	-25.6083	31.1000	*Eragrostis curvula*	Feb-2022	f
LMY_2022_00008	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Sporobolus sp.*		f
LMY_2022_00090	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Sporobolus sp.*		f
LMY_2022_00098	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Sporobolus sp.*		f
LMY_2022_00108	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Sporobolus sp.*		f
LMY_2022_00139	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Panicum maximum*		f
LMY_2022_00152	Tetramesa sp. 1	🟦	🟦	PNS	SA, East London	EC	-32.7039	27.4833	*Sporobolus sp.*		f
LMY_2022_00177	Tetramesa sp. 1	🟦	🟦	NPNS	SA, East London	EC	-32.7039	27.4833	*Panicum maximum*		f
SPY_KZN_058	Tetramesa sp. 2		🟦	NPNS	SA, Mkuze	KZN	-27.6221	32.0247	*Sporobolus pyramidalis*		
SPY_KZN_060	Tetramesa sp. 1	🟦	🟦	PNS	SA, Mkuze	KZN	-27.6221	32.0247	*Sporobolus pyramidalis*	2017	
SPY_KZN_062	Tetramesa sp. 2	🟦	🟦	NPNS	SA, Westwood Mall, Durban	KZN	-29.8291	30.9591	*Sporobolus pyramidalis*	2018	
SPY_KZN_064	Tetramesa sp. 1	🟦	🟦	PNS	SA, Westwood Mall, Durban	KZN	-29.8291	30.9591	*Sporobolus pyramidalis*	2017	
SPY_KZN_068	Tetramesa sp. 1	🟦	🟦	PNS	SA, Westwood Mall, Durban	KZN	-29.8291	30.9591	*Sporobolus pyramidalis*	2017	
SPY_KZN_071	Tetramesa sp. 2	🟦	🟦	NPNS	SA, Mkuze	KZN	-27.6221	32.0247	*Sporobolus pyramidalis*	2017	
SPY_LMP_032	Tetramesa sp. 1	🟦	🟦	PNS	SA, Nylsvlei	LP	-24.5760	28.6432	*Sporobolus pyramidalis*	2017	
SPY_MPA_095	Unknown		🟦	NA	Imvelo Forest, White River	MP	-25.2168	30.9973	*Sporobolus pyramidalis*	2017	
Z01_A	Unknown	🟦	🟦	NPNS	Zimbabwe		-16.92097	30.81323	*Androgpogon gayanus*	2022	f
Z01_B	Unknown	🟦	🟦	NPNS	Zimbabwe		-16.92097	30.81323	*Androgpogon gayanus*	2022	f

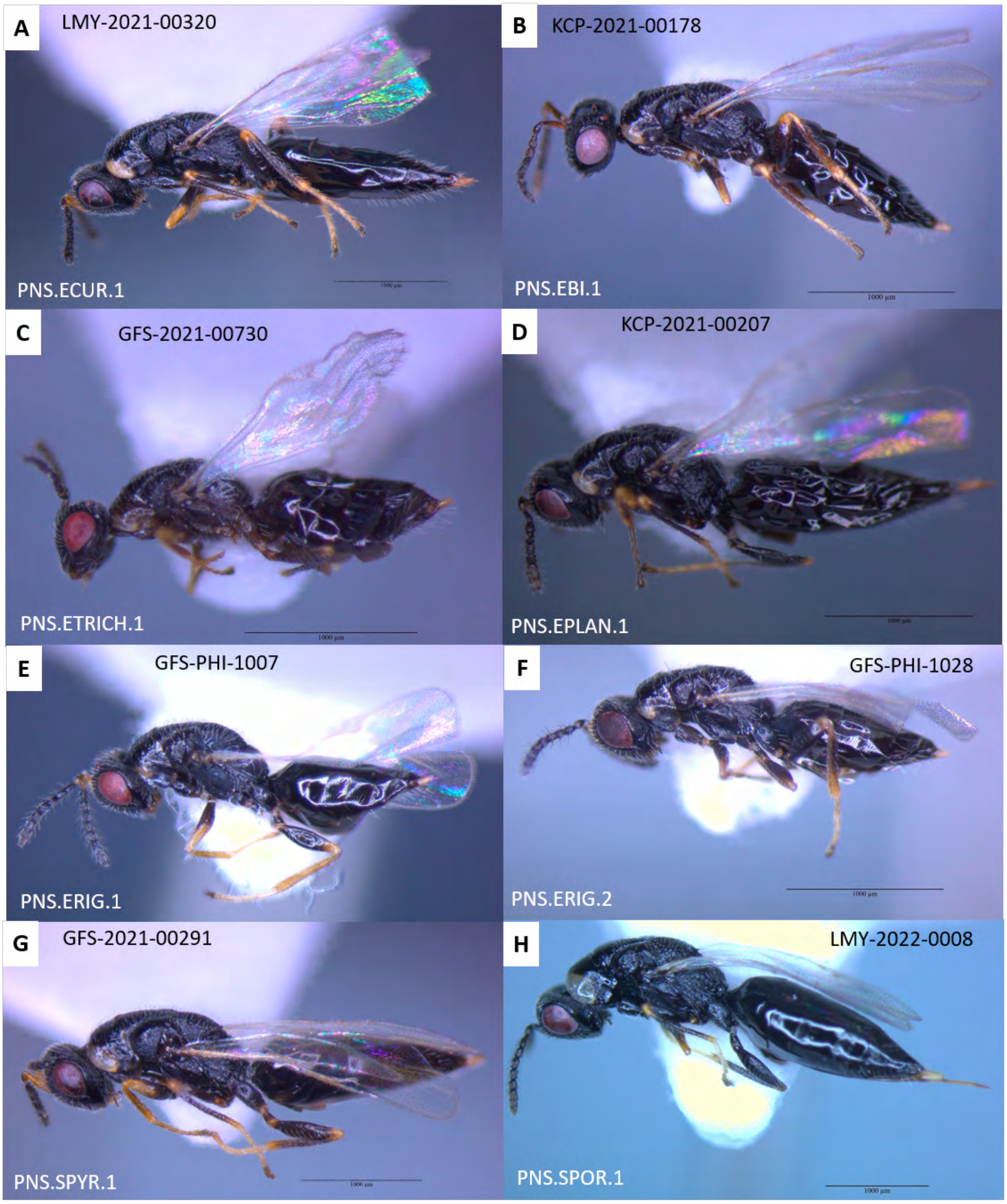
GenBank and BOLD sequences from published and unpublished sources

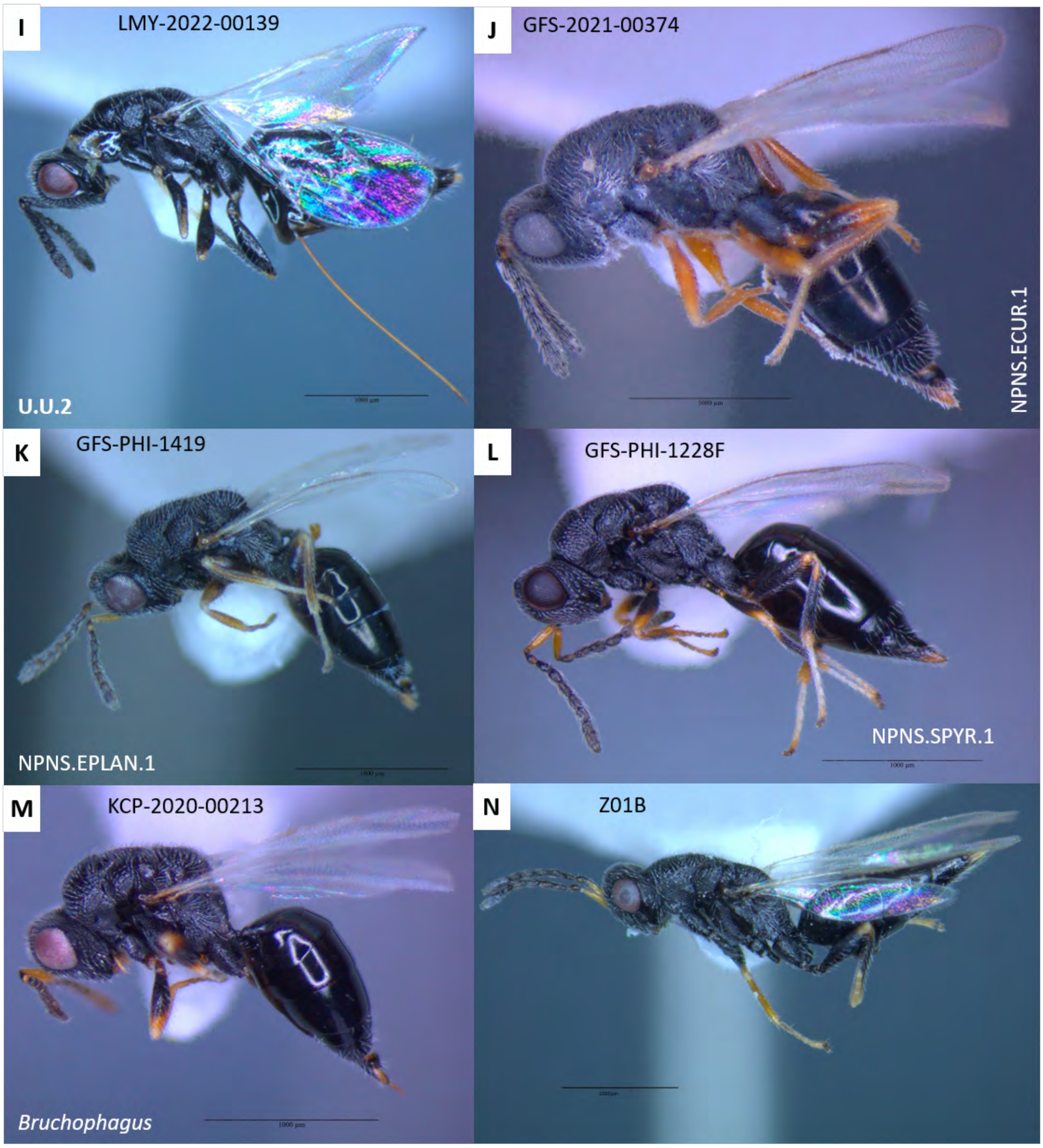
Voucher	Identification	COI	28S	Morphotype	Country	Latitude	Longitude	Reference	Collection_date	Sex
IOZ 020003	Bruchophagus ononis	AY317223	AY317170		China: Fanjing Mt.,Guizhou			Chen et al. 2004		
IOZ 010002	Bruchophagus sp1	AY317226	AY317164		China: Riwoqê Tibet			Chen et al. 2004		
ROM 905047	Bruchophagus sp2	AY317239	AY317169		Guyana: Rupununi district: Kurupukari			Chen et al. 2004		
IOZ 990001	Bruchophagus gibbus	AY317227	AY317155		China: Dayao Mt., Guangxi			Chen et al. 2004		
IOZ 010003	Eurytoma orchidearum	AY317233	AY317163		China: Kangding, Sichuan			Chen et al. 2004		
IOZ 010004	Eurytoma verticillata	AY317225	AY317178		China: Baoxing, Sichuan			Chen et al. 2004		
IOZ 010005	Eurytoma sp. 2	AY317236	AY317166		China: Riwoqê, Tibet			Chen et al. 2004		
IOZ 980001	Eurytoma sp. 3	AY317224	AY317162		China: Tianmu Mt., Zhejiang			Chen et al. 2004		
ROM 905032	Isosomodes gigantenus	AY317235	AY317168		Guyana: Mazaruni-Potaro district: Amatuk fall			Chen et al. 2004		
ROM 920116	Phylloxeroxenus phylloxerae	AY317238	AY317176		Indonesia: East of Kalimantan, Kutai			Chen et al. 2004		
IOZ 990002	Plutarchia indefensa	AY317228	AY317179		China: Dayao Mt., Guangxi			Chen et al. 2004		
IOZ 200001	Sycophila concinna	AY317222	AY317181		China: Napo, Guangxi			Chen et al. 2004		
IOZ 020008		AY317232	AY317167		China: Fanjing Mt., Guizhou			Chen et al. 2004		
IOZ 020009	Sycophila taprobanica	AY317230	AY317159		China: Fanjing Mt., Guizhou			Chen et al. 2004		
IOZ 020010	Sycophila sp. 1	AY317231	AY317158		China: Xinhe, Xinjiang			Chen et al. 2004		
IOZ 200002	Sycophila variegata	AY317234	AY317157		China: Napo, Guangxi			Chen et al. 2004		
ROM 920118	Tenuipetiolus medicaginis	AY317242	AY317174		Indonesia: East of Kalimantan, Kutai			Chen et al. 2004		
IOZ 990003	Tetramesa bambusae	AY317229	AY317180		China: Liuba, Shaanxi			Chen et al. 2004		
ROM 905018	Neorileya meridionalis	AY317243	AY317156		Guyana: Mazaruni-Potaro District: Kaieteur fall			Chen et al. 2004		
IOZ 020006	Megastigmus dorsalis	AY317240	AY317161		China: Fanjing Mt.,Guizhou			Chen et al. 2004		
IOZ 020007	Monodontomerus minor	AY317241	AY317165		China: Fanjing Mt.,Guizhou			Chen et al. 2004		

