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Pre-release evaluation of stem-galling and seed-feeding midges (Diptera: Cecidomyiidae) from South Africa as biological control agents for Guinea grass (*Megathyrsus maximus*) in Texas, United States

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Abstract

Guinea grass (*Megathyrsus maximus*) is a tall-statured C_4 grass with a native range spanning sub-Saharan Africa. Intentionally exported for centuries for its grazing value, it has become a near-global invasive. Texas, USA is the first territory to initiate a biological control program for Guinea grass, targeting an invasive population with genetic origins that have been traced to South Africa. This study focuses on the identification and evaluation of biological control agents for Guinea grass. Native-range natural enemy surveys were performed in South Africa, resulting in the identification of several undescribed species of phytophagous midges (Diptera: Cecidomyiidae) on Guinea grass, including stem-galling taxa (gall midges) and seed-feeding taxa (seed midges). These taxa are expected to be highly host-specific based on the known biology of this family of insects, but the difficulty of rearing them in the laboratory necessitated field-based assessments. Field host range assessments of stem gall midges resulted in the rejection of one candidate (the horn gall midge, undescribed sp.) due to feeding on a relative of the target plant, *Batochloa deusta*, and the prioritization of another candidate (the blister gall midge, *Arabukodiplosis vesicaria*) that feeds only on *Megathyrsus maximus.* Combined field host range evaluations and phylogenetic analysis of a cryptic species complex of seed midges resolved this as a new genus containing up to seven species, with five species that are likely to be specific to their respective Panicoideae grass hosts, including *Megathyrsus maximus* and *Cenchrus ciliaris* (buffelgrass – also invasive in the USA). Climate matching models for the blister gall midge and seed midge on Guinea grass predicted high likelihood of establishment and nearly complete coverage of invaded sites in Texas, and identified northern KwaZulu-Natal, South Africa as an ideal source region for Guinea grass biocontrol agents. The nascent sub-field of grass biocontrol research has developed slowly because of inaccurate perceptions that grasses lack host-specific herbivores, and faces a severe taxonomic impediment given the large number of undescribed insect species feeding on grasses, especially in Africa. This study has contributed to resolving both challenges by identifying several new host-specific insect species, showing that the prospects for future success and discovery in grass biological control are promising.

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List of abbreviations and units

In order of appearance in this manuscript:

IUCN = International Union for the Conservation of Nature (World Conservation Union)
US = United States of America
USD = US Dollars
ZAR = South African Dollars
UTex = University of Texas
USDA = US Department of Agriculture
GBIF = Global Biodiversity Information Facility
CBC = Centre for Biological Control
GLMM = Generalized Linear Mixed Model
sp. = species (singular)
mm = millimetres
msp. = morphospecies
spp. = species (plural)
EC = Eastern Cape province
KZN = KwaZulu-Natal province
GA = Gauteng province
SA = South Afirca
DNA = Deoxyribonucleic acid
Cox1 = cytochrome oxidase gene subunit 1
28S = 28S ribosomal RNA domain D2-D3
RNA = ribonucleic acid
PCR = polymerase chain reaction
µL = microliter
°C = degrees Celsius
min = minutes
Taq = *Thermophilum aquaticum*
MEGA = Molecular Evolutionary Genetic Analysis software

AIC = Akaike Information Criterion

p-dist = pairwise distance (nucleotide)

MCMC = Markov Chain Monte Carlo analysis

GTR = General Time Reversible model

+ G = with gamma parameter

+ I = with a proportion of invariable sites

HKY = Hasegawa-Kishino-Yano model

OTU = Operational Taxonomic Unit

PTP = Poisson Tree Process

BPTP = Bayesian Poisson Tree Process

PTPML = Maximum Likelihood Poisson Tree Process

ABGD = Automatic Barcode Gap Discovery

ASAP = Assemble Species by Automatic Partitioning

ENM = Ecological Niche Model

SDM = Species Distribution Model

GLM = Generalized Linear Model

BIO# = Wordclim climate variable number #

km = kilometre

m^2 = cubic metre

CoverT = Total cover

CoverGG = Guinea grass cover

Hmax = Maximum height

Have = Average height

Till = Tiller count

Dist = Distance

Prov = Province

r = Pearson’s correlation index

PCA = Principal Components Analysis

PC = Principal Component (axis)

AIC_C = Akaike Information Criterion Corrected for small sample sizes

Δ = difference in (e.g. ΔAIC_C)

β = slope (beta) coefficient

βnbinom = slope (beta) coefficient for negative binomial distribution

βbinom = slope (beta) coefficient for binomial distribution

AUCtest = Area Under (Receiver-Operator) Curve test value

AUCdiff = minumum difference in AUC

CBI = Continuous Boyce Index

OR10 = 10th percentile Omission Rate

BAL = Balanced model

SSS = Sum of model Sensitivity and Specificity

MESS = Multivariate Environmental Surfaces

Sum Wt = Sum of Akaike weights

N models = Number of included models

Chapter 1: General Introduction

1.1. Invasive alien species

1.1.1. What are invasive alien species?

Invasive alien species are one of the greatest threats to global biodiversity after habitat destruction (Bellard et al., 2016). Species are defined as *alien* or *introduced* wherever they have been transported by humans (intentionally or unintentionally) outside of their natural range, and *invasive* when they spread rapidly and cause substantial harm to natural ecosystems and/or to human health, societies or economies (Pyšek et al., 2020). The spread of invasive alien species is in many cases promoted by human-mediated disturbance and anthropogenic climate change, meaning that the number of invasions and the extent of their effects are likely to grow in the future (Pyšek et al., 2020).

1.1.2. Examples of invasive alien plant impacts

Invasive alien plants have the ability to drastically alter the structure and function of ecosystems, with negative consequences for biodiversity, ecosystem services and human societies (Walsh et al., 2023). The cumulative economic losses incurred by invasive species since records began has been estimated at over 600 million USD in the United States (for a small list of 15 selected invasive plants) as of 1991, and 14.45 billion USD in China (for a more comprehensive list of species, mostly terrestrial plants) as of 2000, according to estimates cited in a recent review (Office of Technology Assessment, 1993; Xu et al., 2006; Kumar Rai and Singh, 2020). Highly competitive plant invaders can replace native vegetation and form monospecific stands, or dense populations of a single species. The impacts depend on the invader species and the habitat invaded. For example, water hyacinth *Pontederia crassipes* Mart. (Pontederiaceae) invades low-flow aquatic systems in South Africa such as lakes, dams and slow-flowing rivers, forming mats which reduce sunlight penetration to submerged aquatic plants, resulting in food chain collapse (Van Wilgen et al., 2020, 2022). Water hyacinth invasions also incur economic losses by making water channels less accessible for fishing and recreational purposes, with over 42 million ZAR (about 3 million USD) spent on herbicides from 2010 to 2018 (Van Wilgen et al., 2020). An invasive tree, the broad-leaved paperbark *Melaleuca quinquenervia* Blake (Myrtaceae), rapidly establishes dense monospecific stands in the wetlands of the Florida Everglades; these stands alter nutrient cycling by depositing large amounts of leaf litter in the undercanopy, which also suppresses many native plants (Rayamajhi et al., 2010). Resulting reductions in biodiversity of up to 80 % are recorded, a tragedy in one of the world's most valued ecozones, but exploited by prospective land developers to attempt to classify large tracts of invaded land as ecologically "useless" (Turner et al., 2010). The tree's prolific production of above-ground biomass also increases the risk of destructive fires (Turner et al., 2010). On farmland and pastures in several dozen countries, famine weed *Parthenium hysterophorus* L. (Asteraceae) inundates the soil seed bank, allowing monospecific stands to form rapidly after any disturbance (Adkins and Shabbir, 2014). All tissues of famine weed contain parthenin, a compound that reinforces monospecies dominance with its allelopathic effects on native plants and crops, and a dangerous allergen to some people (Adkins and Shabbir, 2014; Nigatu et al., 2010). The economic impacts from the abandonment of invaded farmland, reduced pasture productivity and tainted crops, milk and meat are widespread and extremely costly, justifying the classification of famine weed as one of the world's worst invasive plants (Adkins and Shabbir, 2014).

1.2. Invasive grasses

1.2.1. Human importance of grasses

Grasses (Poaceae) are part of the foundation of human civilization. Cereals – grain products including rice, wheat, maize and sorghum – make up the bulk of the average human diet, bred from wild forms and cultivated for millennia by farming cultures in nearly every part of the world (Sheaffer and Moncada, 2012; Pickersgill, 2016; Pascoe, 2018; Liu et al., 2019). Grasses in the form of cereals, pasture or hay are the basis of most animal protein production in the United States, South Africa, Ethiopia and other countries (Institute for Feed Education and Research, 2020; South African Department of Agriculture, Land Reform and Rural Development, 2021; Gebremariam and Belay, 2021). The food value of this family of plants in the United States alone is on the order of tens of billions of dollars per year (Sheaffer and Moncada, 2012). Not all uses are dietary: grasses are used as building material for rural houses, as biofuel, as ground cover for erosion control and mine rehabilitation, as decorative plants and lawns, and as recreational turf (Nábrádi, 2007).

1.2.2. Impacts of invasive grasses

Grasses are diverse and successful in almost every ecosystem on Earth, and clearly of great importance for humans. Invasive grasses on the other hand, have a disproportionate impact among invasive alien plants, affecting both natural ecosystems and humans. Of the roughly 10,000 grass species in the world, at least 481 have naturalized outside their native range and at least 246 are invasive (Pertierra et al., 2023). On the World Conservation Union (IUCN) list of the 100 worst globally invasive species, which includes both plants and animals, three of the worst species are grasses (*Arundo donax* L., *Imperata cylindrica* Beauv. and *Spartina anglica* Hubb) (Global Invasive Species Database, 2024). Invasive grasses are a problem in every inhabited part of the world, including Africa (Sutton et al., 2021a), Australia (Read et al., 2020; Steinrucken and Vitelli, 2023), North and South America (Ellison and Evans, 1992; Fusco et al., 2019), continental Asia (Udumann et al., 2023) and the Malay Archipelago (Walpole, 2003), Europe (Rodríguez et al., 2021), New Zealand (Anderson et al., 2017) and Caribbean, Pacific and sub-Antarctic islands (Cole, 2022; Soti and Thomas, 2022; Canavan and Paterson, 2023). There is no single trait common to all invasive grasses that makes them invasive, other than the group’s diversity and adaptability, but intentional spread by humans for various uses plays a role in many cases (Pertierra et al., 2023).

Grasses used as pasture for livestock have become particularly successful human-facilitated invaders. Many introduced grasses are perennials of African origin, which are attractive to farmers because of their high productivity, high palatability to livestock, and tolerance of both heavy grazing and drought (Parsons, 1972; Rhodes et al., 2021, 2022a). Although these traits are the natural result of a long evolutionary association with large herbivores and competitive leguminous trees in climates where long dry periods are typical, human selection for the most productive cultivars and other valued traits has created forms with increased invasive potential (Jank et al., 2002; Rhodes et al., 2021; Rhodes et al., 2022a; Van Klinken and Friedel, 2017). Australia, for example, hosts 339 naturalised alien grasses. Of the 17 alien grasses which have the highest environmental impact in Australia, 16 were intentionally introduced by state agricultural agencies in the early 1900s – all but one of them as pasture grasses (Van Klinken and Friedel, 2017). In the Americas, many widespread alien grasses are thought to have arrived accidentally in the early colonial period, and subsequently to have been spread intentionally by cattle farmers who found them valuable; such has been the case with the widespread Guinea grass *Megathyrsus maximus* Simon & Jacobs (ex. *Panicum maximum* L.) (Parsons, 1972). The early 1900s saw the proliferation of state-funded pasture development research stations across the Americas (Parsons, 1972). The ubiquitous kikuyu grass *Cenchrus clandestinus* Morrone (ex. *Pennisetum clandestinum* Hochst.) is thought to have entered Brazil and other South American countries as an escapee from one or more of these research stations, but is no longer considered invasive there because of its value as a lawn and pasture grass (Parsons, 1972). Guinea grass, on the other hand, is now considered invasive in the United States, although new cultivars were introduced for pasture development in Texas as recently as the 1950s (Rhodes et al., 2022a). This species would be extensively bred in Australia, Brazil, India, South Africa and Zimbabwe in the 1970s and 1980s to produce highly productive cultivars for fertilized and irrigated growing systems (Sukhchain and Sidhu, 1992; Pieterse et al., 1997; Jank et al., 2002).

Tall perennial grasses such as Guinea grass are an effective class of invaders. They quickly outgrow and outshade competitor plants, changing the vegetation structure and reducing biodiversity in grasslands and forests (Flory and Clay, 2010; Canavan et al., 2019; Guido et al., 2019). In the process, they produce abundant, quick-drying biomass that increases the intensity and frequency of fires; since the resprouting of such grasses is promoted by fire and similar disturbances, this creates a positive feedback loop that facilitates their dominance (Rossiter et al., 2003; Canavan et al., 2019; Fusco et al., 2019). Gamba grass *Andropogon gayanus* Kunth invasions in Australia, for example, alter fire regimes by increasing fuel load and heat intensity as much as eightfold (Rossiter et al., 2003). In Australia's Litchfield National Park, these intense fires threaten the well-being of visitors, can damage valuable assets, and result in lost revenue from park closures and evacuations (Rossiter-Rachor et al., 2023). Gamba grass is spreading rapidly, and management costs are increasing in turn, making local eradication increasingly costly (Rossiter-Rachor et al., 2023). In South Africa, infestations of the giant reed *Arundo donax* in riparian areas were estimated by Le Maitre *et al.* (2000) to consume about 4.35 million litres of water per year countrywide. This estimate is severely outdated; the current water consumption by *A. donax* is likely to be far higher, considering that 79 % of waterways across South Africa, Lesotho and Swaziland are potentially invasible by this plant (Canavan et al., 2017). In the Rio Grande Basin of the United States and Mexico, *A. donax* was estimated to consume 56.2 million litres of water per year in 2009, valued at over 9.5 million USD (Seawright et al., 2009; Goolsby et al., 2023).