Sequence ID	Morphospecies assignment	COI	28S	Morphotype	Country	Province	Latitude	Longitude	Host	Collection_date	Sex
IOZ 020002	Brachymeria lasus	AY317221	AY317177		China: Fanjing Mt.,Guizhou				Chen et al. 2004		
IOZ 020001	Aiolomorphus rhopaloides		AY317172		China: Fanjing Mt.,Guizhou				Chen et al. 2004		
ROM 920118	Bephratelloides cubensis		AY317173		Indonesia: East of Kalimantan, Kutai				Chen et al. 2004		
IOZ 010001	Bruchophagus sp. 3		AY317160		China: Oamdo Tibet				Chen et al. 2004		
IOZ 020005	Eurytoma manilensis		AY317171		China: Beijing				Chen et al. 2004		
IOZ 020004	Eurytoma sp. 1		AY317175		China: Fanjing Mt.,Guizhou				Chen et al. 2004		
GDEL1626	Bruchophagus sp. 2	MH878935							Unpublished		
GDEL1616	Bruchophagus sp. 2	MH878926							Unpublished		
GDEL1029	Bruchophagus gibbus	KC960021							Delavare et al. 2014		
BIOUG22728-C12	Bruchophagus gibbus	MG340126							Unpublished		
GDEL1288	Bruchophagus caucasicus	JQ756607							Cruaud et al. 2013		
BC-ZSM-HYM-24067-B02	Tetramesa sp.	AMTPA1154-15		PNS	Germany: Bavaria		47.39200	10.37100	Unpublished	6-Aug-2014	f
BC-ZSM-HYM-24059-G10	Tetramesa sp.	AMTPA462-15			Germany: Bavaria		47.38800	10.34400	Unpublished	1-June-2014	f
BC-ZSM-HYM-24062-A09	Tetramesa sp.	AMTPA674-15			Germany: Bavaria		47.38700	10.34400	Unpublished	1-June-2014	m
BC-ZSM-HYM-24062-D05	Tetramesa sp.	AMTPA706-15			Germany: Bavaria		47.39200	10.34100	Unpublished	1-June-2014	
BC-ZSM-HYM-24062-D06	Tetramesa sp.	AMTPA707-15			Germany: Bavaria		47.39200	10.34100	Unpublished	1-June-2014	f
BC-ZSM-HYM-24064-D07	Tetramesa sp.	AMTPA898-15		PNS	Germany: Bavaria		47.38800	10.34400	Unpublished	17-May-2014	m
BC-ZSM-HYM-24064-D08	Tetramesa sp.	AMTPA899-15		PNS	Germany: Bavaria		47.38800	10.34400	Unpublished	17-May-2014	m
BC-ZSM-HYM-24115-H05	Tetramesa sp.	AMTPB1039-15			Germany: Bavaria		47.39200	10.34100	Unpublished	1-June-2014	f
BC-ZSM-HYM-24115-H09	Tetramesa sp.	AMTPB1043-15			Germany: Bavaria		47.39200	10.34100	Unpublished	1-June-2014	f
BC-ZSM-HYM-24118-B05	Tetramesa sp.	AMTPB1252-15		PNS	Germany: Bavaria		47.38800	10.34400	Unpublished	1-June-2014	f
BC-ZSM-HYM-24118-B07	Tetramesa sp.	AMTPB1254-15		PNS	Germany: Bavaria		47.38800	10.34400	Unpublished	1-June-2014	f
BC-ZSM-HYM-24106-E08	Tetramesa sp.	AMTPB151-15		PNS	Germany: Bavaria		47.39400	10.36600	Unpublished	10-June-2014	
BIOUG11906-B01	Tetramesa sp.	KR416842			Canada: Quebec, Forillon NP		48.85700	-64.37600	Unpublished	15-July-2013	
BIOUG42677-C06	Tetramesa sp.	GMGMP2393-18			Germany: Bavaria		48.16500	11.48500	Unpublished	5-June-2017	
BIOUG42916-B01	Tetramesa sp.	GMGMP7221-18			Germany: Bavaria		48.16500	11.48500	Unpublished	5-June-2017	
BIOUG55106-G08	Tetramesa sp.	GMGMT1030-20			Germany: Bavaria		48.16500	11.48500	Unpublished	8-May-2017	
BIOUG33331-G01	Tetramesa sp.	OPPEC4539-17			Canada, Ontario, Owen Sound		44.63370	-80.69830	Unpublished	12-June-2014	
BIOUG34713-F07	Tetramesa sp.	OPPFM1893-17			Canada, Ontario, Windsor		42.26280	-83.07250	Unpublished	11-June-2014	
BIOUG34781-C07	Tetramesa sp.	OPPFM3406-17			Canada, Ontario, Windsor		42.26280	-83.07250	Unpublished	11-June-2014	
BIOUG34699-B12	Tetramesa sp.	OPPFM520-17			Canada, Ontario, Windsor		42.26280	-83.07250	Unpublished	11-June-2014	
BIOUG10641-H10	Tetramesa sp.	KR408765			Canada: Ontario, Georgian Bay Islands NP		44.74180	-79.85010	Unpublished	23-May-2013	
BIOUG10613-H05	Tetramesa sp.	KR405666			Canada: Ontario, Georgian Bay Islands NP		44.74180	-79.85010	Unpublished	23-May-2013	
BIOUG10613-D11	Tetramesa sp.	KR402173			Canada: Ontario, Georgian Bay Islands NP		44.74180	-79.85010	Unpublished	23-May-2013	
BIOUG05588-G03	Tetramesa sp.	MG344135			Canada: Ontario, Burlington		43.33200	-79.84000	Unpublished	3-May-2013	
BIOUG31750-F03	Tetramesa sp.	MG344033			Canada: Ontario, Guelph		43.51870	-80.17090	Unpublished	15-May-2015	
BIOUG21916-E07	Tetramesa sp.	MG340113			Canada: Ontario, Scarborough, Woburn		43.77750	-79.22680	Unpublished	8-May-2015	
BIOUG00786-G04	Tetramesa sp.	MG339825			Canada: Ontario, Wellington County		43.53700	-80.13400	Unpublished	9-May-2010	
BIOUG21953-D02	Tetramesa sp.	MG335372			Canada: Ontario, Toronto		43.68980	-79.53120	Unpublished	8-May-2015	
BIOUG31770-F09	Tetramesa sp.	MG334647			Canada: Ontario, Guelph		43.51870	-80.17090	Unpublished	22-May-2015	
BIOUG21911-C05	Tetramesa sp.	MG339505			Canada: Ontario, Toronto, Zion Heights		43.79800	-79.37000	Unpublished	8-May-2015	
BIOUG03191-A06	Tetramesa sp.	MF903065			Canada: Alberta, Elk Island NP		53.68500	-112.86000	Unpublished	15-June-2012	
BIOUG03349-F04	Tetramesa sp.	KR800436			Canada: Manitoba, Riding Mountain NP		50.67600	-99.89800	Unpublished	26-June-2012	
10BBCHY-1098	Tetramesa sp.	KR799792			Canada: Saskatchewan, Prince Albert NP		53.90500	-106.07400	Unpublished	18-June-2010	
KNWR 5327	Tetramesa sp.	KU496847			USA: Alaska, Kenai Peninsula		60.58400	-150.68200	Unpublished	13-June-2006	
D1502	Tetramesa sp. 1		AY599281		USA: MD: Wicomico Co.				Gillespie et al. 2005		
LK-2005	Tetramesa sp.		DQ080114		Germany (?)				Unpublished		
UCRC_ENT 161286	Tetramesa sp. 2		JN623677		USA: MD: Prince Georges Co.				Munro et al. 2011		

Table S2: Divergence values (K2P), as percentages, for subsets of the 28S sequence data. Bold values along the diagonal are intraspecific distances. Bruch. = *Bruchophagus*, PNS = pronotal spot, NPNS = non-pronotal spot. Cells in grey represent putative conspecific groups, with divergenc values < 3.5%.

	A. gayanus	*Bruch.*	*Eurytoma*	*PNS A. donax*	*PNS E. curvula*	*PNS Sporobolus*	*NPNS E. curvula*	*NPNS hirta*	*NPNS Sporobolus*	*T. bambusae*
A. gayanus	**0**									
Bruch.	4.7	**5.1**								
Eurytoma	3.9	3.7	**1.6**							
PNS A. donax	2.6	7	6	**0.22**						
PNS E. curvula	1.3	5.4	4.3	2	**0.06**					
PNS Sporobolus	1.2	5	4	2.6	0.6	**0.22**				
NPNS E. curvula	4.5	5.3	3.2	6.5	4.7	4.9	**0.04**			
NPNS hirta	0.4	5.6	4.7	2.7	1.3	1.5	4.4	**0.07**		
NPNS Sporobolus	4.2	5.1	2.9	6.4	4.8	4.7	1	4.6	**0.87**	
T. bambusae	2	5.5	4.8	0.3	2	2.2	6.2	2.6	5.8	**N/A**


A
LMY-2021-00320
PNS.ECUR.1
1000 µm
B
KCP-2021-00178
PNS.EBI.1
1000 µm
C
GFS-2021-00730
PNS.ETRICH.1
1000 µm
D
KCP-2021-00207
PNS.EPLAN.1
1000 µm
E
GFS-PHI-1007
PNS.ERIG.1
F
GFS-PHI-1028
PNS.ERIG.2
1000 µm
G
GFS-2021-00291
PNS.SPYR.1
1000 µm
H
LMY-2022-0008
PNS.SPOR.1
1000 µm


I
LMY-2022-00139
U.U.2
J
GFS-2021-00374
NPNS.ECUR.1
K
GFS-PHI-1419
NPNS.EPLAN.1
L
GFS-PHI-1228F
NPNS.SPYR.1
M
KCP-2020-00213
Bruchophagus
N
Z01B

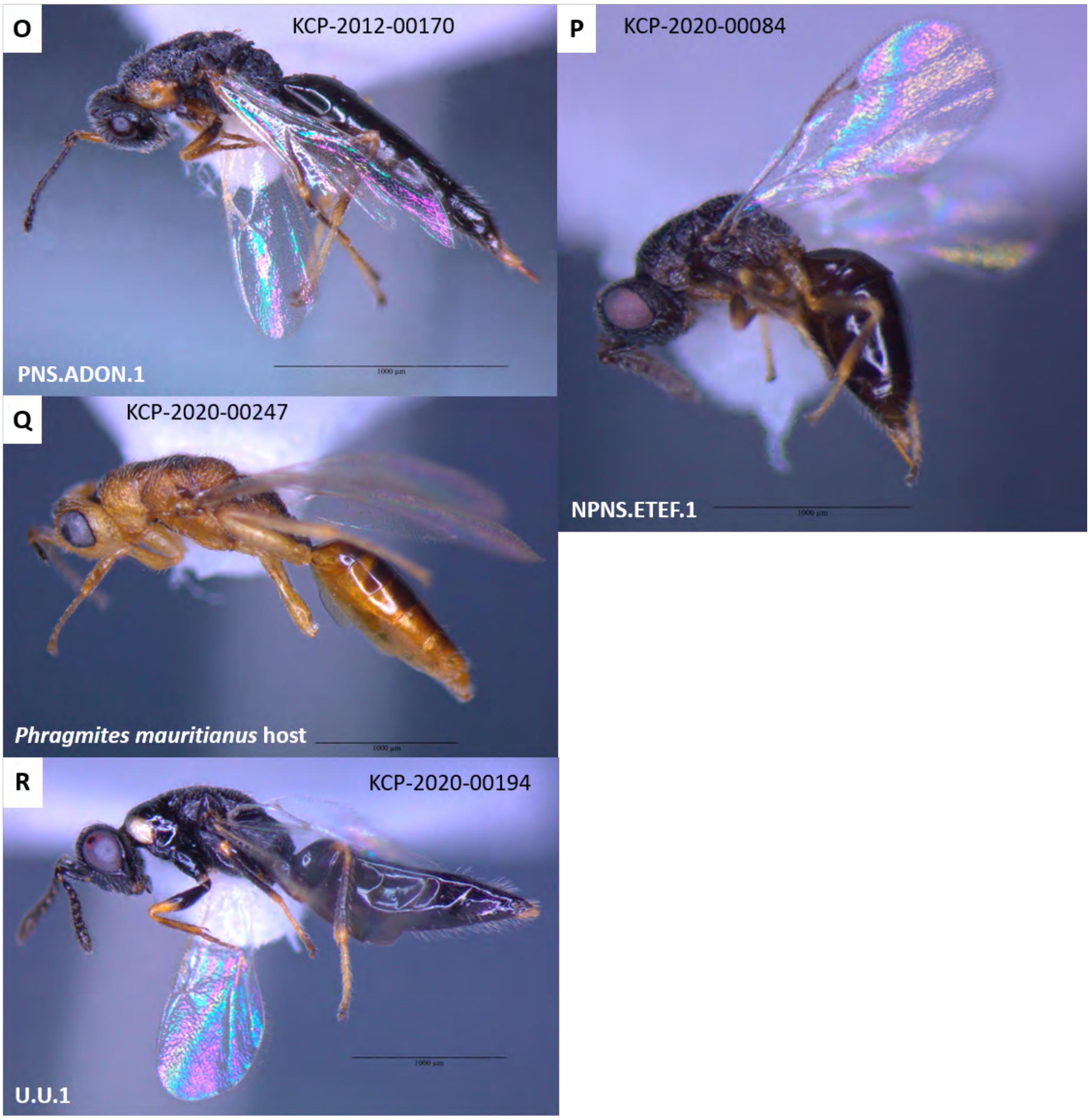


Figure S1: A - Q: microscope images of reference specimens appearing in the COI and 28S phylogenetic trees (Fig. 2.11 and Fig. 2.9). Sample codes and clade IDs are shown at the top and bottom of each image, respectively.

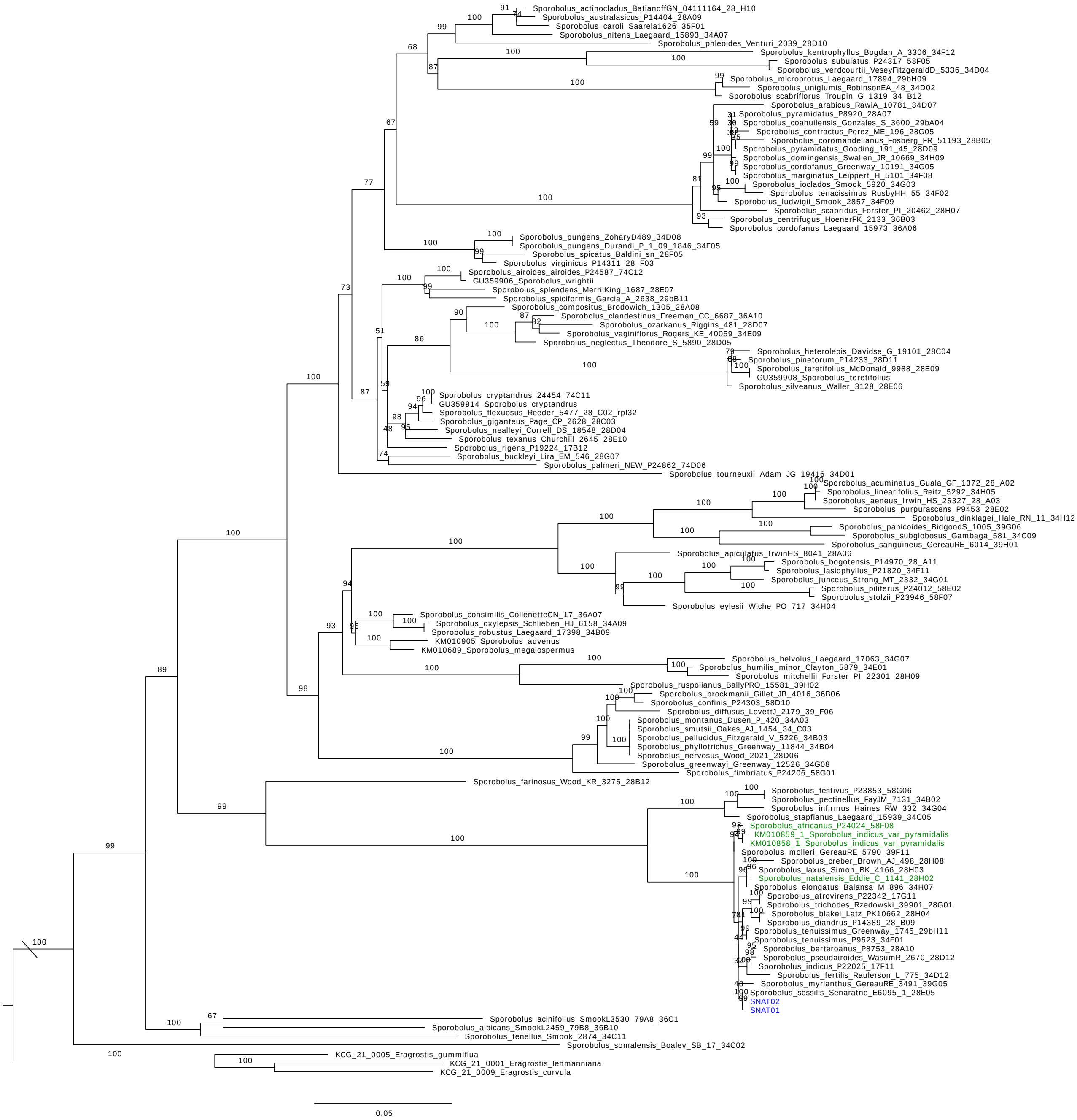


Figure S2: Maximum likelihood phylogeny (IQTREE) for *Sporobolus* ITS sequences from Peterson *et al.* (2014), with the unknown *Sporobolus* samples SNAT01 and SNAT02 shown in blue text. *Sporobolus africanus*, *S. natalensis*, and *S. pyramidalis* are in green text. The best-fit BIC model was SYM + I + Γ, and the log-likelihood of the consensus tree was -21198.414. Three *Eragrostis* samples served as an outgroup (sourced from Peterson *et al.* (2010)).