1.2.3. Management of invasive grasses

Given the immense costs associated with grass invasions, there is an obvious need for policy and intervention programs to manage them. Policy-making is, however, complicated by conflicts of interest: introduced grasses can be considered problematic or useful (or both) by different parties, particularly where conservation priorities conflict with agricultural priorities (Grice et al., 2012; Rhodes et al., 2021). The declaration of valuable pasture grasses as invasive species, for example, has faced opposition from some landowners (Wapshere, 1990). The control of invasive grasses raises other unique challenges compared to other invasive plants: infestations are often patchily distributed across vast areas, making finding and removing the plant with minimal collateral damage a difficult and expensive task, as with *Spartina* grasses in the western United States. By 2003, the smooth cordgrass *Spartina alterniflora* Loisel (and its hybrids with native *Spartina foliosa* Trin.) had invaded almost 9000 hectares total of ecologically sensitive mudflats in Willapa Bay, Washington, greatly exceeding early estimates of its rate of spread (Major et al., 2003; Washington State Department of Agriculture, 2017). Hybridization with the native S*partina foliosa* Trin., which *S. alterniflora* threatens to drive to extinction, is a further complicating factor (Ayres et al., 2004). An eradication program using combined mechanical and chemical methods has since achieved massive reductions, with the expenditure of several million USD in labour and equipment, and ongoing costs exceeding 700,000 USD per year (Washington State Department of Agriculture, 2017). *Spartina* and many other grasses are tolerant of general herbicides, requiring repeated applications to suppress the remaining patches (Chandramohan et al., 2002; Grevstad et al., 2003). Besides the financial costs of large-scale herbicide use, concerns about environmental impact have also led to legal challenges, making this an unsustainable approach to management in most cases (Grevstad et al., 2003). The relative success of *Spartina* control has only been possible due to the limited spatial extent of the invasion. Gamba grass in Australia's Northern Territory, on the other hand, covers almost 30,000 hectares of Litchfield National Park alone, and is spreading at a rate of over 1,000 hectares per year (Rossiter-Rachor et al., 2023). The cost of a local eradication program has been estimated at 6.6 million Australian dollars (about 4.1 million USD) over the next five years (Rossiter-Rachor et al., 2023). Most of this expenditure will go towards ground-based herbicide applications. The adaptation of Gamba grass and many other African grasses to regular fire and herbivory in their native range makes them highly tolerant of disturbance – a trait that aids their invasiveness – and also renders mechanical removal and controlled burning largely ineffective as control methods (Rossiter, 2003; Canavan et al., 2019; Sutton et al., 2019).

1.3. Biological control

1.3.1. What is biological control?

Biological control, or biocontrol, is the use of an invasive alien organism's natural enemies as control agents, such as herbivorous insects on invasive plants and microbial pathogens of insect pests (Van Driesche and Bellows, 1996). Biocontrol is a natural alternative to mechanical and chemical management tools, pairing persistent reduction in invasive species populations with low cost and low environmental harm, as it relies on self-reproducing and self-propagating live organisms (Hinz et al., 2020). Classical biological control refers to the importation of natural enemies of an invasive species from its native range into the invaded range (McFadyen, 1998). It is rooted in the enemy release hypothesis, which states that introduced species become invasive partly because they are removed from the pressure of their native specialist feeders – those that feed exclusively on one species or its close relatives (Keane and Crawley, 2002). Because of this close association, specialist feeders tend to be more damaging to their hosts over time than generalist feeders that are only an occasional source of damage (Keane and Crawley, 2002). There is evidence that some plants in their invasive range decrease their allocation of resources to defences against specialist feeders (but not generalists) while increasing their allocation to growth and reproduction (the key prediction of the Evolution of Improved Competitive Ability hypothesis) (Joshi and Vrieling, 2005; Sun et al., 2023), and experience increased density and decreased herbivory associated with a low species richness of associated invertebrates (Baso et al., 2024).

Because populations of herbivores and predators are dependent on the population density of their food source, biocontrol does not eradicate the target species (except, perhaps, very locally) but rather reduces populations to tolerable levels (Samways, 1981). The definition of “tolerable levels” of an invasive species depends on the thresholds of economic and ecological impact for each species (Moran et al., 2021). For example, an invasive plant, the prickly pear cactus *Opuntia ficus-indica* Miller (Cactaceae), has been evaluated as under complete control by its biocontrol agents (most notably the cochineal bug *Dactylopius opuntiae* Cockerell [Hemiptera: Dactylopidae]) in South Africa, on the basis that plant density, biomass, area and rate of spread have all been reduced below a threshold where any other interventions are required to reduce impacts on agriculture (Moran et al., 2021). On the other hand, complete control in all respects is not necessarily desirable for all species. Australian *Acacia* (Fabaceae) species are considered invasive in the wild in South Africa, but some species (such as *Acacia cyclops* A. Cunn.) remain valuable for timber and other uses. It is therefore not economically favourable to introduce biocontrol agents that significantly reduce the biomass of valued *Acacia* species (Post et al., 2010). Instead, biocontrol agents (such as the flower bud gall midge *Dasineura dielsi* Rübsaamen [Diptera: Cecidomyiidae]) have been introduced that reduce rate of spread by reducing seed set, which has been sufficient to keep the ecological impacts of some *Acacia* species below a tolerable threshold (Impson et al., 2021; Moran et al., 2021).

Classical biocontrol of invasive plants has been implemented in 90 countries, with substantial results reported in 50-60 % of programs globally (Schwarzländer et al., 2018; Winston et al., 2024). Since its recorded origins in the late 18th century and particularly since its rapid uptake in the latter half of the 20th century, the discipline has become increasingly refined in terms of scientific theory, research techniques and application, resulting in a steady increase in the rate of agent establishment and negligible impacts on nontarget plants (Hinz et al., 2020, 2024).

1.3.2. Agent selection in classical biocontrol programs

Classical biocontrol programs for invasive plants typically begin with surveys on the plant in its region of origin to generate lists of candidate biocontrol agents (Goolsby et al., 2006a). The focus of these initial surveys is on finding herbivorous insects, mites, phytopathogenic fungi or other pathogens associated with the target plant that are likely to be host-specific and that have the potential to be damaging, at an individual level (e.g. vegetative tissue loss) and/or or a population level (e.g. decreased reproductive output). About 80 % of all plant biocontrol agents are insects, mainly because host-specific insects are common (Samways, 1981; Schwarzländer et al., 2018). Evidence that an agent is host-specific enough to not cause damage to native species or agriculturally important plants in the region of introduction, and is therefore safe to use, is the first criterion determining whether or not it will accepted for release (Wapshere, 1989).

Host-specificity tests are structured experiments, normally done in indoor laboratories, quarantine facilities or experimentally manipulated field settings, that assess the host range and preference of candidate agents (Samways, 1981). It is generally accepted that the closer the phylogenetic relationship of a non-target plant species to the target plant species, the higher the risk of non-target attack will be, because of similar chemical, morphological and phenological cues which determine herbivore feeding preferences (Kelch and McClay, 2003). Current best practice in host-specificity testing of biocontrol agents is the centrifugal phylogenetic method, as described by Wapshere (1974). Rather than testing all possible non-target species of concern, the most closely related species are prioritized, followed by fewer tests on progressively more distant relatives (Lesieur et al., 2020).

Human manipulation of the sequence of sensory cues involved in host selection by insects (intentionally or unintentionally) in the laboratory may distort their behaviour and produce responses that would not be observed in the field (Heard, 1999; Blossey et al., 2018). For example, two species of *Galerucella* beetle were initially refused for release in California, United States as biocontrol agents for purple loosestrife *Lythrum salicaria* L. (Lythraceae) based on unacceptable damage to a popular ornamental plant, crepe myrtle *Lagerstroemia indica* L. (Lythraceae) in indoor tests. Field tests showed that this damage was only associated with *L. indica* grown in close proximity to *L. salicaria*, which rarely occurs in nature (Hinz et al., 2014). Assessments of host range through field tests or field surveys are therefore an important complement to laboratory tests (Goolsby et al., 2006a; Sutton et al., 2021b). Field surveys are exploratory, rather than experimental, but can also make use of the centrifugal phylogenetic method (Yell et al., 2024).

The potential effectiveness of an agent is an important factor to consider before release, once its host-specificity has been assessed; releasing agents that are host-specific but not effective is a waste of resources and increases the risk of unpredicted ecological impacts (McClay and Balciunas, 2005). The ability of an agent to damage its host – both at an individual level and a population level – is determined by traits such as fecundity, voltinism and mode of feeding, as well as the suitability of the climate (Hill and Olckers, 2001; Wapshere, 1989). Climate matching between the native range and the proposed introduced range, using ecological niche models, can identify where an agent can establish and be most effective (Kass et al., 2021; Cowie et al., 2023). An agent that is sufficiently host-specific, likely to be damaging and, ideally, well-suited to the climate in its new native range is a good candidate for export and release (Van Driesche and Bellows, 1996).

1.3.3. Biological control of invasive grasses

Grasses historically have not been considered suitable targets for biocontrol, and few biocontrol programs have been initiated against them. This is mainly due to a perception that grasses, lacking many of the secondary compounds thought to drive herbivorous insect coevolution and speciation, do not generally have host-specific agents, making the risk of damage to valuable pasture grasses and graminaceous crops too high; it is also partly due to concerns that grass agents are too difficult to rear or not damaging enough (Wapshere, 1990; Sutton et al., 2019, 2021a; Olckers et al., 2021). There is strong evidence to the contrary from biocontrol programs in the United States. The biocontrol program against the giant reed *Arundo donax* L., initiated in 1998, was among the first to use an arthropod agent against an invasive grass. The stem-galling wasp *Tetramesa romana* Walker (Hymenoptera: Eurytomidae), the armoured scale *Rhizaspidiotus donacis* Leonardi (Hemiptera: Diaspidae), and the leaf mining gall midge *Lasioptera donacis* Coutlin (Diptera: Cecidomyiidae) all completed their lifecycles exclusively on hosts in the genus *Arundo* in host-specificity trials and were released in the Rio Grande Valley of Texas and Mexico from 2009 to 2017 (Goolsby et al., 2023). *Tetramesa romana* and *R. donacis* both established with considerable success (Marshall et al., 2018a; Goolsby and Moran, 2019). By 2019, the estimated live biomass of *A. donax* along an invaded length of the Rio Grande river had been halved compared to pre-biocontrol levels in 2009; the amount of water saved has been valued at 4.4 million USD per year, or 22 million USD by 2024 (Goolsby et al., 2023). For comparison, the total cost of research and the rearing and release of agents for the program from 1998 to 2009 is estimated at 14.2 million USD in today's currency – a cost : benefit ratio of 1 : 1.5, not accounting for additional benefits other than water savings (Patrick Moran [USDA], personal communication).

The planthopper *Prokelisia marginata* Van Duzee (Hemiptera: Delphacidae), which feeds and reproduces on a narrow range of *Spartina* species, was released in Willapa Bay, Washington from 2000 to 2001 to control *S. alterniflora* (Grevstad et al., 2003). The planthopper proved able to reduce *S. alterniflora* seed production by up to 90 % and caused leaf necrosis when high in numbers, although reduction in the area of infestation by the planthopper alone was not achieved, possibly due to interference from herbicide applications early in the program that affected agent populations (USDA, 2007). Without this interference, millions of dollars in herbicides might have been saved (USDA, 2007).

The successes achieved in grass biocontrol in the United States in the 2000s contrast with perceptions from the decade prior, when concerns that grass-feeding agents would damage cultivated sugarcane in Hawaii prevented biocontrol programs being launched against known fire hazards and biodiversity threats, such as fountain grass *Cenchrus setaceus* Morrone (ex. *Pennisetum setaceum* Forssk.) (Gardner et al., 1995). Perceptions have changed, and biocontrol is now being seriously considered as a management tool for invasive grasses in Hawaii (Johnson, 2023).

Prospects for grass biocontrol continue to improve. Two species of stem-mining moth in the genus *Archanara* (Lepidoptera: Noctuidae) were recently released as biocontrol agents of invasive biotypes of the reed *Phragmites australis* Trin. in Canada and are being evaluated for release in the United States (Blossey et al., 2018; deJonge et al., 2022; McTavish et al., 2024). In another example, two species of rust fungi have been evaluated as promising biocontrol agents for needlegrass *Nasella neesiana* Barkworth, a species invasive in New Zealand (Anderson and Barton, 2022). One of these, *Uromyces pencanus* Arth. & Holw. was approved for release, and Argentina, the country of origin of the rust, recently granted permission to export (Anderson et al., 2017; Den Breeyen, 2021). Several other potentially suitable biocontrol agents for invasive grasses have been discovered in native range surveys, particularly within the last 20 years, and it is likely that there are many more agents to be found (Sutton et al., 2019; Lotzfalideh et al., 2020).

In South Africa, several candidate biocontrol agents for locally invasive grasses are under evaluation (Sutton et al., 2021a). The highest priority grass targets for biocontrol in South Africa either have effective biocontrol programs in the Americas (*Arundo donax*) or are native to South America (*Cortaderia* and *Nasella* species) (Sutton et al., 2021a). Meanwhile, South Africa has “donated” many invasive plant species to other countries, especially grasses (Olckers et al., 2021). International collaboration on biocontrol research programs sets a firm basis for the equitable sharing of knowledge and biocontrol agents, benefiting all parties (Mason et al., 2023). Grass biocontrol research in South Africa has accelerated in the last five years thanks to such collaborations, and is either based on successful overseas programs (Canavan et al., 2017) or focuses on native grasses for the benefit of partner countries. The latter include programs for African lovegrass *Eragrostis curvula* Nees, the rat’s tail grasses *Sporobolus pyramidalis* Beauv. and S*porobolus natelensis* Durand & Schinz, Gamba grass *Andropogon gayanus*, and the focal plant of this study, Guinea grass *Megathyrsus maximus* Simon & Jacobs (Sutton et al. 2019, 2021a; Olckers et al., 2021).

1.4. Guinea grass

1.4.1. Phylogeny and taxonomy

Megathyrsus maximus (formerly *Urochloa maximus* Webster, *Panicum maximum* Jacq.), commonly known as Guinea grass, is a member of the Paniceae subtribe within the subfamily Panicoideae of the Poaceae. The subfamily includes several economically important grasses, including maize (*Zea mays* L.) and sugarcane (*Saccharum offinicinarum* L.) (Burke et al., 2016). Initially placed in the genus *Panicum* along with many other grasses with a similar open paniculate inflorescence, Guinea grass has since undergone several taxonomic revisions based on phylogenetic techniques (Simon and Jacobs, 2003). Presently, together with its three described congeners, *M. infestus* Simon & Jacobs, *M. trichocladus* Salariato & Zuloaga, and *M. vollesini* Salariato & Zuloaga, it is sister to the *Urochloa*-*Brachiaria* complex (Torres González and Morton, 2005; Soreng et al., 2017; Kew Royal Botanic Gardens, 2024) with which it shares some ecologically relevant characteristics, such as a tendency for vertical tillers to bend and set root on contact with the soil (Matson, 2018). The generic name *Megathyrsus* means “large staff” or “large stem”.

1.4.2. Biology

Guinea grass is a perennial C_4 bunch grass, and is highly variable in its growth form. Although bunch-forming, it may have a more spreading habit depending on growth conditions (Hosaka, 1956; Rhodes et al., 2021). The plant can reach up to 3.5 metres in height, depending on genotype and water availability (Aganga and Tshwenyane, 2004). Two biotypes exist in the wild, one short and narrow-leafed (“short form”) and one tall and broad-leafed (“tall form”); these represent conspecific, but genetically distinct subspecies that may be somewhat reproductively isolated (Fig. 1.1) (Rhodes et al., 2021; Gaskin et al., 2022). In the United States, short-form Guinea grass is predominant in the seasonally dry rangelands of south Texas, while tall-form Guinea grass is more moisture-dependent and occurs in Hawaii, southern Florida, and in Rio Grande Valley of Texas (Rhodes et al., 2021). Phylogenetic studies have indicated that the probable source population for Texan short-form Guinea grass is south-eastern Africa, the best genetic match being populations in the KwaZulu-Natal province of South Africa (Bon et al., 2011; Gaskin et al., 2022).

Short-form Guinea grass in South Africa bear dense, translucent silicate hairs on the lower stems, leaf sheaths, ligules and nodes, while tall-form Guinea grass in South Africa and both biotypes in Texas and Kenya usually lack these hairs except on the ligules (Fig. 1.2) (Aaron Rhodes [UTex], personal communication). The root system is deep and dense, affording drought and fire tolerance (Aganga and Tshwenyane, 2004; Soti and Thomas, 2022). In its native distribution, the grass thrives under the canopies of leguminous trees, which may be attributed to its shade tolerance and relatively high nitrogen requirements (Pieterse et al., 1997; Rhodes et al., 2021). Primary productivity can be extremely high; in southern Florida, vertical growth of over 15 centimeters (6 inches) a day is possible for tall-form Guinea grass (Matson, 2018). Crude protein levels are relatively high, depending on the cultivar, irrigation and fertilization, and the grass is generally considered one of the best for beef production (Benabderrahim and Elfalleh, 2021).

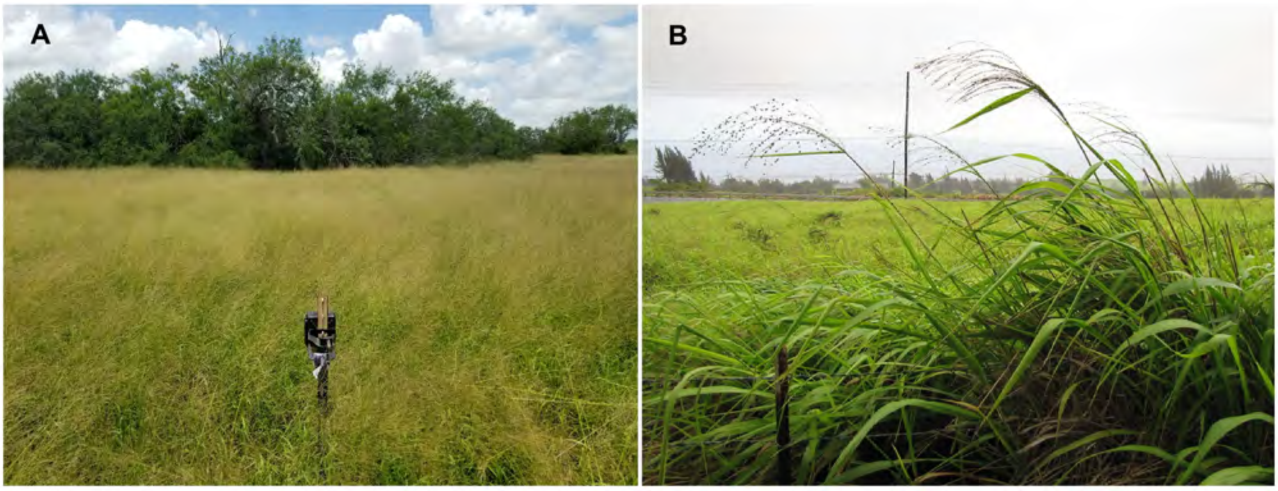

Figure 1.1. Infestations of Guinea grass *Megathyrsus maximus* biotypes: short-form (A) in a field in Kenedy county, Texas, and tall-form (B) in flower on a roadside in Hawaii (Credit: Aaron Rhodes [A], Forest Starr & Kim Starr [B])

Guinea grass can reproduce clonally from stolons and rhizomes, but its primary means of spread is from animal and human dispersal of seeds (Rhodes et al., 2021). The panicles are distinctive and sometimes very large (10-60 cm long), growing in proportion to the size of the plant. The whorl of racemes (at least three, usually five or more) at the base of the panicle is a key identifying feature of short form Guinea grass in the field in South Africa and is also found in Texan populations (Van Oudtshoorn, 2012; Colin Morrison [UTex], personal communication). After number of panicles per plant, number of racemes per panicle and panicle length are the biggest predictors of plant seed set (Sukhchain and Sidhu, 1992). Seed production is high – each plant may produce several thousand seeds a year – but seed survival and germination rates are relatively low (Rhodes et al., 2021; Sukhchain and Sidhu, 1992). Some cultivars have been selected for higher seedling viability, among other traits favourable to growers (Sukhchain and Sidhu, 1992). The reproductive modes of Guinea grass varieties range from sexual to facultatively apomictic to obligately apomictic. Sexual and apomictic diploid and tetraploid populations exist in the wild, with varying chromosome counts, and some cultivars have been bred to higher ploidy levels (Rhodes et al. 2021).

Guinea grass produces allelochemicals – including 2-hydroxyphenylacetic acid or 2HPPA, a growth inhibitor for a wide range of crops and wild plants – in its roots, culms and leaves. In greenhouse experiments, Guinea grass phenolic extracts inhibit the growth and seedling recruitment of some shrubs and grasses (Morrison et al., 2023a). Allelochemicals in soil and leaf litter combined with shading from tall, dense stands of Guinea grass likely contribute to its dominance in invaded habitats and negative effects on native plants and crops (Morrison et al., 2023a).

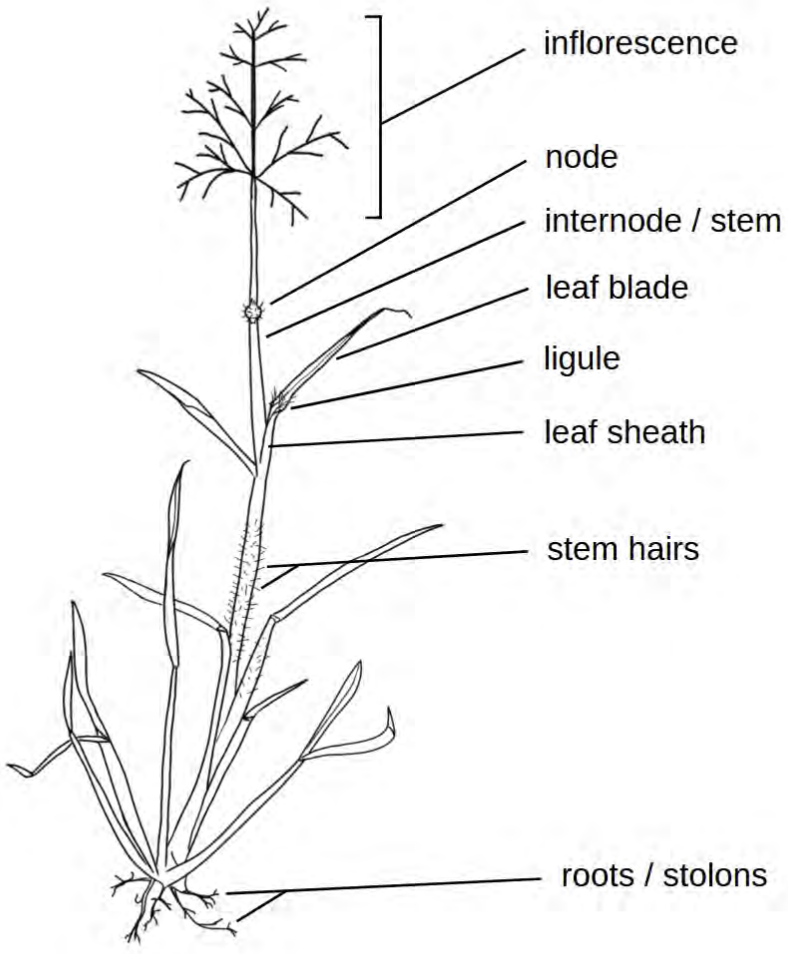

Figure 1.2. Structural diagram of a Guinea grass tiller (short-form, South African wild type).

1.4.3. Distribution and history

Guinea grass is an Afrotropical grass widely cultivated as fodder for livestock (Rhodes et al., 2021). From its native range spanning virtually all of sub-Saharan Africa, the grass and its domesticated cultivars have been introduced or have spread to a vast number of countries and territories across the Americas, southeast Asia, Australia and Oceania. Only the polar and boreal regions, most desert and cold temperate regions, and most of non-tropical Asia have no recorded occurrences (GBIF, 2024; Fig. 1.3). A combination of repeated intentional introductions over several centuries and adventive spread by seed has led to its current distribution (Rhodes et al., 2021).

Guinea grass was already widespread in parts of the Caribbean in the early 1700s (Cole, 2022) and was introduced to India in 1793 (Sukhchain and Sidhu, 1992) and to Sri Lanka soon after (Udumann et al., 2023). Introduction to other former European colonies in the Americas began in the early 1800s (Parsons, 1972). Although already present in Mississippi in 1813, its introduction to south Texas began more recently, in the 1950s (Parsons, 1972; Rhodes et al., 2022a). Rapid adventive spread since 1970 brought the grass to central Texas by around 2020, and the front of invasion now threatens to advance into northern Texas (Rhodes et al., 2022b; Bannin, 2023).

Although wild-type Guinea grass is officially considered invasive across most of its introduced range, the cultivation of Guinea grass is not necessarily prohibited, owing to the economic value of certain cultivars in some countries (Queensland Government, 2020; Rhodes et al., 2021; Goolsby et al. 2022). Cultivars ‘Gatton’ and ‘Petrie’ are grown in Australia, ‘Vencidor’ in South America, ‘Mutale’ in parts of South Africa, and other cultivars in their respective countries (Pieterse et al., 1997; Aganga and Tshwenyane, 2004). Current United States legislation does not distinguish between invasive and agriculturally useful varieties (John Goolsby [USDA], personal communication).

Guinea grass is now considered invasive throughout the Caribbean, although its importation and culturing as feed for cattle once supported local economies (Prieto et al., 2012; Cole, 2022). Among West Africans and the West African diaspora in the Caribbean, traditional uses of Guinea grass as thatching and for ceremonial and agricultural purposes continued through the colonial era; the application of mulched Guinea grass to agricultural fields as a fertilizer and drought mitigation strategy is still practised in Ghana, Jamaica and Antigua (Daisley et al., 1988; FAO, 2008; Cole, 2022; Kaigbanja et al., 2020).