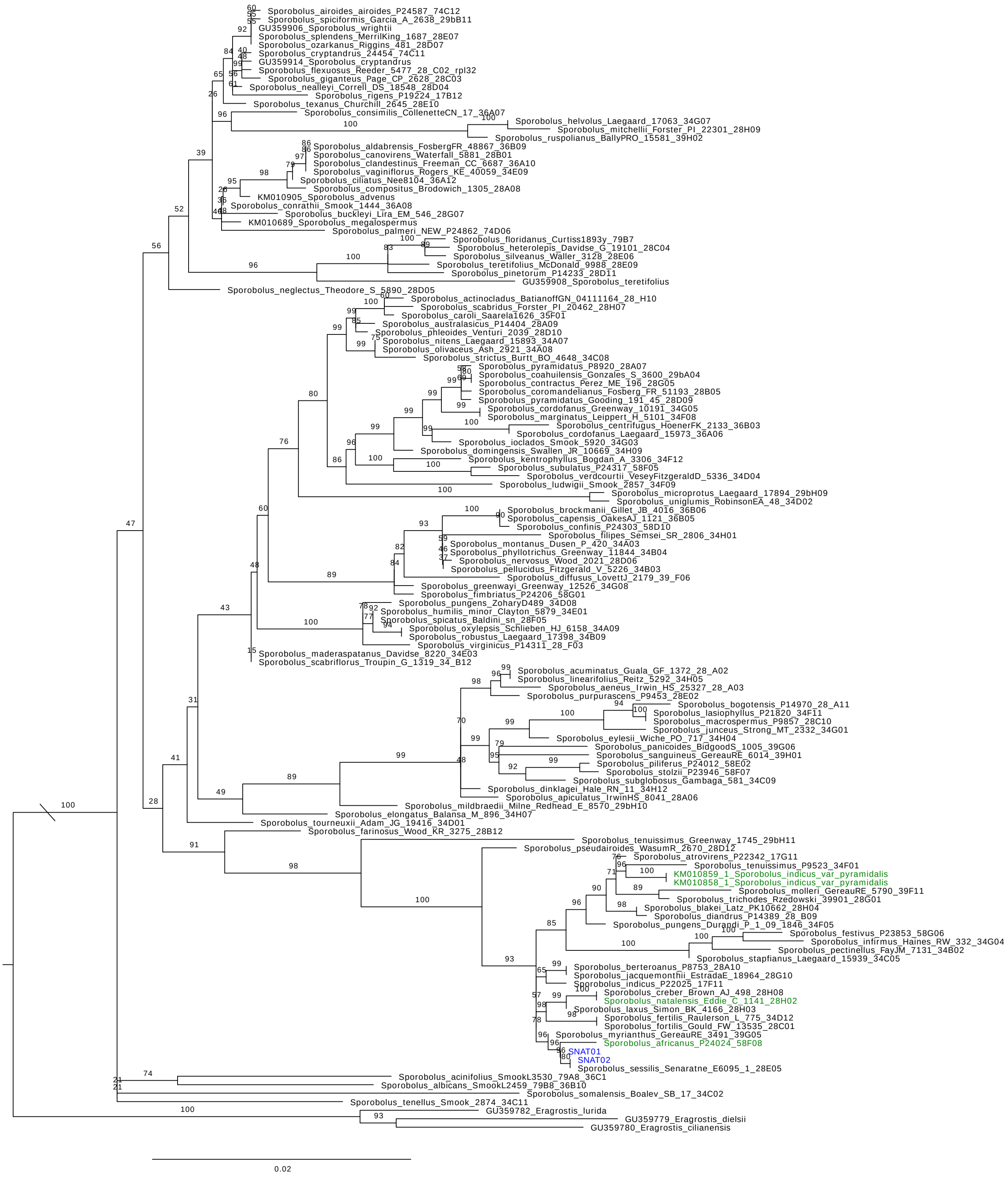


Figure S3: Maximum likelihood phylogeny (IQ-TREE) for *Sporobolus* chloroplast (trnL and trnK) sequences from Peterson *et al.* (2014), with the unknown *Sporobolus* samples SNAT01 and SNAT02 shown in blue text. *Sporobolus africanus*, *S. natalensis*, and *S. pyramidalis* are in green text. The best-fit BIC models for trnL and trnK was TPM3 + F + Γ and TPM3u + F + Γ, respectively, and the log-likelihood of the consensus tree was -7792.919. Three *Eragrostis* samples served as an outgroup (sourced from Peterson *et al.* (2010)).

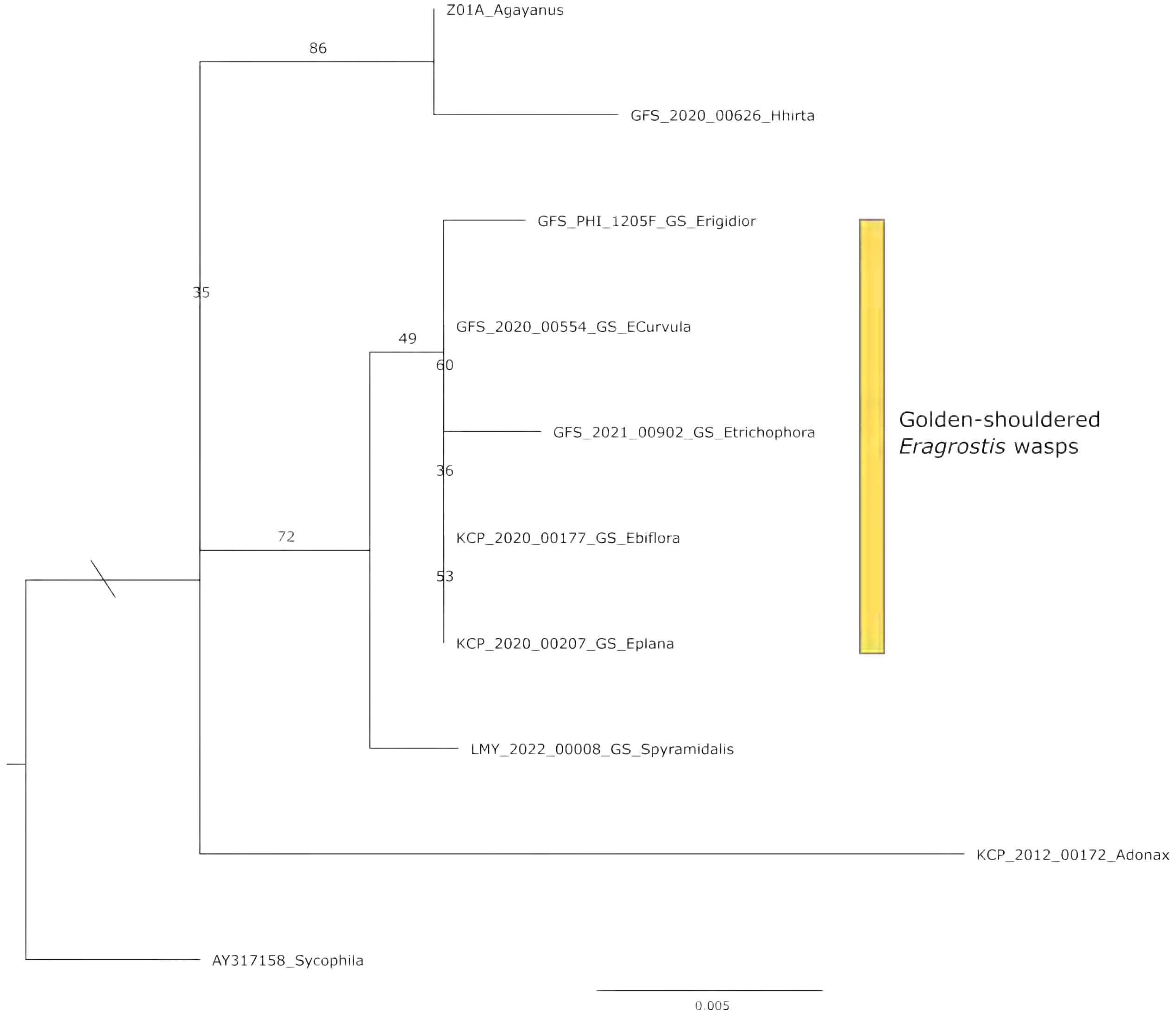


Figure S4: A maximum likelihood phylogeny for representative 28S sequences, showing that the pronotal spot (PNS) *Eragrostis Tetramesa* wasps were not resolved. The best-fit BIC model for this tree was TNe + I (log-likelihood of the consensus tree was -1011.932)