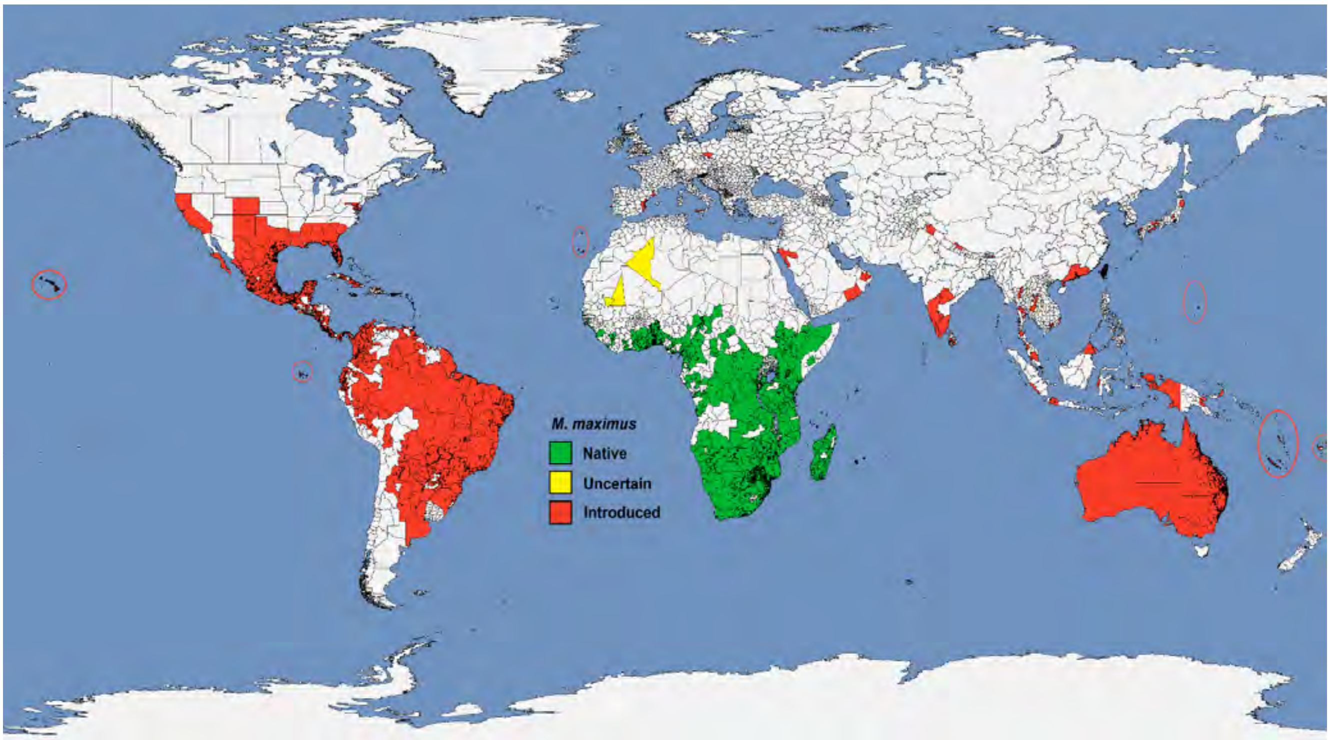

Figure 1.3. Distribution of Guinea grass (*M. maximus*) across administrative units (states / provinces) of world countries based on Global Biodiversity Information Facility records (gbif.org). Green areas indicate the native range, red areas the introduced range, yellow areas uncertain status, and light grey areas no records. Black points in shaded areas indicate recorded occurrences. Some major island chains with recorded introductions are circled.

1.4.4. Impact

In the United States, where considerable research has been done on the effects of Guinea grass, it is rated as a serious weed of several crops, including citrus, cotton and sugarcane (Rhodes et al., 2021). In citrus orchards in Florida, the grass may smother young trees and affects fruit production by competing with the crop for nutrients; mechanical or chemical removal carries the risk of damaging the crop (Chandramohan et al., 2022). Guinea grass is a particularly serious weed of sugarcane in the United States, partly because it is an alternate host for the sugarcane aphid, *Melanaphis sacchari* Zehntner (Hemiptera: Aphidae), and the sugarcane leaf spot fungus, *Bipolaris yamadae* Shoemaker (Ascomycota: Pleosporales: Pleosporaceae) (Rhodes et al., 2021). Guinea grass is also a weed of surgarcane plantations in Argentina, Brazil and Cuba (Soti and Thomas, 2022), and of coconut plantations in Sri Lanka (Udumann et al. 2023). In Sri Lanka, the Philippines and Hawaii, Guinea grass is a notable invader of native forests and a pest of forestry plantations (Walpole, 2003; Weerawardane and Dissanayake, 2003; Rhodes et al., 2021). The August 2023 fires in Maui, Hawaii, are regarded as the deadliest wildfires in the United States in over a century, and caused billions of US dollars in property damage (Reuters, 2023). Fire experts consider the fires to have been worsened by climate change and invasive grasses (The Guardian, 2023). Stands of Guinea grass and buffelgrass can facilitate forest fires by increasing fuel loads on the forest floor and helping flames reach vertically into the canopy (Sands, 2018; Johnson, 2023). Guinea grass has also contributed to the decline of native plant species in Hawaii (Rhodes et al., 2021). In south Texas, Guinea grass and other invasive grasses threaten at least one native vertebrate species: the northern bobwhite quail (*Colinus virginianus* L.). Although bobwhites sometimes prefer the cover offered by dense stands of Guinea grass, the displacement of native grass species that support their insect prey is likely contributing to a decline in the birds' population (Kuvlesky et al., 2017).

1.4.5. Control

A combination of managed burning and grazing has proven effective as a cultural method of controlling Guinea grass in Texan pastures (Soti and Thomas, 2022), but this method carries its own costs and risks, and cannot be applied in the wild. General herbicides can be used to control Guinea grass on a small scale, most effectively when combined with other control methods, but the long-term financial and environmental cost of large-scale intervention of this nature is prohibitive (Matson, 2018; Soti and Thomas, 2022). Another complicating factor is the tolerance of Guinea grass to several herbicides and the risk of selecting for herbicide-resistant varieties (Chandramohan et al., 2002). An effective biocontrol agent for Guinea grass could augment current management tools and provide ongoing, large-scale and safe control in the wild.

The classical biological control program for Guinea grass began with a survey by Mercadier et al. (2009) in Cameroon, which identified a number of herbivorous insects as candidate agents, although none went on to be evaluated. Identification of the correct species and biotype of target plant is important to make sure that candidate agents found in the native range are the best match (Van Driesche and Bellows, 1996; Goolsby et al., 2006b). Failure to match the correct biotypes of weed and agent can result in time and resources being wasted on agents that cannot feed or reproduce effectively on the target plant. For example, two species of stem-boring *Buakea* moth larvae (Lepidoptera: Noctuidae) are exclusively associated with Guinea grass in Kenya (Moyal et al. 2011) but attempts to cultivate the moths on Texan Guinea grass in the laboratory failed, possibly due to a biotype mismatch (Vacek et al., 2021; Gaskin et al., 2022).

It has since been established that South African short-form Guinea grass is well-matched genetically to Texan populations, and is more likely to yield suitable biocontrol agents. This contributed to a research partnership being established between the University of Texas (Austin) in the United States and the Centre for Biological Control (CBC) at Rhodes University (Makhanda) in South Africa in 2019. Biotype matching is especially important for the Guinea grass biocontrol program: while short-form Guinea grass is widely considered a noxious weed, tall-form Guinea grass is economically valuable in several countries (Gaskin et al., 2022). The possibility that suitable biocontrol agents may be required to be damaging to the short form but not the tall form must be considered during evaluation. The herbivorous mites *Abacarus* sp. (Acari: Eriophyidae) from Kenya and *Diptacus* sp. (Acari: Diplomatidae) from South Africa were shelved as biocontrol agents because they developed on both biotypes of Guinea grass in laboratory trials (Gaskin et al., 2022). Surveys have, however, found additional agents of interest that have not yet been evaluated for host-specificity.

1.5. Cecidomyiidae as grass biocontrol agents

1.5.1. Role in this study

Several species of stem-galling and seed-feeding cecidomyiid midges (Diptera: Cecidomyiidae) have been found on Guinea grass in surveys in Kenya and, more recently, in South Africa (Chapter 2). Because the host-specificity of cecidomyiids can generally expected to be high (Carneiro et al., 2009), this group of insects was prioritized for evaluation as biocontrol agents, and is the primary focus of this study. Detailed information on the insects associated with Guinea grass is provided in Chapter 2.

1.5.2. Importance of the Cecidomyiidae

The Cecidomyiidae are a cosmopolitan group of small (2-10 mm long), delicate flies with varied biology. Most are phytophagous, although many are mycophagous, and some are predators of aphids, scale insects and mites (Gagné and Jaschhof, 2021). The larvae of most members of the most diverse subfamily, Cecidomyiinae, are plant endophages; many species initiate and inhabit abnormal growths called galls or *cecidia* on the stems, leaves or floral structures of a very wide range of plant taxa – hence their common name, gall midges (Dorchin et al., 2017). The gall serves as a source of food and protection from predators until the young midge completes pupation. Adults are weak fliers, do not feed, and live for only a few days during which they disperse and reproduce (Hall et al., 2012).

There are over 6,600 described cecidomyiid species across over 800 genera (Gagné and Jaschof, 2021). The number of extant species on Earth is estimated to be far in excess of this number; new species and even genera are continually being found (Dorchin et al., 2019a; Dorchin et al., 2022). The group is of great economic interest for two reasons, the first being that certain species are among the most destructive pests of globally important crops, especially grains: the hessian fly *Mayetiola destructor* Say and the wheat blossom midge *Sitodiplosis mosellana* Géhin on wheat (Sadeghi et al., 2021; Cheng et al., 2020), the sorghum midge *Stenodiplosis sorghicola* Coquillet (ex. *Contarinia sorghicola*) on grain sorghum, and the African rice gall midge *Orseolia oryzivora* Harris & Gagné and the Asian rice gall midge *Orseolia oryzae* Kolesik & Gagné on rice in West Africa and tropical Asia, respectively. *Chilophaga virgati* Gagné is a pest of switchgrass *Panicum virgatum* L., a fodder grass and biofuel crop (Calles Torrez et al., 2014). The lentil midge (*Contarinia Lentis* Aczél), jujube midge (*Dasineura jujubifolia* Jiao & Bu), swede midge (*Contarinia nasturtii* Kieffer), pear midge (*Contarinia pyrivora* Riley) and clover seed midge (*Dasineura leguminicola* Lintner) also damage their respective crop plants (Wigglesworth, 1946; Kolesik, 2000; Jiao et al., 2017).

The second reason for economic interest in this group is their potential as biocontrol agents for invasive plants. Most are either monophagous or feed on a few related species within a genus (Dorchin et al., 2019a), a critical trait for biocontrol agents. At least 10 species of cecidomyiids have been released as classical biological control agents and established successfully (Winston et al., 2024). The shoot-tip galler Z*euxodiplosis giardi* Kieffer was the first cecidomyiid to be exported to control an invasive species, St John's Wort *Hypericum perforatum* L. (Hypericaceae), in Australia, with later releases in Canada, the United States, and South Africa (Winston et al., 2024; Gordon and Kluge, 1991). The stem galler *Lophodiplosis trifida* Gagné, released to control broad-leaved paperbark in Florida, United States in 2008, achieved dramatic reductions in growth of the target plant; together with other biocontrol agents, this has led to gradual ecosystem recovery in parts of the Everglades (Pratt et al., 2013; Rayamajhi et al., 2013). The damage caused by the leaf miner *Lasioptera donacis* to the giant reed *Arundo donax* in its native range in Europe suggests similar biocontrol outcomes are possible along infested parts of the Rio Grande in Texas, United States; the agent's establishment there is currently being attempted (Marshall et al., 2018b; Goolsby et al., 2023). In South Africa, the flower bud gallers *Dasineura dielsi* and *Dasineura rubiformis* Kolesik have established widely on the invasive Australian wattles *Acacia cyclops* A. Cunn. and *Acacia mearnsii* De Wild, respectively, and introduction of a third *Dasineura* species to control other invasive *Acacia* species has been attempted (Impson et al., 2021). Biocontrol of honey mesquite *Prosopis glandulosa* Torr. (Fabaceae) in South Africa using another flower bud galler in the *Asphondylia prosopidis* species complex is under consideration (Park and Thompson, 2019; Kleinjan et al., 2021).

Cecidomyiids are understudied, despite their economic importance, owing to a combination of factors – their small size, the crypticity of the larvae and the ephemerality of the adults, and in particular the very small number of specialists in their taxonomy and biology (Dorchin et al., 2015). Most of the estimated diversity of the group has never been sampled (Gagné and Jaschof, 2021). The taxonomy is mainly morphological, but most subgroups and their defining characters are undersampled; large phylogenetic studies have only been undertaken recently (Dorchin et al., 2019a; Gagné and Jaschof, 2021). A lack of information on the subfamily Cecidomyiinae in particular is a scientific stumbling block that limits research into their potential as biocontrol agents.

1.6. Study aims and components

The aim of this study was to identify suitable biocontrol agents for Guinea grass, and particularly to evaluate the suitability of cecidomyiid species as biocontrol agents for Guinea grass in Texas. The study has the following components:

(1) Surveys were done in South Africa to identify natural enemies of Guinea grass. Candidate biocontrol agents in the family Cecidomyiidae are selected as a focus group. Survey findings, laboratory rearing trials and studies of seasonal abundance and parasitism were used to build an initial knowledge base on the biology of the candidate agents **(Chapter 2).**

(2) Host-specificity of the stem gall-inducing candidate agents was studied in native-range field host range surveys **(Chapter 3)**. Native grass species were prioritized according to their phylogenetic relationship to Guinea grass, and were sampled at sites with sympatric short-form Guinea grass as a natural control group.

(3) Host-specificity of the seed-feeding candidate agents was studied in further native-range field host range surveys **(Chapter 4)**. Because the species of interest occurring on Guinea grass and other grasses are morphologically similar and have yet to be formally described, specimens collected in surveys were characterized genetically using multiple marker loci. Field host range and species delimitation using phylogenetic distances were used as evidence for the existence of distinct, host-specific species on related grasses.