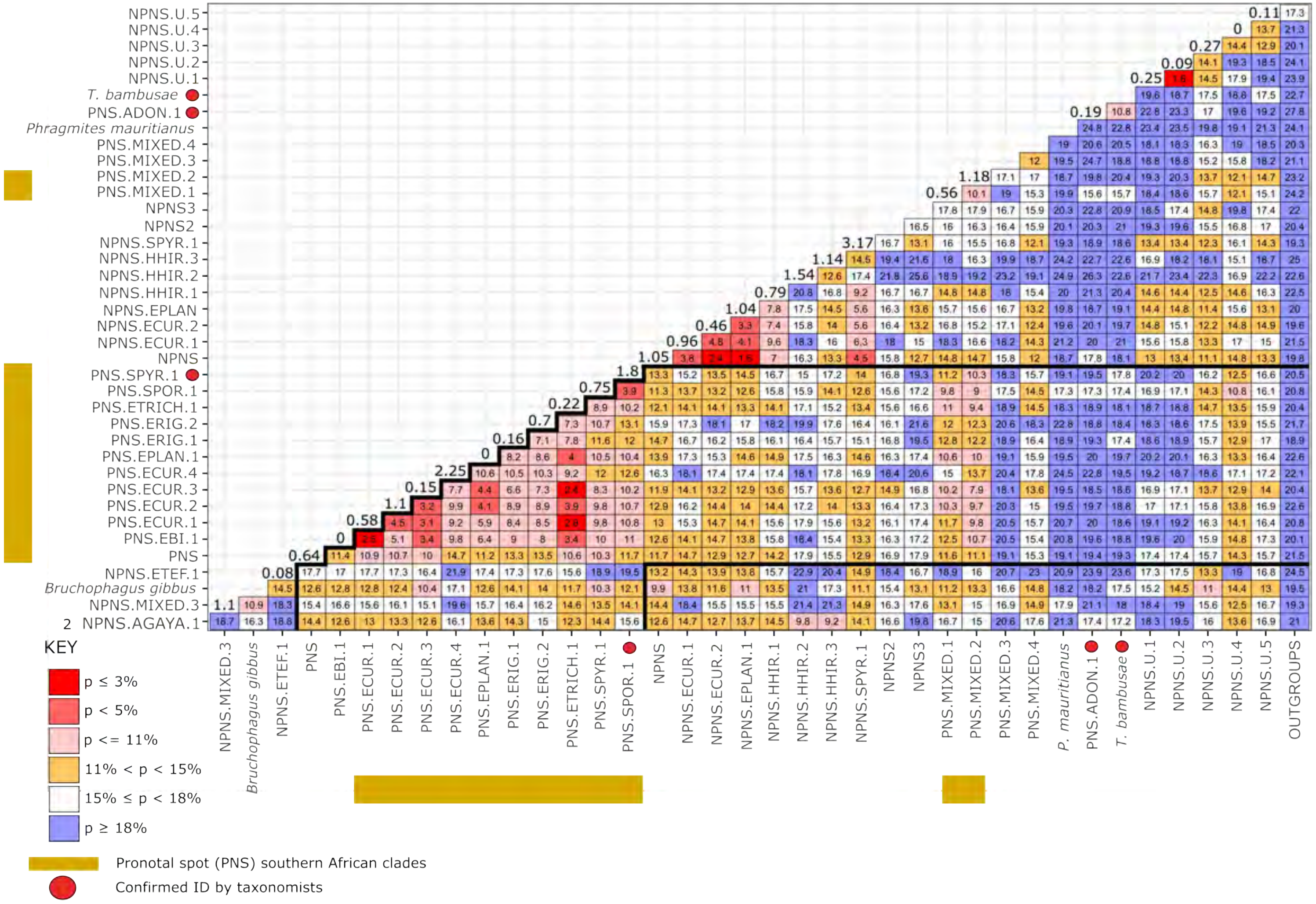
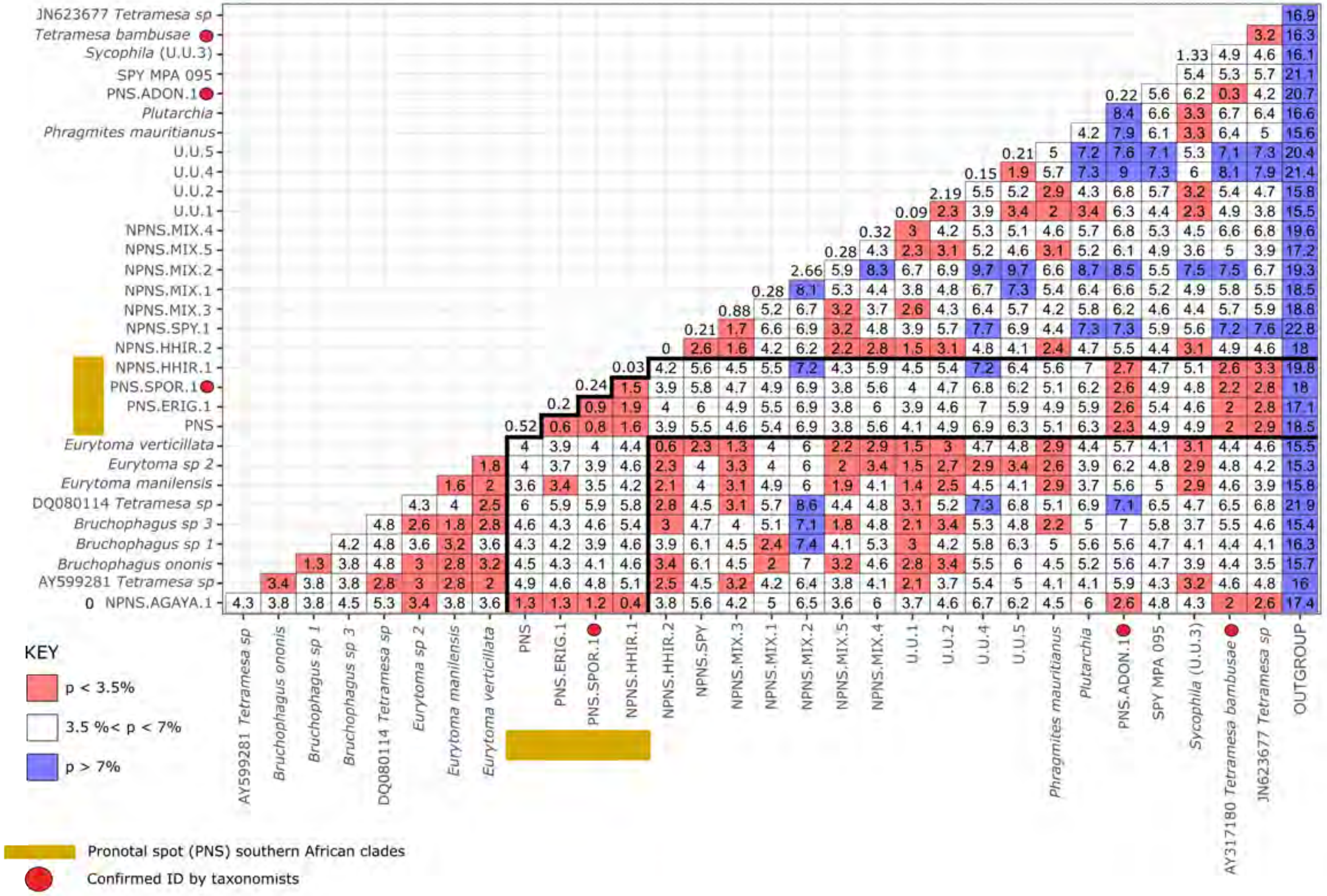


Figure S5: Between-group p-distance matrix for the COI mitochondrial region (500 bootstrap repeats with pairwise deletion, applying the K2P model) showing the average number of base substitutions per site (n = 255 nucleotide sequences, 545 bp length). See Fig. 2.9 for acronyms. Values are displayed as percentages. Values above each vertical column are intraspecific divergence values. Black borders surround the PNS clades.



	AY599281 *Tetramesa sp*	*Bruchophagus ononis*	*Bruchophagus sp 1*	*Bruchophagus sp 3*	DQ080114 *Tetramesa sp*	*Eurytoma sp 2*	*Eurytoma manilensis*	*Eurytoma verticillata*	PNS	PNS.ERIG.1	PNS.SPOR.1 ●	NPNS.HHIR.1	NPNS.HHIR.2	NPNS.SPY	NPNS.MIX.3	NPNS.MIX.1	NPNS.MIX.2	NPNS.MIX.5	NPNS.MIX.4	U.U.1	U.U.2	U.U.4	U.U.5	*Phragmites mauritianus*	*Plutarchia*	PNS.ADON.1 ●	SPY MPA 095	*Sycophila* (U.U.3)	AY317180 *Tetramesa bambusae* ●	JN623677 *Tetramesa sp*	OUTGROUP
JN623677 *Tetramesa sp*																															16.9
Tetramesa bambusae ●																														3.2	16.3
Sycophila (U.U.3)																												1.33	4.9	4.6	16.1
SPY MPA 095																												5.4	5.3	5.7	21.1
PNS.ADON.1 ●																										0.22	5.6	6.2	0.3	4.2	20.7
Plutarchia																										8.4	6.6	3.3	6.7	6.4	16.6
Phragmites mauritianus																									4.2	7.9	6.1	3.3	6.4	5	15.6
U.U.5																							0.21	5	7.2	7.6	7.1	5.3	7.1	7.3	20.4
U.U.4																						0.15	1.9	5.7	7.3	9	7.3	6	8.1	7.9	21.4
U.U.2																					2.19	5.5	5.2	2.9	4.3	6.8	5.7	3.2	5.4	4.7	15.8
U.U.1																				0.09	2.3	3.9	3.4	2	3.4	6.3	4.4	2.3	4.9	3.8	15.5
NPNS.MIX.4																			0.32	3	4.2	5.3	5.1	4.6	5.7	6.8	5.3	4.5	6.6	6.8	19.6
NPNS.MIX.5																		0.28	4.3	2.3	3.1	5.2	4.6	3.1	5.2	6.1	4.9	3.6	5	3.9	17.2
NPNS.MIX.2																	2.66	5.9	8.3	6.7	6.9	9.7	9.7	6.6	8.7	8.5	5.5	7.5	7.5	6.7	19.3
NPNS.MIX.1																0.28	8.1	5.3	4.4	3.8	4.8	6.7	7.3	5.4	6.4	6.6	5.2	4.9	5.8	5.5	18.5
NPNS.MIX.3															0.88	5.2	6.7	3.2	3.7	2.6	4.3	6.4	5.7	4.2	5.8	6.2	4.6	4.4	5.7	5.9	18.8
NPNS.SPY.1														0.21	1.7	6.6	6.9	3.2	4.8	3.9	5.7	7.7	6.9	4.4	7.3	7.3	5.9	5.6	7.2	7.6	22.8
NPNS.HHIR.2													0	2.6	1.6	4.2	6.2	2.2	2.8	1.5	3.1	4.8	4.1	2.4	4.7	5.5	4.4	3.1	4.9	4.6	18
NPNS.HHIR.1												0.03	4.2	5.6	4.5	5.5	7.2	4.3	5.9	4.5	5.4	7.2	6.4	5.6	7	2.7	4.7	5.1	2.6	3.3	19.8
PNS.SPOR.1 ●											0.24	1.5	3.9	5.8	4.7	4.9	6.9	3.8	5.6	4	4.7	6.8	6.2	5.1	6.2	2.6	4.9	4.8	2.2	2.8	18
PNS.ERIG.1										0.2	0.9	1.9	4	6	4.9	5.5	6.9	3.8	6	3.9	4.6	7	5.9	4.9	5.9	2.6	5.4	4.6	2	2.8	17.1
PNS									0.52	0.6	0.8	1.6	3.9	5.5	4.6	5.4	6.9	3.8	5.6	4.1	4.9	6.9	6.3	5.1	6.3	2.3	4.9	4.9	2	2.9	18.5
Eurytoma verticillata									4	3.9	4	4.4	0.6	2.3	1.3	4	6	2.2	2.9	1.5	3	4.7	4.8	2.9	4.4	5.7	4.1	3.1	4.4	4.6	15.5
Eurytoma sp 2								1.8	4	3.7	3.9	4.6	2.3	4	3.3	4	6	2	3.4	1.5	2.7	2.9	3.4	2.6	3.9	6.2	4.8	2.9	4.8	4.2	15.3
Eurytoma manilensis							1.6	2	3.6	3.4	3.5	4.2	2.1	4	3.1	4.9	6	1.9	4.1	1.4	2.5	4.5	4.1	2.9	3.7	5.6	5	2.9	4.6	3.9	15.8
DQ080114 *Tetramesa sp*						4.3	4	2.5	6	5.9	5.9	5.8	2.8	4.5	3.1	5.7	8.6	4.4	4.8	3.1	5.2	7.3	6.8	5.1	6.9	7.1	6.5	4.7	6.5	6.8	21.9
Bruchophagus sp 3					4.8	2.6	1.8	2.8	4.6	4.3	4.6	5.4	3	4.7	4	5.1	7.1	1.8	4.8	2.1	3.4	5.3	4.8	2.2	5	7	5.8	3.7	5.5	4.6	15.4
Bruchophagus sp 1				4.2	4.8	3.6	3.2	3.6	4.3	4.2	3.9	4.6	3.9	6.1	4.5	2.4	7.4	4.1	5.3	3	4.2	5.8	6.3	5	5.6	5.6	4.7	4.1	4.4	4.1	16.3
Bruchophagus ononis			1.3	3.8	4.8	3	2.8	3.2	4.5	4.3	4.1	4.6	3.4	6.1	4.5	2	7	3.2	4.6	2.8	3.4	5.5	6	4.5	5.2	5.6	4.7	3.9	4.4	3.5	15.7
AY599281 *Tetramesa sp*		3.4	3.8	3.8	2.8	3	2.8	2	4.9	4.6	4.8	5.1	2.5	4.5	3.2	4.2	6.4	3.8	4.1	2.1	3.7	5.4	5	4.1	4.1	5.9	4.3	3.2	4.6	4.8	16
0 NPNS.AGAYA.1	4.3	3.8	3.8	4.5	5.3	3.4	3.8	3.6	1.3	1.3	1.2	0.4	3.8	5.6	4.2	5	6.5	3.6	6	3.7	4.6	6.7	6.2	4.5	6	2.6	4.8	4.3	2	2.6	17.4

KEY

🟥 p < 3.5%

☐ 3.5 %< p < 7%

🟦 p > 7%

▬ Pronotal spot (PNS) southern African clades

● Confirmed ID by taxonomists

Figure S6: Between-group p-distance matrix for the 28S nuclear region (500 bootstrap repeats with pairwise deletion, applying the K2P model) showing the average number of base substitutions per site (n = 230 nucleotide sequences, 605 bp length). Values are displayed as percentages. Values above each vertical column are intraspecific divergence values. Black borders surround the PNS clades.

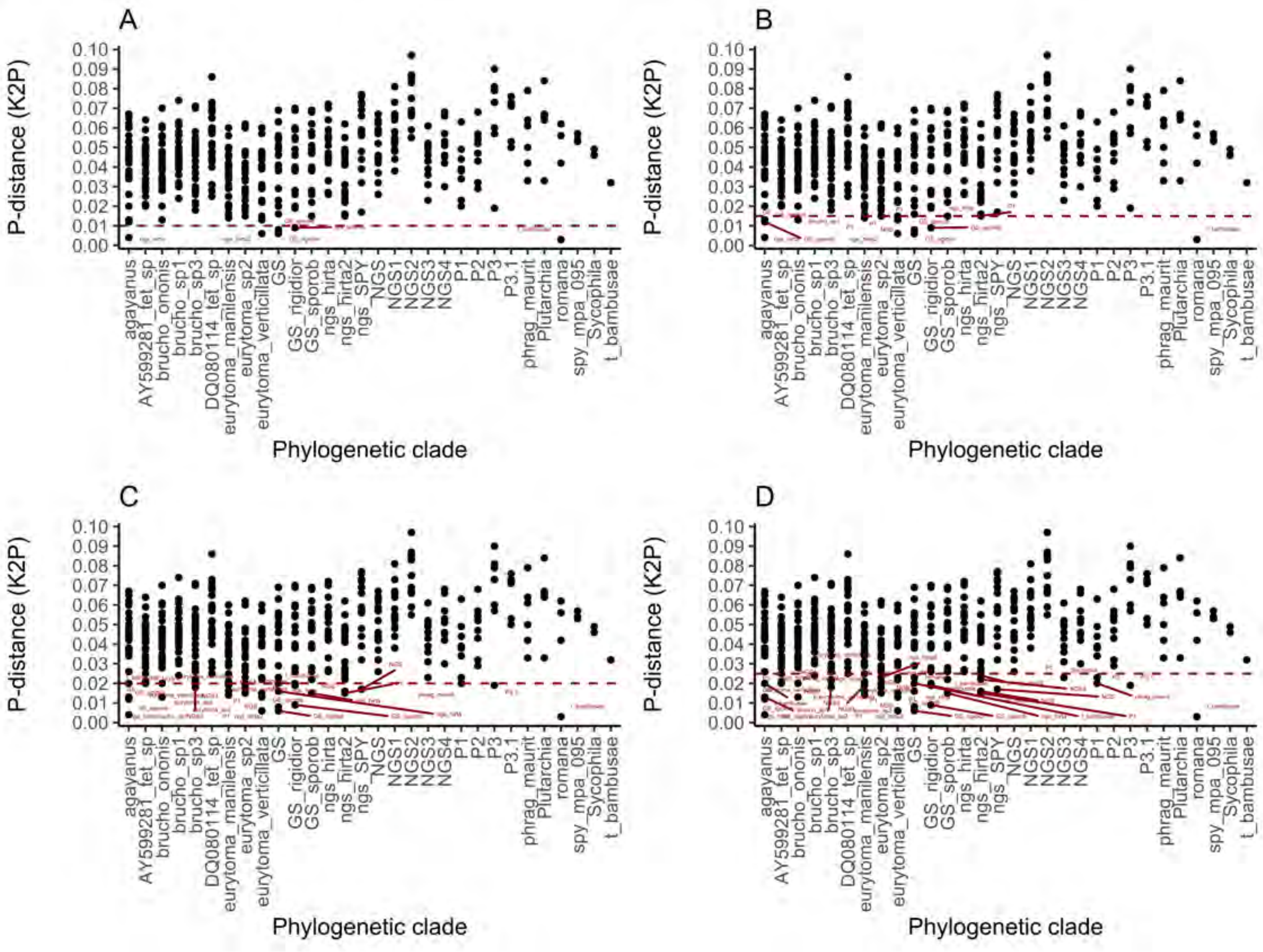


Figure S7: Genetic distance plots for the 28S gene region, showing the pairwise p-distances between groups at threshold intervals of (A) 1%, (B) 1.5%, (C) 2%, and (D) 2.5%. The groups labelled in red in each vertical column are considered to belong to the same taxonomic group as the corresponding phylogenetic clade on the x-axis. GS = PNS, NGS = NPNS.

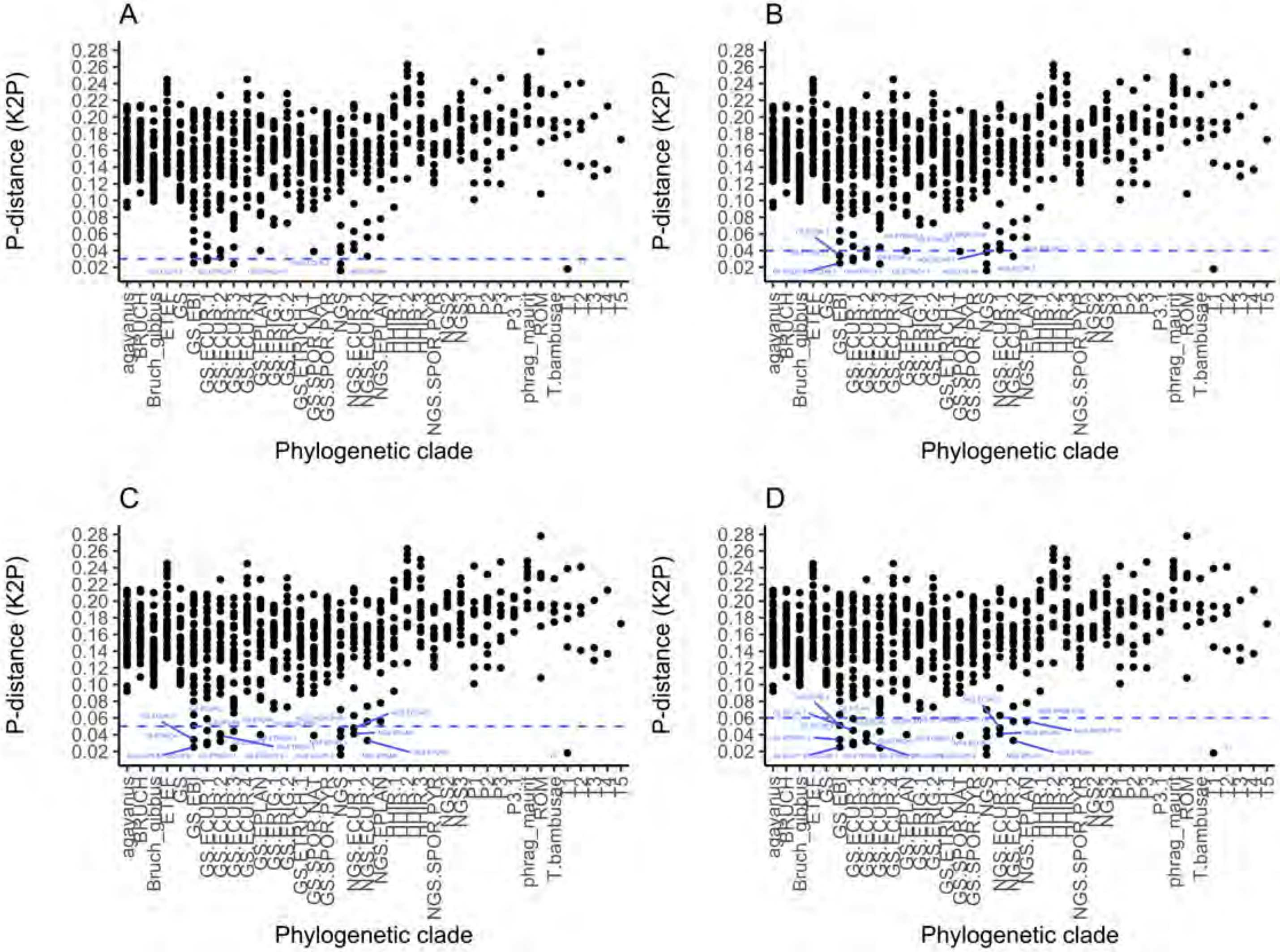


Figure S8: Genetic distance plots for the COI gene region, showing the pairwise p-distances between groups at threshold intervals of (A) 3%, (B) 4%, (C) 5%, and (D) 6%. The groups labelled in blue in each vertical column are considered to belong to the same taxonomic group as the corresponding phylogenetic clade on the x-axis. GS = PNS, NGS = NPNS.