(4) Statistical models and predictive machine-learning-based models were built to characterize the habitat / climate niche of the two most promising candidate agents and predict climate suitability for these agents in Texas, using data from native-range field surveys **(Chapter 5)**.

(5) The findings of this study and related studies were synthesized in a section that discusses their implications and the prospects for the biological control of Guinea grass and invasive grasses in general **(Chapter 6)**.

Chapter 2: Natural enemy surveys

2.1. Introduction

2.1.1. Natural enemy surveys

A classical biocontrol program for an invasive plant must begin with establishing an inventory of natural enemies of the target plant in its native range, from which the most promising will be selected for evaluation as candidate biocontrol agents (Goolsby et al., 2006a). This may initially be in the form of literature searches and examination of museum specimens, although field surveys in the native range of the plant are almost always necessary (Goolsby et al., 2006a; Egli and Olckers, 2020; Reid et al., 2020). Collecting the full diversity of natural enemies requires surveys at multiple sites across a wide area, at multiple times of year, using a variety of sampling methods (Goolsby et al., 2006a). Not all natural enemies found in these surveys will be considered as candidate biocontrol agents, but only those prioritized based on key criteria: that they are suitably abundant and consistently associated with the target plant, cause some identifiable form of damage or have the potential to be damaging, and are likely to be host-specific (Egli and Olckers, 2020; Sutton et al., 2021b).

Host-specificity is the primary criterion for selecting a candidate agent, determining whether or not an agent is ecologically and economically safe to release (Schaffner, 2001). Priority is therefore given to endophages, such as stem borers, seed borers, leaf miners and gall-inducers, which are often more host-specific than ectophages (Gaston et al., 1992), and representatives of taxa with a high number of host-specific species – often those already used successfully in other biocontrol programs (Harris, 1991). The range of taxa considered includes herbivorous insects, mites and phytopathogenic fungi, although insects are the most commonly used host-specific group (Samways, 1981; Schwarzländer et al., 2018). In many cases, the species found in native range surveys are new to science, and their exact taxonomy is unknown, requiring identification using molecular genetic methods (Goolsby et al., 2006a; Van Steenderen et al., 2023). In addition to host-specificity, other aspects of the life cycle of natural enemies, such as their effects on the target plant, phenology, climate tolerances, and relationships with predators, parasitoids or pathogens are important criteria for prioritizing specific taxa as candidate agents (Egli and Olckers, 2015; Downey et al., 2021; Morrison et al., 2023b).

Damage caused to the target plant is the secondary criterion for selecting candidate agents, as it determines whether the type of damage inflicted matches desired biocontrol outcomes, and whether or not an agent is actually capable of effective biocontrol (Harris, 1991; McClay and

Balciunas, 2005). Agents that are not sufficiently damaging carry more costs and risks than benefits, and should not be used (McClay and Balciunas, 2005). Damage can be measured in the field or in the laboratory, using overt signs of feeding (e.g. feeding scars on leaves, boring of stems or seeds, chlorosis, or formation of galls) or indirect effects on plant growth at an individual level (e.g. stunted height, reduced flowering and seed set) or a population level (eg. reduced density of monospecific stands, or reduced soil seed banks and seedling recruitment) (Briese, 2000; Goolsby et al., 2006a; Sutton et al., 2021b).

In cases where damage is difficult to measure, seasonal abundance may be used as a proxy for herbivore pressure (Egli and Olckers, 2015). The phenology of insects that feed on specific plant tissues can be strongly seasonal, matching not only weather patterns but also the phenology of the host plant (Price and Hunter, 2015). This is no less true for insect herbivores on grasses, particularly widespread perennials of African origin, such as buffelgrass, which are typically adapted to highly seasonal habitats (Morrison et al., 2023). In the case of Guinea grass, where impacts in the invaded range result from a high rate of spread and monospecific stands covering large areas, agents that reduce reproductive output as well as competitiveness during the plant's peak growth phase would be ideal (Rhodes et al., 2021, 2022a).

Tertiary criteria for selecting candidate agents are relationships outside of host plant interactions, such as interactions with the abiotic environment and competitor species. The phenology and climate tolerances of natural enemies affect their likelihood of establishment as well as their damage potential in a new range and must be investigated to some extent (McFadyen, 1998; Goolsby et al., 2000). Predators, parasitoids or pathogens of natural enemies in their native range must be investigated to understand their effects on natural enemy reproduction and feeding, which may have implications for their ability to control the target plant, and to avoid accidentally introducing these undesirable organisms in later stages of the biocontrol program (Harris, 1991; Goolsby et al., 2006a). Herbivorous insects may benefit ecologically from enemy release, ie. being separated from their specialist predators and parasitoids (László et al., 2024). In a biocontrol context, enemy release can improve an agent's chances of establishing at sites of introduction, its rate of spread, and its ability to damage the target plant (Ertle, 1993; Adair, 2005; Goolsby et al., 2006a). Studying the parasitoids of a candidate agent in its native range makes it possible to predict the recruitment and impact of parasitoids in the introduced range, and how this might affect biocontrol efficacy (Paynter, 2010).

2.1.2. Previous surveys on Guinea grass

Guinea grass is host to a similarly diverse arthropod fauna in both East and West Africa, but this diversity is much reduced in Texas, United States, where the plant is invasive (Mercadier et al., 2009; Rhodes et al., 2022a). Field surveys in Texas have so far not shown that any of the arthropods associated with Guinea grass in its native range have been accidentally introduced to the United States (Rhodes et al., 2022a). Three candidate biocontrol agents for short-form Guinea grass in Texas have already been evaluated but were shelved for various reasons: the moth *Buakea keauae* from Kenya, because of low rearing success resulting from a possible genetic mismatch between Kenyan and Texan Guinea grass (Moyal et al. 2011; Vacek et al., 2021), and the mites *Abacarus* sp. and *Diptacus* sp. from Kenya and South Africa, respectively, because of non-target feeding on tall-form Guinea grass (Gaskin et al., 2023).

Conspecific plant populations from different regions may vary genetically as well as morphologically and/or chemically, with implications for the ability of biocontrol agents to establish and feed on different genotypes (Goolsby, 2006a; Paterson et al., 2009). For highly host-specific biocontrol agents, genotype matching between regional populations of the agent and its host plant can determine biocontrol effectiveness to a great extent; for example, regional genotypes of the fern mite *Floracarus perrepae* Knihinicki & Boczek (Acari: Eriophyidae) can only effectively damage certain regional genotypes of its host, the climbing fern *Lygodium microphyllum* R.Br. (Goolsby et al., 2006b). Searching for biocontrol agents in the region of origin of the target plant is therefore highly recommended and is becoming standard practice (Paterson et al., 2009; Mitchell et al., 2024).

Several species of stem gall midge (Cecidomyiidae: Cecidomyiinae) (such as *Arabukodiplosis basalis* Kolesik, which forms characteristic nodal galls) have been collected on Guinea grass in Kenya, and are predicted to be highly host-specific, but it is not certain that they will be suitably matched to Guinea grass genotypes in Texas due to genetic differences between the two populations (Kolesik et al., in press; Gaskin et al., 2023). The research focus for the Guinea grass biocontrol program has accordingly shifted to natural enemies that are more likely to be suitably host-specific as well as suitably matched to the target plant genotype – particularly insects from South Africa, where Guinea grass populations are the closest genetic match to Texan populations (Gaskin et al., 2023).

2.1.3. Chapter aims

The aim of this chapter is to identify natural enemies of Guinea grass in its native range in South Africa, prioritize the most promising candidate agents based on predicted host-specificity, and investigate the life cycle and predicted impact of these candidate agents using laboratory rearing trials and field studies of their phenology. This chapter has the following components:

(1) Surveys for natural enemies were done in two South African provinces: KwaZulu-Natal and the Eastern Cape. A full list of taxa is provided, with notes on priority taxa, their parasitoids, and the types of damage caused to the target plant.

(2) The seasonal abundances and parasitism rates of high-priority candidate agents – the blister gall midge and seed midge species – were investigated and linked to local temperature and rainfall patterns in a series of field surveys over 14 months. These data were used to predict agent impact and the degree to which impact may be affected by seasonal changes and parasitoids.

2.2. Methods

2.2.1. Natural enemy surveys

Arthropods and fungi on Guinea grass were surveyed at sites in the Eastern Cape (n = 15 sites) and KwaZulu-Natal (n = 5 sites) during the summer months (November-April) from 2022 to 2024, on warm, clear days when insects were likely to be most active (Table S1; Figure 2.1). A combination of two or all three of the following sampling methods were used at each site: 2-3 minute net sweeps in large monospecific Guinea grass patches (“sweep” method – n = 8 sites); collections from a sample of 100-200 Guinea grass tillers that were visually inspected and dissected or stored in mesh cages for 1-2 weeks to allow for emergences (“tillers" method – n = 20 sites); and emergences from cut Guinea grass panicles (inflorescences) stored in plastic containers for 1-2 weeks to isolate taxa attacking reproductive parts (“panicles” method – n = 15 sites). Sites were selected on the basis of being suitable for at least two of these methods to ensure adequate sampling. Signs of plant damage were photographed and associated organisms were studied under an optical microscope. Organisms that could not be identified were sent to the South African National Collection at the Agricultural Research Council (ARC-Roodeplaat, Kwa-Mhlanga Road, Pretoria) to be identified by specialists on the appropriate taxa. Identified arthropods and fungi were added to a list of known herbivorous taxa on Guinea grass in South Africa.

2.2.2. Seasonal abundance and parasitism

Repeat surveys for gall midges, seed midges and parasitoids were done at sites with dense monospecific stands of Guinea grass in the coastal city of Port Alfred (Eastern Cape, South Africa) on a monthly to bimonthly basis from October 2022 to January 2024. Standardized gall search times (0.25-1.0 hrs) and panicle collections (10-20 panicles) were used, and searches were done by no more than two people at a time. Gall searches began with an initial 15-20 minute scouting phase: if galls were found in this time, the search continued up to a limit of 1 hour, and if galls were not found, it was assumed that no galls were present. Galls and panicles were placed in separate plastic tubs, and the emergence of cecidomyiids and parasitoid wasps was recorded over two weeks. Weather data for the survey sites were sourced from the South African Weather Service. Monthly temperature maxima and minima, rainfall and relative humidity in Port Alfred were plotted against the monthly recorded abundances of the four insect groups – gall midges, seed midges and the respective parasitoids of each.

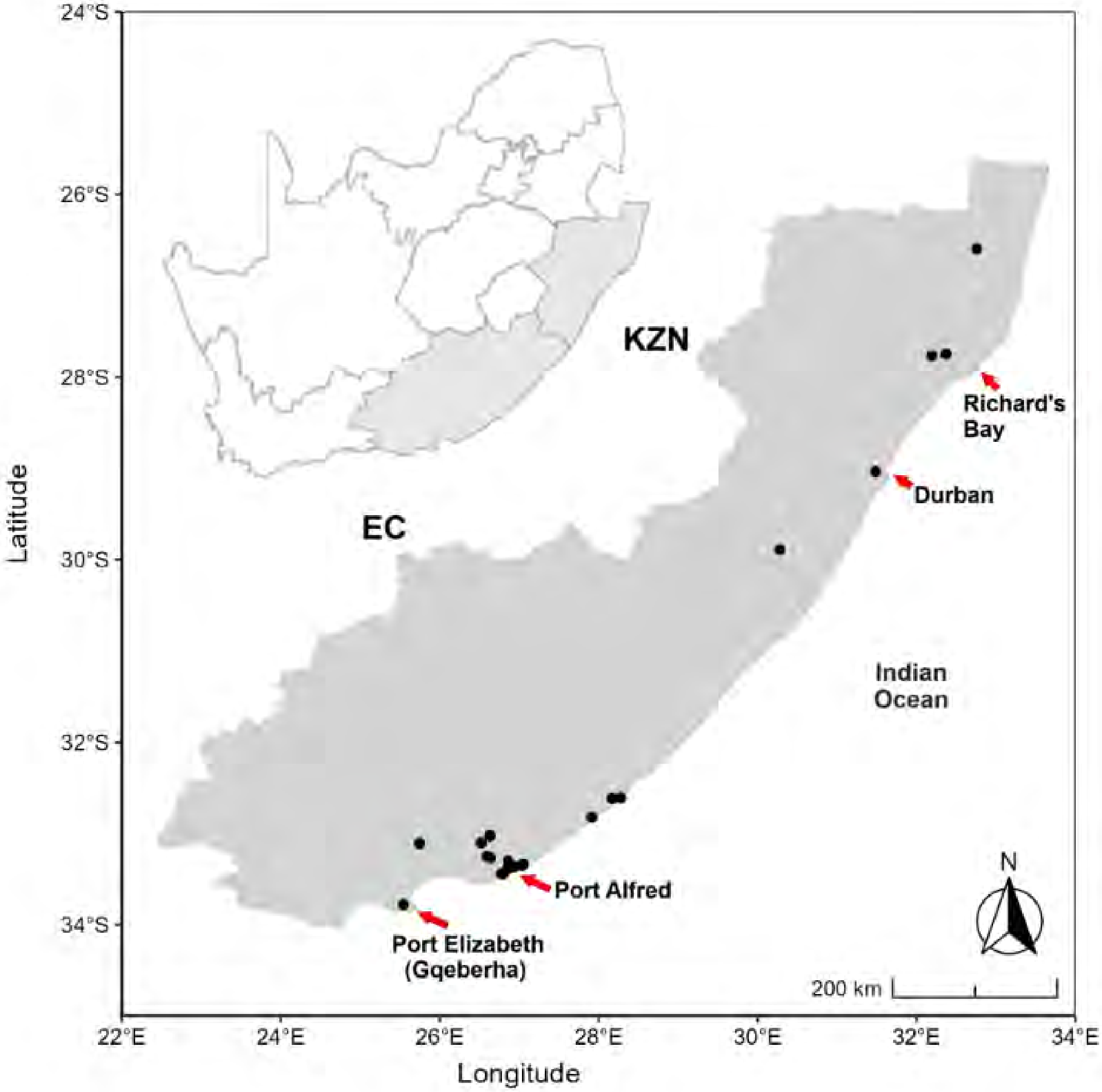

Figure 2.1. Map of natural enemy survey sites in two South African provinces (grey areas in country inset), the Eastern Cape (EC) and KwaZulu-Natal (KZN). Survey sites indicated by filled black circles. Locations of some major cities for reference are indicated by red arrows.

2.2.3. Statistical analyses

To assess whether the sampling effort of the surveys was sufficient, the cumulative numbers of ectophagous taxa, endophagous taxa and all herbivorous arthropod taxa found in successive samples were used to plot three separate species accumulation curves. These curves were inspected visually to see whether or not the curves approached their asymptotes.

Given that Guinea grass and other grasses are capable of rapid growth responses (Matson, 2018), and based on preliminary data on monthly insect abundance, it was presumed that population-level responses of the insects to seasonal changes and resulting plant growth were fairly rapid (within 1-2 months). Generalized Linear Models (GLMs) (stats::glm; R Core Team, 2023) were built in R version 4.3.1 (R Core Team, 2023) to test whether monthly recorded abundances of each candidate agent and its parasitoids were correlated with weather variables for the current month or for a one-month lag period, using a negative binomial distribution. Model fit was evaluated by examining scaled residuals plots (DHARMa::simulateResiduals, Hartig, 2022). Relative humidity in Port Alfred for the study period was very stable (76.6 +/- 0.95) and caused model fitting issues when included as a variable, and was therefore excluded from the analysis.

2.3. Results

2.3.1. Natural enemy surveys

Surveys on Guinea grass in South Africa identified 75 macroscopic arthropod and fungus taxa, most of which were recorded on this plant for the first time in this survey. Of these taxa, there are 34 herbivorous insect morphospecies (10 endophagous) from 26 families, comprising a range of functional groups, including sap suckers, leaf chewers, leaf cell feeders, stem borers, seed feeders and gall-inducers (Table 2.1). The family Cecidomyiidae was the most morphospecies-rich, containing four morphospecies of gall-inducers and three morphospecies of seed feeders. Three morphospecies of phytopathogenic fungi induce galls on the florets, and another morphospecies induces galls on the stems. The remaining taxa include detritivorous mites and detritivorous, predatory or parasitic insects. The species accumulation curves for ectophagous, endophagous and all herbivorous insects approached their asymptotes, indicating that the sampling effort was sufficient to identify most taxa present (Fig. 2.2).

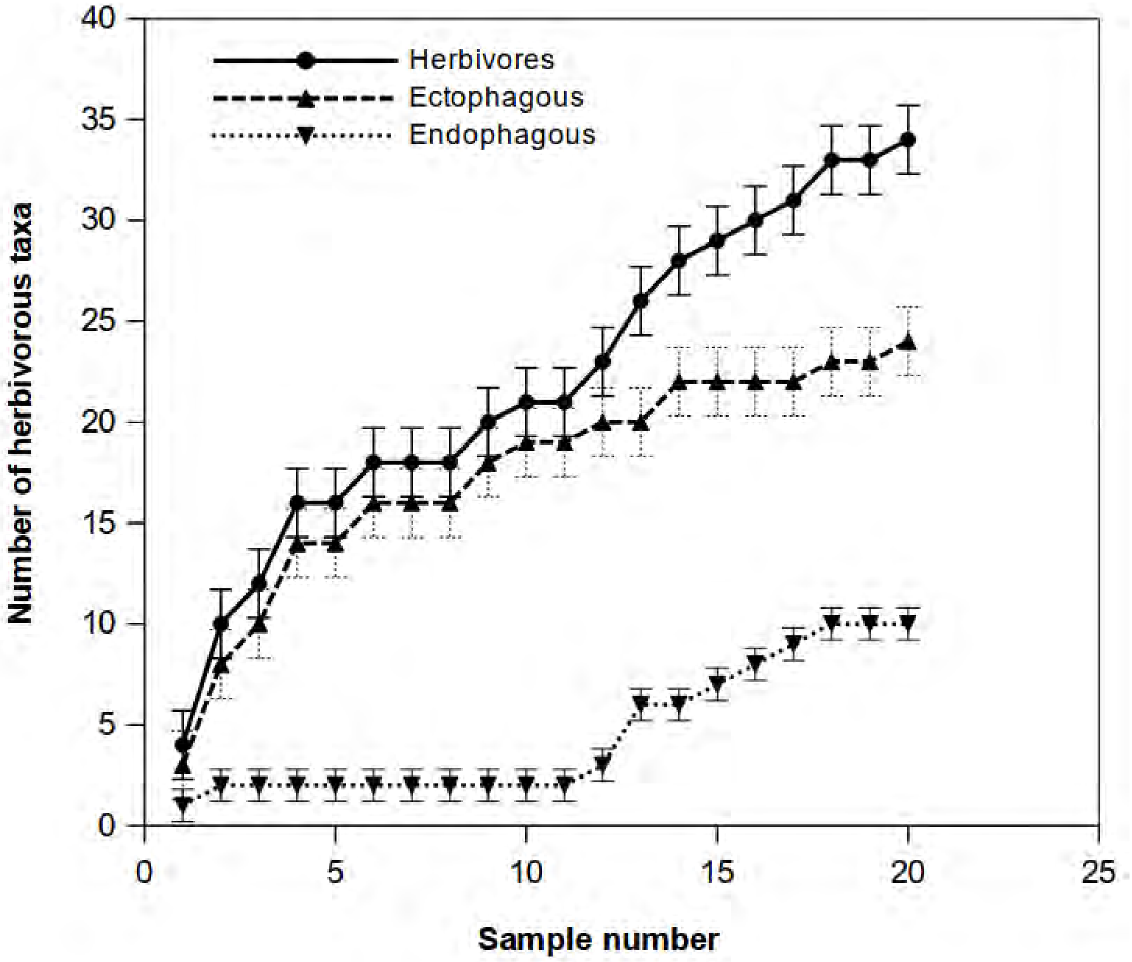

Figure 2.2. Species accumulation curves for all herbivorous arthropod taxa and for ectophagous and endophagous arthropod taxa identified in natural enemy surveys on Guinea grass. Error bars represent standard deviation for each group of taxa.

2.3.2. Notable taxa

Endophagous insect taxa such as gall-inducers, and herbivorous cecidomyiids in particular, are often highly host-specific (Crawley, 1989; Carneiro, 2009; Dorchin et al., 2019a). Notable endophagous taxa and their parasitoids identified in natural enemy surveys are summarized below, with an emphasis on cecidomyiids as a priority group. Other taxa found in these surveys with potential biocontrol applications are discussed in Chapter 6.

Arabukodiplosis vesicaria (“Blister gall midge”) (Cecidomyiidae: Cecidomyiinae)

The blister gall midge *Arabukodiplosis vesicaria* forms thin-walled, bright green “blister” galls on the stem internodes of short-form Guinea grass (Fig. 2.3 A). The galls are up to 11 mm long and up to

6 mm in diameter; they may occur singly, in pairs or in clusters of 3-10 galls per internode. This species has the widest known distribution of the known stem gall midges on Guinea grass in South Africa, occurring in several provinces up to 180 km from the coast, and is the most frequently collected (Chapter 3). This species has also been recorded on short-form Guinea grass in Kenya (Kolesik et al., in press).

The life cycle of this species has been studied in the laboratory. Adult blister gall midges live for one to three days and do not feed. The sex ratio is female-skewed; 61 % of recorded adult emergences have been female (Chi-squared test $\chi2 = 4.84$; $p = 0.028$; $n = 100$). Adult females are generally larger than adult males (5-7 mm versus 4-5 mm, from head to tip of abdomen) and longer lived (< 72 hours versus < 24 hours) (Fig. 2.4).The swollen, pink-orange abdomen of females is due to their heavy egg load, which is around 200 eggs. Eggs are translucent pink-orange, elongate and < 2 mm in length. Mating and oviposition usually occurs at night, as in other cecidomyiids whose behaviour has been studied (Cheng et al., 2020). Unmated females survive longer and may eventually oviposit if a fresh grass stem is provided, but the eggs will not hatch. Females preferentially oviposit in clusters on or near the tips of stem hairs or ligular hairs on Guinea grass, and not on the stem surface, leaves or leaf sheaths (Fig. 2.5 A). This is thought to be a defence against egg predation, similar to the behaviour of the moth *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) which lays egg sticks on the spines of *Opuntia* spp. (Varone et al., 2019). Hairs on the surface of the galls may also serve a defensive function for the developing larva or pupa (Bailey et al., 2009). Tall-form Guinea grass in South Africa and both biotypes of Guinea grass in Texas and Kenya generally lack stem hairs, however. Eggs hatch within three to four days at 21 °C, and first instar larvae immediately begin seeking suitable sites for gall initiation (Fig. 2.5 B). Fully-formed galls may be produced within three to four weeks at the same temperature (Fig. 2.5 C). The pupae partially exit the gall using a set of procephalic horns and eclose as winged adults on the exterior of the gall (Fig. 2.5 D). Adults are poor fliers and poor walkers, usually clinging to objects or surfaces between bouts of flight.

“Horn gall midge” (Cecidomyiidae: Cecidomyiinae)

The horn gall midge (undescribed sp.) forms pale galls with red striations or mottling and a leaflike green tip or “horn” at or near stem nodes (Fig. 2.3 B), differing from the related Kenyan basal stem gall midge (*Arabukodiplosis basalis* Kolesik) mainly in that the galls of the latter are smaller, sometimes in dense clusters, and often occur at basal stem nodes (Fig. 2.3 C). On short-form Guinea grass in KwaZulu-Natal, the main body of the gall can be up to 45 mm long and over 10 mm in diameter, and the tip up to 15 mm long. Galls may be found up to 67 km from the coast and usually occur singly but may be paired with one or two other galls. The blister and horn gall midges occur sympatrically in KwaZulu-Natal and Kenya.

Table 2.1. List of arthropods and fungi associated with Guinea grass in South Africa from natural enemy surveys, with feeding modes indicated. An asterisk (*) denotes taxa that have not been identified to the specified taxonomic level. Obligate feeders on live plant tissue are indicated in bold.

Class	**Order**	**Family**	**Lower taxa**	**Feeding mode**
Insecta	Coleoptera	Chrysomelidae	**Galerucinae sp.**	**Leaf chewer**
		Coccinellidae	*Exochomus* sp.	Predator
			Cheilomenes lunata	Predator
			Hippodamia variegata	Predator
		Curculionidae	***Sciobius* sp.**	**Leaf chewer**
			*	
		Lycidae	*Lycus* sp.	Nectar feeder / Predator
		Melyridae	*	Pollen feeder
	Diptera	Asilidae	*	Predator
		Cecidomyiidae	***Arabukodiplosis vesicaria* (blister gall midge)**	**Gall-inducer**
			Cecidomyiinae sp. (horn gall midge)	**Gall-inducer**
			Cecidomyiinae sp. (balloon gall midge)	**Gall-inducer**
			Cecidomyiinae sp. (seed midge msp. 1)	**Seed feeder**
			Cecidomyiinae sp. (seed midge msp. 2)	**Seed feeder**
			Cecidomyiinae sp. (seed midge msp. 3)	**Seed feeder**
			Micromyinae sp.	Detritivore
		Chironomidae	*	Detritivore (as larva)
		Chloropidae	***Parectecephala* sp.**	**Gall-inducer / Inquiline**
			*	
		Conopidae	*	Nectar feeder
		Mycetophilidae	*Epicypta* sp.	Detritivore (as larva)
		Syrphidae	*Allograpta* sp.	Nectar feeder
		Tachinidae	*	Nectar feeder / Parasitoid
	Hemiptera	Alydidae	*	**Sap sucker**
		Aphididae	***Melanaphis pyraria***	**Sap sucker**
		Aphrophoridae	*	**Sap sucker**
		Cicadellidae	***Balclutha petrusa***	**Leaf cell feeder**
		Coccidae	*	**Sap sucker**
		Coreidae	*	**Sap sucker**
		Delphacidae	***Oaristes snelli / Toya sp.***	**Leaf cell feeder**
		Diaspididae	*	**Stem sap sucker**
		Lophodidae	*	**Sap sucker**
		Fulgoridae	*	**Sap sucker**
		Miridae	*	**Sap sucker**
		Pentatomidae	*Agonoscelis* sp.	Sap sucker / Omnivore
			Carbula sp.	Sap sucker / Omnivore
			Pentatomidae sp. 1	Sap sucker / Omnivore