Table S3: Sample information for all the grass sequences (rpl32-trnL, rps16-trnK, rps16, and ITS) included in this work. Unshaded cells in the gene columns denote missing data.

Label	Grass species	Latitude	Longitude	Collection date	rpl32-trnL spacer	rps16-trnK spacer	rps16 intron	ITS
KCG-21-0001	*Eragrostis lehmanniana*	-28.75	27.53	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0002	*Eragrostis lehmanniana*	-28.75	27.53	2/4/2021	🟦			🟦
KCG-21-0003	*Eragrostis lehmanniana*	-28.75	27.53	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0005	*Eragrostis gummiflua*	-28.75	27.53	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0006	*Eragrostis gummiflua*	-28.75	27.53	2/4/2021		🟦	🟦	🟦
KCG-21-0007	*Eragrostis gummiflua*	-28.75	27.53	2/4/2021	🟦		🟦	🟦
KCG-21-0009	*Eragrostis curvula*	-28.75	27.53	2/4/2021	🟦		🟦	🟦
KCG-21-0010	*Eragrostis curvula*	-28.75	27.53	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0011	*Eragrostis curvula*	-28.75	27.53	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0013	*Eragrostis plana*	-28.76	27.55	2/4/2021	🟦	🟦	🟦	🟦
KCG-21-0014	*Eragrostis plana*	-28.76	27.55	2/4/2021		🟦	🟦	🟦
KCG-21-0015	*Eragrostis plana*	-28.76	27.55	2/4/2021		🟦	🟦	🟦
KCG-21-0024	*Eragrostis tef*	-28.73	27.59	6/4/2021	🟦	🟦	🟦	🟦
KCG-21-0025	*Eragrostis tef*	-28.73	27.59	6/4/2021	🟦	🟦	🟦	🟦
KCG-21-0026	*Eragrostis tef*	-28.73	27.59	6/4/2021	🟦	🟦	🟦	🟦
KCG-21-0030	*Eragrostis planiculmis*	-28.73	27.6	6/4/2021	🟦	🟦	🟦	🟦
KCG-21-0031	*Eragrostis planiculmis*	-28.73	27.6	6/4/2021	🟦	🟦	🟦	🟦
KCG-21-0032	*Eragrostis planiculmis*	-28.73	27.6	6/4/2021	🟦		🟦	🟦
LYG_21_0001	*Cymbopogon sp.*	-27.89	32.21	9/2021		🟦	🟦	🟦
LYG_21_0002	*Digitaria tricholoaenoides*	-32.70	27.48	9/2021	🟦		🟦	🟦
LYG_21_0003	*Digitaria tricholoaenoides*	-32.70	27.48	9/2021	🟦		🟦	🟦
LYG_21_0006	*Eragrostis capensis*	-33.28	26.80	9/2021	🟦	🟦	🟦	🟦
LYG_21_0011	*Hyparrhenia hirta*	-26.05	28.16	9/2021			🟦	🟦
LYG_22_0001	*Panicum maximum*	-25.03	31.04	2022	🟦	🟦	🟦	🟦
LYG_22_0002	*Panicum maximum*	-25.03	31.04	2022	🟦	🟦	🟦	
KCG_22_0001	*Eragrostis biflora*	-28.77	27.53	31/1/2022	🟦		🟦	
KCG_22_0002	*Eragrostis capensis*	-28.77	27.53	31/1/2022	🟦	🟦	🟦	

Table S4: GMYC species assignments on the full 28S data set. EC = Eastern Cape, FS = Free State, KZN = Kwa-Zulu Natal, LP = Limpopo, MP = Mpumalanga.

GMYC species	Sample ID	Morphospecies	Latitude	Longitude	Province
1	GFS_2019_00019	*Tetramesa sp. 4*	-33.282	26.486	EC
1	GFS_2020_00614	*Tetramesa sp. 4*	-33.969	25.592	EC
1	GFS_2019_00079	*Tetramesa sp. 4*	-33.282	26.486	EC
1	GFS_2021_00354	*Tetramesa sp. 4*	-33.280	26.799	EC
1	GFS_2020_00667	*Tetramesa sp. 4*	-33.963	24.954	EC
1	GFS_2021_00805	*Tetramesa sp. 8*	-28.033	32.283	KZN
1	GFS_2020_00055	*Tetramesa sp. 4*	-33.969	25.592	EC
1	KCP_2020_00092	*Tetramesa sp. 17*	-28.442	27.362	FS
1	GFS_2020_00492	*Tetramesa sp. 4*	-33.963	24.954	EC
1	KCP_2020_00061	*Tetramesa sp. 4*	-28.395	27.262	FS
1	KCP_2020_00019	*Tetramesa sp. 4*	-28.395	27.262	FS
1	KCP_2020_00052	*Tetramesa sp. 4*	-28.452	27.332	FS
1	GFS_2021_00846	*Tetramesa sp. 10*	-27.622	32.025	KZN
1	KCP_2020_00055	*Tetramesa sp. 4*	-28.395	27.262	FS
1	GFS_2021_00902	*Tetramesa sp. 10*	-27.622	32.025	KZN
2	GFS_2020_00055	*Tetramesa sp. 4*	-33.969	25.592	EC
2	KCP_2020_00092	*Tetramesa sp. 17*	-28.442	27.362	FS
3	GFS_2020_00492	*Tetramesa sp. 4*	-33.963	24.954	EC
3	KCP_2020_00061	*Tetramesa sp. 4*	-28.395	27.262	FS
3	KCP_2020_00019	*Tetramesa sp. 4*	-28.395	27.262	FS
3	KCP_2020_00052	*Tetramesa sp. 4*	-28.452	27.332	FS
3	GFS_2021_00846	*Tetramesa sp. 10*	-27.622	32.025	KZN
3	KCP_2020_00055	*Tetramesa sp. 4*	-28.395	27.262	FS
3	GFS_2021_00902	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2019_00035	*Tetramesa sp. 4*	-33.969	25.592	EC
4	GFS_2019_00041	*Tetramesa sp. 4*	-33.282	26.486	EC
4	GFS_2021_00329	*Tetramesa sp. 1*	-29.762	30.692	KZN
4	GFS_2020_00130	*Tetramesa sp. 4*	-33.969	25.592	EC
4	KCP_2020_00012	*Tetramesa sp. 4*	-28.452	27.333	FS
4	KCP_2020_00032	*Tetramesa sp. 4*	-28.395	27.262	FS
4	GFS_2020_00155	*Tetramesa sp. 4*	-33.969	25.592	EC
4	GFS_2021_00883	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2020_0003	*Tetramesa sp. 4*	-33.969	25.592	EC
4	GFS_2020_00184	*Tetramesa sp. 4*	-33.969	25.592	EC
4	GFS_2020_00680	*Tetramesa sp. 4*	-33.963	24.954	EC
4	GFS_2021_00745	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00918	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00773	*Tetramesa sp. 10*	-28.033	32.283	KZN
4	GFS_2021_00730	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2020_00091	*Tetramesa sp. 4*	-33.969	25.592	EC
4	GFS_2020_00233	*Tetramesa sp. 4*	-32.877	28.095	EC
4	KCP_2020_00048	*Tetramesa sp. 4*	-28.452	27.333	FS
4	GFS_2021_00887	*Tetramesa sp. 13*	-28.033	32.283	KZN
4	GFS_2021_00901	*Tetramesa sp. 12*	-29.762	30.692	KZN
5	GFS_2019_00069	*Tetramesa sp. 4*	-33.282	26.486	EC
5	GFS_2020_00102	*Tetramesa sp. 4*	-32.877	28.095	EC
5	KCP_2020_00045	*Tetramesa sp. 4*	-28.452	27.332	FS
6	GFS_2019_00039	*Tetramesa sp. 4*	-33.282	26.486	EC
6	GFS_PHI_1196F	*Tetramesa sp. 10*	-28.033	32.283	KZN
6	GFS_PHI_1416F	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	KCP_2020_00238	*Tetramesa sp. 4*	-28.756	27.558	FS
6	KCP_2020_00239	*Tetramesa sp. 24*	-28.756	27.558	FS
6	GFS_2020_00554	*Tetramesa sp. 4*	-33.963	24.954	EC
6	GFS_2021_00810	*Tetramesa sp. 10*	-27.622	32.025	KZN
6	KCP_2020_00177	*Tetramesa sp. 23*	-28.769	27.527	FS
6	GFS_2021_00762	*Tetramesa sp. 8*	-28.033	32.283	KZN
6	LMY_2022_00139	*Tetramesa sp. 1*	-32.704	27.483	EC
6	GFS_PHI_1196M	*Tetramesa sp. 11*	-28.033	32.283	KZN
6	KCP_2020_00044	*Tetramesa sp. 4*	-28.452	27.332	FS
6	KCP_2020_00036	*Tetramesa sp. 4*	-28.452	27.332	FS
6	GFS_PHI_1027_2	*Tetramesa sp. 1*	-27.891	32.210	KZN
6	GFS_2020_00015	*Tetramesa sp. 4*	-33.969	25.592	EC
6	GFS_2020_00086	*Tetramesa sp. 4*	-32.877	28.095	EC
6	GFS_PHI_1132M	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	KCP_2020_00062	*Tetramesa sp. 4*	-28.452	27.333	FS
6	KCP_2020_00201	*Tetramesa sp. 25*	-28.769	27.527	FS
6	KCP_2020_00217	*Tetramesa sp. 23*	-28.769	27.527	FS
6	KCP_2020_00207	*Tetramesa sp. 25*	-28.756	27.558	FS
6	LMY_2021_00220	*Tetramesa sp. 4*	-30.347	24.563	NC
6	GFS_2020_00087	*Tetramesa sp. 4*	-33.969	25.592	EC
6	GFS_PHI_1132	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	LMY_2021_00117	*Tetramesa sp. 4*	-25.608	31.100	MP
6	GFS_PHI_1447	*Tetramesa sp. 18*	-27.891	32.210	KZN
6	LMY_2021_00197	*Tetramesa sp. 4*	-30.347	24.563	NC
6	GFS_PHI_1027_1	*Tetramesa sp. 1*	-27.891	32.210	KZN
6	LMY_2021_00308	*Tetramesa sp. 4*	-25.608	31.100	MP
6	GFS_PHI_1238M	*Tetramesa sp. 11*	-28.033	32.283	KZN
6	KCP_2020_00199	*Tetramesa sp. 25*	-28.769	27.527	FS
6	GFS_PHI_1007	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	GFS_PHI_1080F	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	GFS_PHI_1205F	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	GFS_PHI_1232F	*Tetramesa sp. 26*	-27.891	32.210	KZN
6	GFS_PHI_1028F	*Tetramesa sp. 26*	-27.891	32.210	KZN
7	GFS_2020_00043	*Tetramesa sp. 1*	-32.872	28.084	EC
7	LMY_2022_00008	*Tetramesa sp. 1*	-32.704	27.483	EC
7	LMY_2022_00108	*Tetramesa sp. 1*	-32.704	27.483	EC
7	LMY_2022_00098	*Tetramesa sp. 1*	-32.704	27.483	EC
7	GFS_2020_00053	*Tetramesa sp. 1*	-32.872	28.084	EC
7	LMY_2022_00152	*Tetramesa sp. 1*	-32.704	27.483	EC
7	GFS_PHI_1101F	*Tetramesa sp. 1*	-27.891	32.210	KZN
7	LMY_2022_00090	*Tetramesa sp. 1*	-32.704	27.483	EC
7	GFS_2020_00116	*Tetramesa sp. 1*	-32.872	28.084	EC
7	SPY_KZN_060	*Tetramesa sp. 1*	-27.622	32.025	KZN
7	SPY_KZN_064	*Tetramesa sp. 1*	-29.829	30.959	KZN
7	SPY_LMP_032	*Tetramesa sp. 1*	-24.576	28.643	LP
7	GFS_2020_00152	*Tetramesa sp. 1*	-32.872	28.084	EC
8	KCP_2011_00169	*Tetramesa romana*	-28.432	28.013	FS
8	KCP_2011_00173	*Tetramesa romana*	-28.401	27.253	FS
8	KCP_2012_00170	*Tetramesa romana*	-28.511	27.302	FS
8	KCP_2012_00172	*Tetramesa romana*	-28.421	27.264	FS
9	GFS_PHI_1101M	*Tetramesa sp. 1*	-27.891	32.210	KZN
10	GFS_PHI_1447M	*Tetramesa sp. 18*	-27.891	32.210	KZN