			Pentatomidae sp. 2	Sap sucker / Omnivore
		Pseudococcidae	***Antonina graminis***	**Root sap sucker**
		Plataspidae	*****	**Sap sucker**
		Reduviidae	*****	**Predator**
		Thripidae	*****	**Leaf/stem scraper**
	Hymenoptera	Apidae	*Allodapula* sp.	Nectar feeder / Pollen feeder
		Crabronidae	*Philanthus* sp.	Predator
		Eulophidae	Tetrastichinae sp.	Parasitoid
			Neotrichoporoides sp. 1	Parasitoid
			Neotrichoporoides sp. 2	Parasitoid
			Eulophidae sp.	Parasitoid
		Eupelmidae	*Eupelmus* sp.	Parasitoid
			*	Parasitoid
		Eurytomidae	*Eurytoma* sp.	Parasitoid
			***Tetramesa* sp.**	**Stem borer**
		Formicidae	*Agraulomyrmex* sp.	Omnivore
			Camponotus sp.	Omnivore
			Cataulacus sp.	Omnivore
			Crematogaster peringueyi	Omnivore
			Lepisiota capensis	Omnivore
		Platygastridae	*Platygaster* sp.	Parasitoid
		Pteromalidae	*Amotura* sp.	Parasitoid
		Sphecidae	*	Predator / Nectar feeder
		Torymidae	*	Parasitoid
	Lepidoptera	**Geometridae**	*	**Leaf chewer (?)** / Nectar feeder
		Scythrididae	*	**Leaf chewer (?)** / Nectar feeder
	Mantodea	Mantidae	*	Predator
	Orthoptera	**Acrididae**	*****	**Leaf chewer**
		Gryllidae	***Oecanthus* sp.**	**Leaf chewer**
		Tettigoniidae	*****	**Leaf chewer**
	Psocoptera	*	*	Detritivore
Arachnida	Araneae	Araneidae	*	Predator
		Salticidae	*	Predator
		Tetragnathidae	*	Predator
		Thomosidae	*	Predator
Exobasidiomycetes	Tilletiales	Tilletiaceae	***Conidiosporomyces ayresii***	**Flower smut fungus**
Sordariomycetes	Hypocreales	Clavicipitaceae	***Claviceps* sp. 1**	**Flower ergot fungus**
			***Claviceps* sp. 2**	
			***Balansia / Myriogenospora* sp.**	**Stem ergot fungus**

“Balloon gall midge” (Cecidomyiidae: Cecidomyiinae)

The balloon gall midge (undescribed sp.) has itself never been collected, and is only known from galls found on short-form Guinea grass in KwaZulu-Natal up to 56 km from the coast. Because the galls are attacked by the same parasitoids as the blister and horn gall midges, it is presumed to be a fourth cecidomyiid species. The galls are dark green, elongated, hollow protuberances (‘balloons’) resembling budding tillers, and are restricted to shoots at the base of the tussocks (Fig. 2.3 D). They are up to 30 mm long and up to 6 mm in diameter.

Other gall-inducers

Grass flies (Diptera: Chloropidae) have been reared from cecidomyiid galls (n = 3), with one specimen identified as a *Parectecephala* species. Although many chloropids are themselves gall-inducers on many grass species, others are also opportunistic inquilines of other gall-inducers (László et al., 2024; Nartshuk, 2014). It is likely that several chloropid species are associated with Guinea grass in Africa; six chloropid morphospecies (not identified to species level) have been collected on Guinea grass in Kenya, although their feeding modes and host relationships are not known (Rhodes et al., 2022b).

Four species of fungi forming gall-like structures were recorded on Guinea grass. Fungi which destroy fertile spikelets are the smut fungus *Conidiosporomyces ayresii* Vánky & R. Bauer (ex. *Tilletia ayresii* Berke.) (Exobasidiomycetes: Tilletiales: Tilletiaceae) and an ergot fungi in the genus *Claviceps* (Sordariomycetes: Hypocreales: Clavicipitacae) (one producing clear “honeydew” with an orange mycelial growth, likely *Claviceps maximensis* Theis, and one producing dark sclerotia, likely *Claviceps africana* Fre., Man. & De Mill.) and a *Balansia* / *Myriogenospora* sp. (Sordariomycetes: Hypocreales: Clavicipitacae) that induces black growths on young stems, which become severely stunted and apparently incapable of flowering (CABI, 2009; Pažoutová et al., 2011; Cruz-Laufer et al., 2019). Fungi in these genera are generally not host-specific, and have been recorded on several grass species other than Guinea grass (USDA, 2024).

Conidiosporomyces ayresii is a minor emerging pest of Guinea grass seed production, first reported in Japan in 2011 and in the United States in 2019 (Tsukiboshi et al., 2011; Rosskopf et al., 2019)

Tetramesa (Hymenoptera: Eurytomidae)

Stem-boring wasps in the genus *Tetramesa*, which are associated with the Poaceae, are generally host-specific, and include biocontrol agents for invasive grass species, have been collected in previous surveys on Guinea grass (Wapshere, 1990; Goolsby et al., 2023; Yell et al., 2024).

Although specimens were not collected in this survey, damage typical of *Tetramesa* spp. (hollowed stems with circular exit holes) was used to determine the presence of the wasps as done by Yell *et al.* (2024). In another ongoing study, preliminary cross-incoculation trials and field abundance records comparing *Tetramesa* spp. from Guinea grass and *Setaria* grasses have suggested that *Setaria* spp. are the primary hosts (Guy Sutton [CBC], personal communication).

Seed midges (Cecidomyiidae: Cecidomyiinae)

A complex of undescribed seed midge species was reared from Guinea grass panicles. The larvae of seed midges feed on the contents of developing seeds, resulting in seed inviability. Attacked seeds are not deformed, but turn straw-coloured prematurely compared to newly-formed healthy seeds on the same panicle, which appear green under a microscope (Fig. 2.6 A-B1). The pupae exit the seed through a fissure in the seed coat before eclosing as adults (Fig. 2.6 B2-B4). Adults and pupae are 1-2 mm in length, slightly smaller than their host seeds.

Two seed midge morphospecies are the most commonly reared from Guinea grass, distinguished by differences in the antennae, female abdomen and external genitalia, limb length and (visible without magnification) integument colour (Fig. 2.7). The two morphs often occur on the same panicle, but seed midge msp. 1 (“light morph”) is more abundant than seed midge msp. 2 (“dark morph”). Large numbers emerge from seeds collected in warm months. A third, rare morphospecies has also been collected. This undescribed species complex is studied in detail in Chapter 4.

Parasitoids (Hymenoptera)

Many of the insects associated with Guinea grass are parasitoids of herbivorous taxa. Stem gall midges and seed midges are attacked by a diverse assemblage of parasitoid wasps during their immature life stages. Large numbers of wasps in the genus *Platygaster* sp. (Platygastridae) have been reared from blister galls containing gall midge pupae; wasps in this genus are specialist gregarious parasitoids of cecidomyiids (Matsuo et al., 2018) and include *Platygaster diplosisae* Risbec, a biological control agent of the African rice gall midge *Orseolia oryzivora* Harris & Gagné (Ogah et al., 2010) and *Platygaster californica* Ashmead, a parasitoid of a biocontrol agent, the groundsel bush gall midge *Rhopalomyia californica* Felt (Briggs and Latto, 2000). Other parasitoids of the stem gall midges are representatives of the genera *Eurytoma* (Eurytomidae) and *Eupelmus* (Eupelmidae) and the families Eulophidae, Pteromalidae and Torymidae, all of which include known parasitoids of cecidomyiids and other endophagous herbivorous insects (Fig. 2.8) (Briggs and Latto, 2000; Calles Torres et al., 2014; Al-Khatib et al., 2016).

Parasitoids reared from panicles with seed midges are Eulophidae, Eurytomidae, Eupelmidae, and the egg parasitoid *Trichogramma* sp. (Hymenoptera: Trichogrammatidae). Parasitoid larvae consume the host late-stage larva or pupa within the gall or seed, expel a meconium before pupation (Fig. 2.9 A), and eclose before exiting the gall or seed. Gall midge parasitoids exit the host gall by chewing a circular hole in the wall with their mandibles (Fig. 2.9 B); seed midge parasitoids either chew a hole in the seed coat or exit through a natural fissue in the seed coat like their hosts (Fig. 2.6 C2-C3). The meconium of the seed midge parasitoid is visible as a dark spot visible through the seed coat, and can be used to determine if a seed has been parasitized (Fig. 2.6 C1). Since it is not currently possible to distinguish which seed midge morphospecies has parasitized a seed before it emerges, it is not known whether any parasitoids attack only one seed midge host or multiple hosts.

2.3.3. Seasonal abundance and parasitism

Blister gall midge abundance was positively correlated with higher minimum temperature and lower maximum temperature in both the current month (min. $\beta = 2.809$, $\chi^2 = 16.112$, df = 1, $p < 0.001$; max. $\beta = -3.145$, $\chi^2 = 17.808$, df = 1, $p < 0.001$) and the lag month (min. $\beta = 3.495$, $\chi^2 = 4.789$, df = 1, $p = 0.0286$; max. $\beta = -3.879$, $p = 0.017$), and slightly lower rainfall in the current month ($\beta = -0.130$, df = 1, $p = 0.001$), but not rainfall in the lag month ($\beta = -0.004$, df = 1, $p = 0.55$). Gall midge parasitoid abundance was also positively correlated with slightly lower rainfall in the current month ($\beta = -0.0254$, $\chi^2 = 7.866$, df = 1, $p = 0.005$).

Seed midge abundance, similarly, was positively correlated with higher minimum temperature and lower maximum temperature in the current month (min. $\beta = 4.769$, $\chi^2 = 12.614$, df = 1, $p < 0.001$, max. $\beta = -5.179$, $\chi^2 = 10.689$, df = 1, $p = 0.001$) and in the lag month (min. $\beta = 32.960$, $\chi^2 = 47.375$, df = 1, $p < 0.001$, max. $\beta = -27.290$, $\chi^2 = 49.462$, df = 1, $p = 0.001$) and slightly lower rainfall in the current month ($\beta = -0.078$, $\chi^2 = 6.563$, df = 1, $p = 0.010$) and in the lag month ($\beta = -0.0230$, $\chi^2 = 21.492$, df = 1, $p < 0.001$). Seed midge parasitoid abundance was positively correlated with slightly higher rainfall in the lag month ($\beta = 0.0578$, $\chi^2 = 18.798$, df = 1, $p < 0.001$).

The patterns of abundance of the stem and seed midges and their respective parasitoids were similar and extremely seasonal. At the Port Alfred sites, there were no new galls formed and no adult seed midge emergences in April-October (late autumn to winter). This was followed by a sudden upsurge to peak abundance in November-December (late spring to early summer) concurrent with the onset of the rainy season, and then a gradual decline in abundance (Fig. 2.10). Parasitoid abundance initially increased in step with the hosts, but peaked dramatically in January-March (mid to late summer), when parasitoids outnumbered unparasitized hosts (2.6 : 1 for gall midge parasitoids and 2.3 : 1 for seed midge parasitoids). The rate of parasitism of the blister gall midge ranged 50-100 % (average 73 %) depending on the time of year. The collective rate of parasitism of both seed midge species (no data are available on rates of parasitism for each species) ranged 0-94 % (average 17 %).

The emergence of adult seed midges in warm months (November-April) was highly variable. The number of seed midge msp. 1 emergences in warm months was nearly 11 adults per panicle at a maximum, but the average was slightly over one adult per panicle. Seed midge msp. 2 was less abundant, occurring at an average 1 adult per five panicles in warm months.

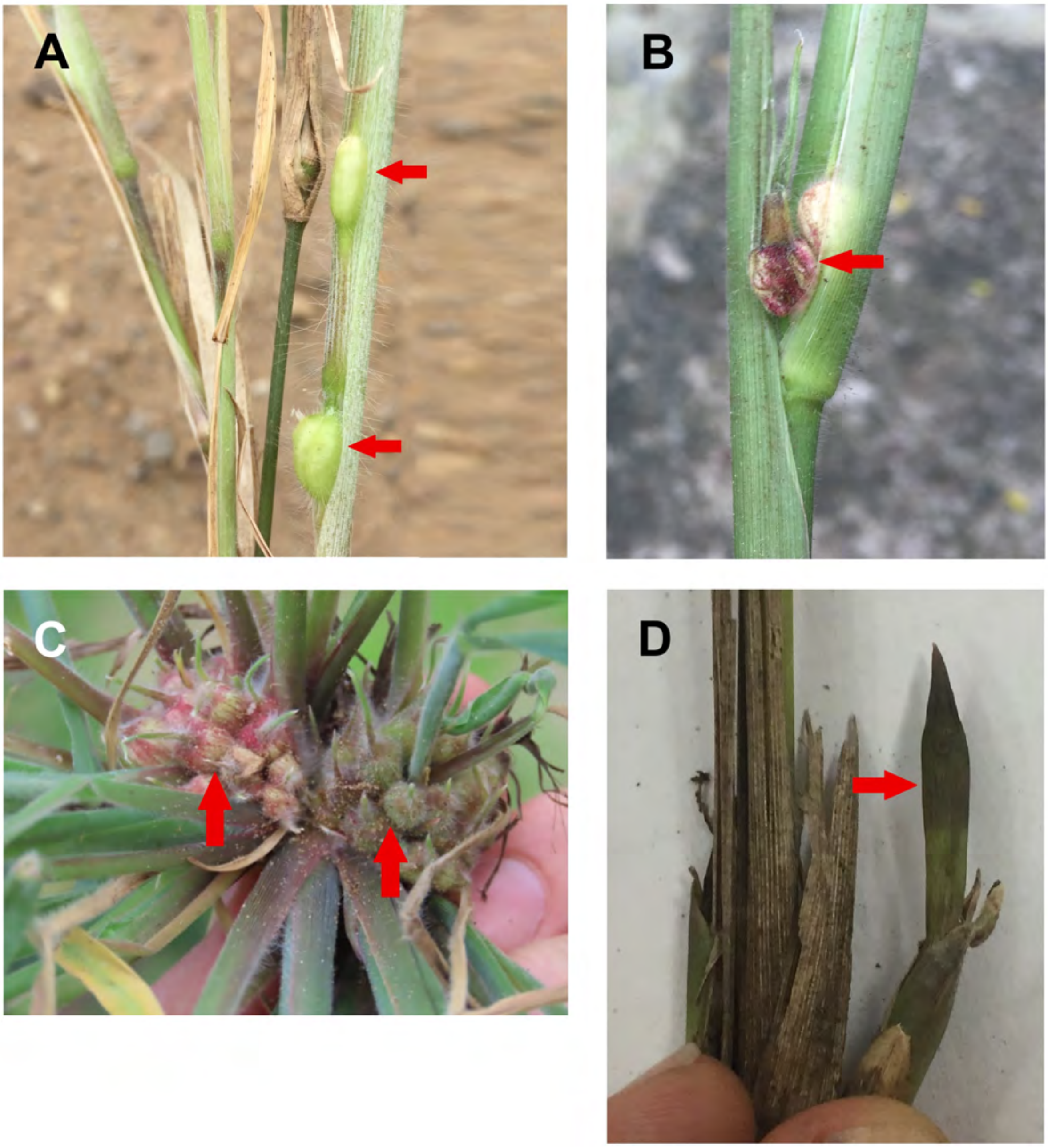

Figure 2.3. Stem gall types on Guinea grass induced by (A) Blister gall midge (*Arabukodiplosis vesicaria*); (B) Horn gall midge (undescribed sp.); (C) Basal stem gall midge (*Arabukodiplosis basalis*); (D) Balloon gall midge (undescribed sp.). Galls are indicated by red arrows. (Image C credit: Iain Paterson).