Table S5: GMYC species assignments on the full COI data set. EC = Eastern Cape, FS = Free State, KZN = Kwa-Zulu Natal, LP = Limpopo, MP = Mpumalanga.

GMYC species	Sample ID	Morphospecies	Latitude	Longitude	Province
1	CVS0002	*Tetramesa sp. 1*	-27.891	32.210	KZN
1	SPY_KZN_060	*Tetramesa sp. 1*	-27.622	32.025	KZN
1	SPY_KZN_064	*Tetramesa sp. 1*	-29.829	30.959	KZN
1	SPY_KZN_68	*Tetramesa sp. 1*	-29.829	30.959	KZN
1	SPY_LMP_032	*Tetramesa sp. 1*	-24.576	28.643	LP
1	GFS_2020_00053	*Tetramesa sp. 1*	-32.872	28.084	EC
1	GFS_2020_00152	*Tetramesa sp. 1*	-32.872	28.084	EC
1	GFS_2020_00116	*Tetramesa sp. 1*	-32.872	28.084	EC
2	LMY_2022_00008	*Tetramesa sp. 1*	-32.704	27.483	EC
2	LMY_2022_00090	*Tetramesa sp. 1*	-32.704	27.483	EC
2	LMY_2022_00152	*Tetramesa sp. 1*	-32.704	27.483	EC
2	LMY_2022_00098	*Tetramesa sp. 1*	-32.704	27.483	EC
2	LMY_2022_00108	*Tetramesa sp. 1*	-32.704	27.483	EC
3	GFS_2019_00019	*Tetramesa sp. 4*	-33.282	26.486	EC
3	GFS_2020_00055	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2019_00079	*Tetramesa sp. 4*	-33.282	26.486	EC
3	GFS_2019_00069	*Tetramesa sp. 4*	-33.282	26.486	EC
3	GFS_2020_00015	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2020_00087	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2020_00614	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2020_00091	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2020_00155	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2019_00035	*Tetramesa sp. 4*	-33.969	25.592	EC
3	LMY_2021_00117	*Tetramesa sp. 4*	-25.608	31.100	MP
3	LMY_2021_00308	*Tetramesa sp. 4*	-25.608	31.100	MP
3	GFS_2019_00039	*Tetramesa sp. 4*	-33.282	26.486	EC
3	GFS_2020_00130	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2021_00354	*Tetramesa sp. 4*	-33.280	26.799	EC
3	GFS_2019_00041	*Tetramesa sp. 4*	-33.282	26.486	EC
3	GFS_2020_00102	*Tetramesa sp. 4*	-32.877	28.095	EC
3	GFS_2020_00184	*Tetramesa sp. 4*	-33.969	25.592	EC
3	GFS_2020_00233	*Tetramesa sp. 4*	-32.877	28.095	EC
3	GFS_2020_0003	*Tetramesa sp. 4*	-33.969	25.592	EC
3	LMY_2021_00220	*Tetramesa sp. 4*	-30.347	24.563	NC
3	KCP_2020_00177	*Tetramesa sp. 23*	-28.769	27.527	FS
3	KCP_2020_00178	*Tetramesa sp. 23*	-28.769	27.527	FS
4	GFS_2021_00730	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00773	*Tetramesa sp. 10*	-28.033	32.283	KZN
4	GFS_2021_00810	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00883	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00846	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_2021_00902	*Tetramesa sp. 10*	-27.622	32.025	KZN
4	GFS_PHI_1196F	*Tetramesa sp. 10*	-28.033	32.283	KZN
4	GFS_PHI_1238M	*Tetramesa sp. 11*	-28.033	32.283	KZN
4	GFS_PHI_1196M	*Tetramesa sp. 11*	-28.033	32.283	KZN
5	KCP_2020_00048	*Tetramesa sp. 4*	-28.452	27.333	FS
5	KCP_2020_00055	*Tetramesa sp. 4*	-28.395	27.262	FS
5	KCP_2020_00062	*Tetramesa sp. 4*	-28.452	27.333	FS
6	KCP_2020_00036	*Tetramesa sp. 4*	-28.452	27.332	FS
6	KCP_2020_00044	*Tetramesa sp. 4*	-28.452	27.332	FS
6	KCP_2020_00045	*Tetramesa sp. 4*	-28.452	27.332	FS
6	KCP_2020_00239	*Tetramesa sp. 24*	-28.756	27.558	FS
6	KCP_2020_00052	*Tetramesa sp. 4*	-28.452	27.332	FS
6	LMY_2021_00197	*Tetramesa sp. 4*	-30.347	24.563	NC
6	KCP_2020_00061	*Tetramesa sp. 4*	-28.395	27.262	FS
6	KCP_2020_00238	*Tetramesa sp. 4*	-28.756	27.558	FS
7	KCP_2020_00199	*Tetramesa sp. 25*	-28.769	27.527	FS
7	KCP_2020_00207	*Tetramesa sp. 25*	-28.756	27.558	FS
7	KCP_2020_00201	*Tetramesa sp. 25*	-28.769	27.527	FS
8	GFS_2020_00492	*Tetramesa sp. 4*	-33.963	24.954	EC
8	GFS_2020_00680	*Tetramesa sp. 4*	-33.963	24.954	EC
8	GFS_2020_00554	*Tetramesa sp. 4*	-33.963	24.954	EC
9	GFS_PHI_1007	*Tetramesa sp. 26*	-27.891	32.210	KZN
9	GFS_PHI_1205F	*Tetramesa sp. 26*	-27.891	32.210	KZN
9	GFS_PHI_1447	*Tetramesa sp. 18*	-27.891	32.210	KZN
9	GFS_PHI_1028F	*Tetramesa sp. 26*	-27.891	32.210	KZN
9	GFS_PHI_1080F	*Tetramesa sp. 26*	-27.891	32.210	KZN
9	GFS_PHI_1232F	*Tetramesa sp. 26*	-27.891	32.210	KZN
10	GFS_PHI_1132	*Tetramesa sp. 26*	-27.891	32.210	KZN
10	GFS_PHI_1416F	*Tetramesa sp. 26*	-27.891	32.210	KZN
10	GFS_PHI_1132M	*Tetramesa sp. 26*	-27.891	32.210	KZN
11	KCP_2020_00012	*Tetramesa sp. 4*	-28.452	27.333	FS
11	KCP_2020_00217	*Tetramesa sp. 23*	-28.769	27.527	FS
11	KCP_2020_00019	*Tetramesa sp. 4*	-28.395	27.262	FS
11	KCP_2020_00032	*Tetramesa sp. 4*	-28.395	27.262	FS
11	KCP_2020_00194	*Tetramesa sp. 24*	-28.769	27.527	FS
12	GFS_2020_00563	*Tetramesa sp. 7*	-33.963	24.954	EC
12	GFS_2020_00626	*Tetramesa sp. 7*	-33.963	24.954	EC
12	GFS_2020_00576	*Tetramesa sp. 7*	-33.963	24.954	EC
12	GFS_2020_00702	*Tetramesa sp. 7*	-33.963	24.954	EC
13	Z01A	*Tetramesa agaya1*	-16.921	30.813	ZIM
13	Z01B	*Tetramesa agaya1*	-16.921	30.813	ZIM
14	GFS_2020_00574	*Tetramesa sp. 7*	-33.963	24.954	EC
14	GFS_2020_00645	*Tetramesa sp. 7*	-33.963	24.954	EC
15	KCP_2011_00169	*Tetramesa romana*	-28.432	28.013	FS
15	KCP_2012_00172	*Tetramesa romana*	-28.421	27.264	FS
15	KCP_2012_00170	*Tetramesa romana*	-28.511	27.302	FS
15	KCP_2011_00173	*Tetramesa romana*	-28.401	27.253	FS
16	GFS_2021_00887	*Tetramesa sp. 13*	-28.033	32.283	KZN
17	KCP_2020_00092	*Tetramesa sp. 17*	-28.442	27.362	FS
18	KCP_2020_00219	*Tetramesa sp. 17*	-28.769	27.527	FS