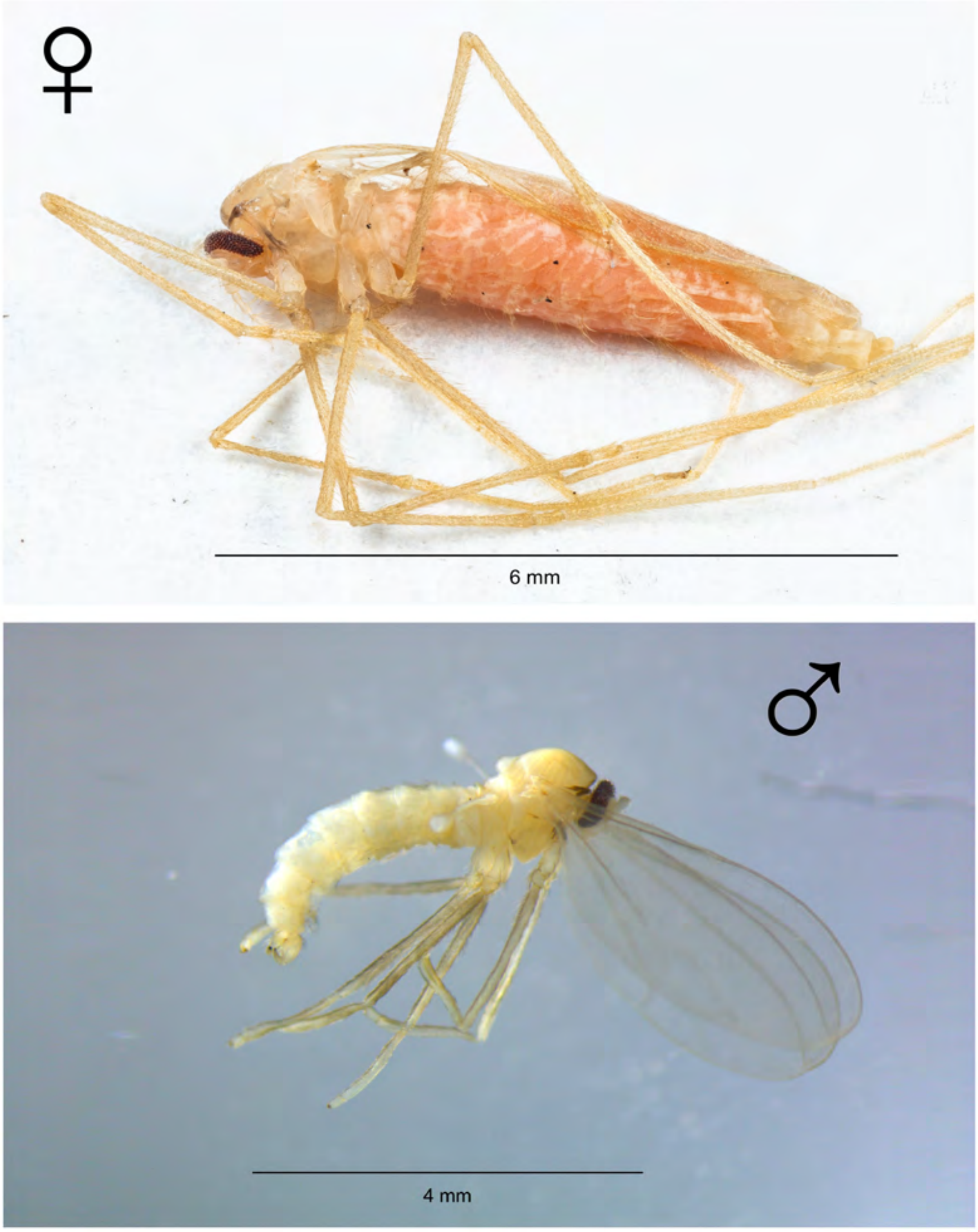

Figure 2.4. Adult blister gall midge female (top) and male (bottom). (Image top credit: David Taylor)

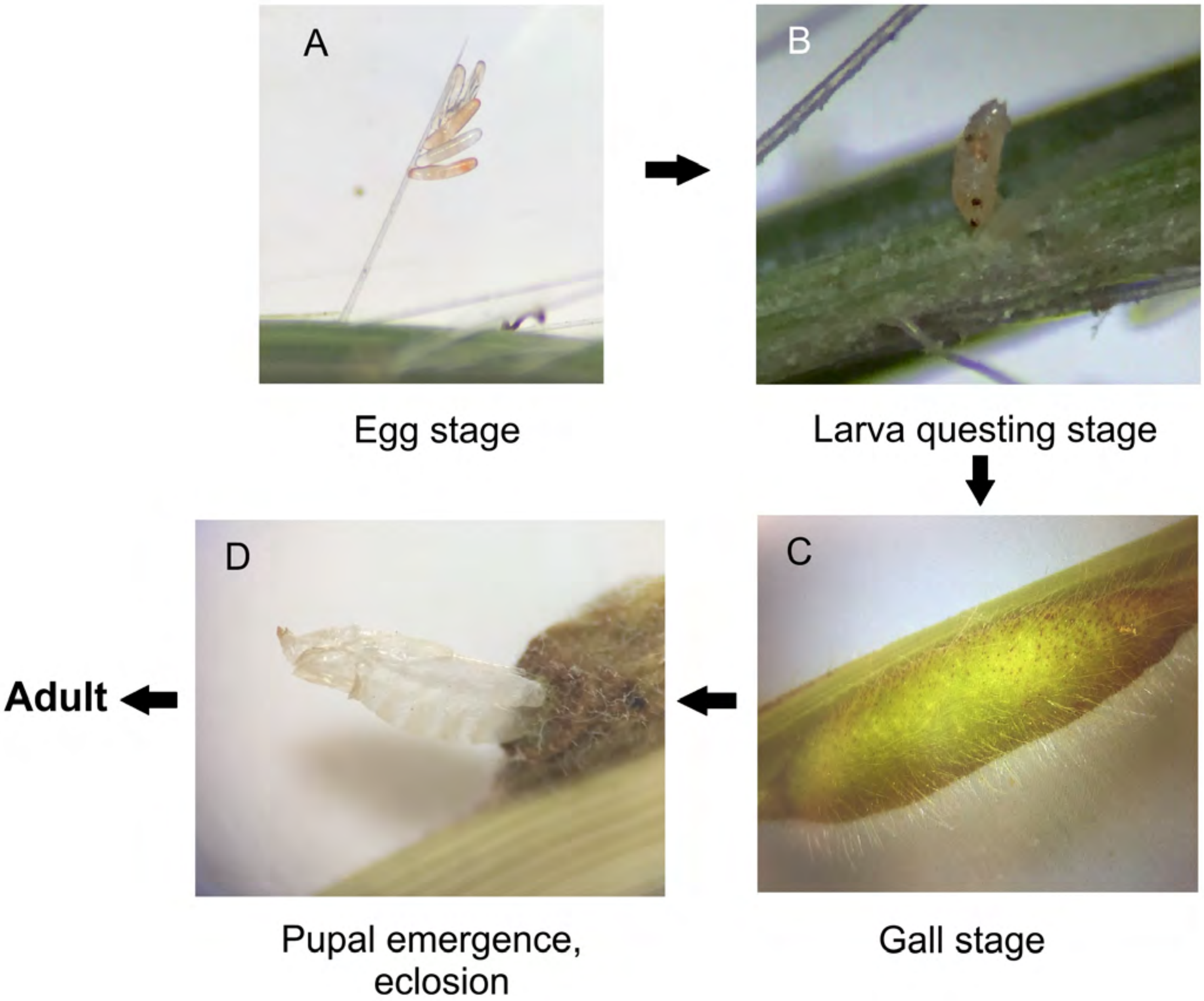

Figure 2.5. Blister gall midge immature life stages in chronological sequence (A-D).

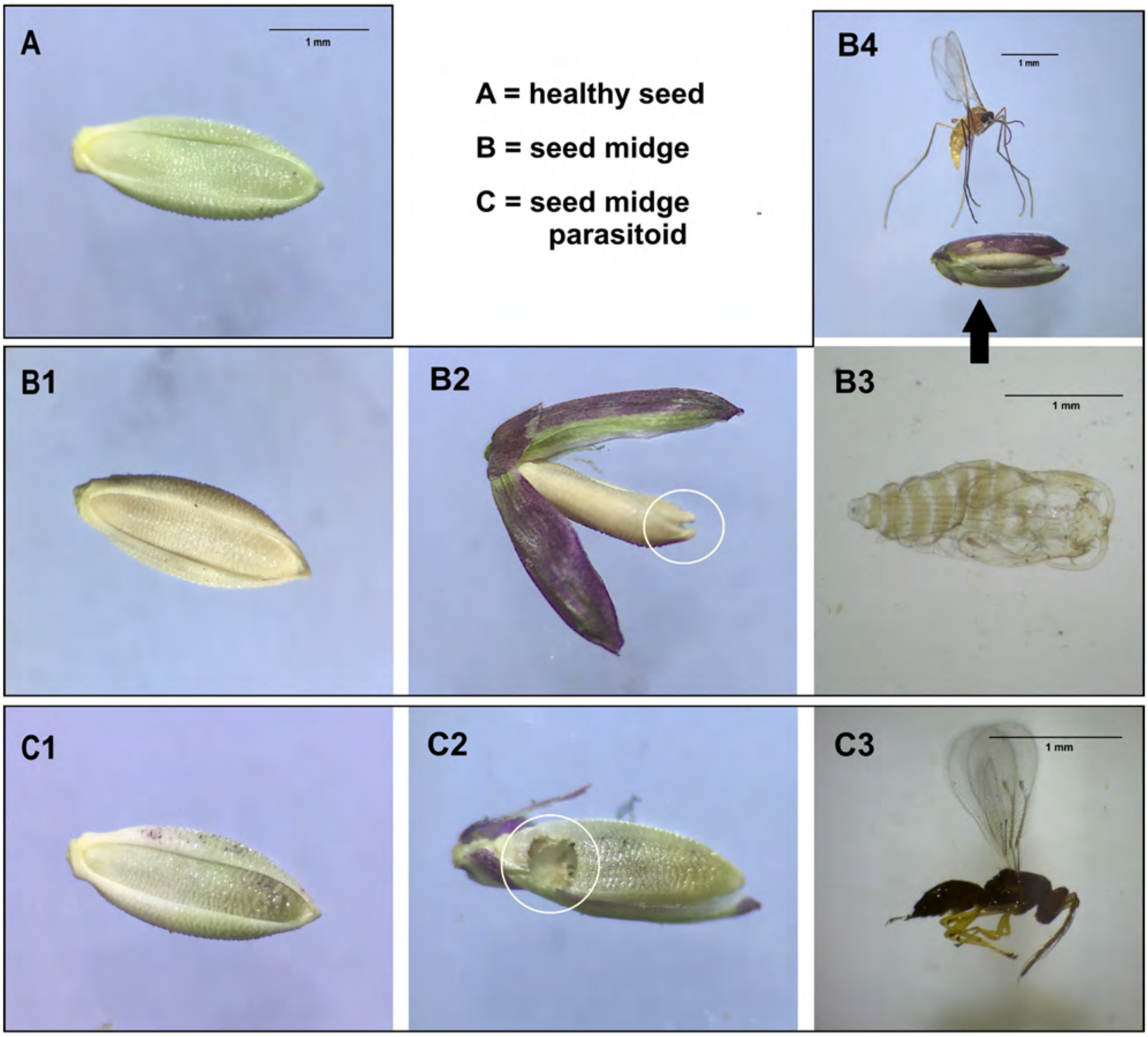

Figure 2.6. Healthy Guinea grass seed (A) versus seeds damaged by seed midge msp. 1 (B) and seeds with seed midge parasitoids (C). Seed midges exit the seed through a natural fissure in the seed coat (B2) as pupae (B3) before eclosing as adults (B4). Parasitoids may be indicated by a dark meconium (C1) and typically exit the seed by chewing a circular exit hole in the seed coat (C2) before emerging as fully-formed adults (C3).

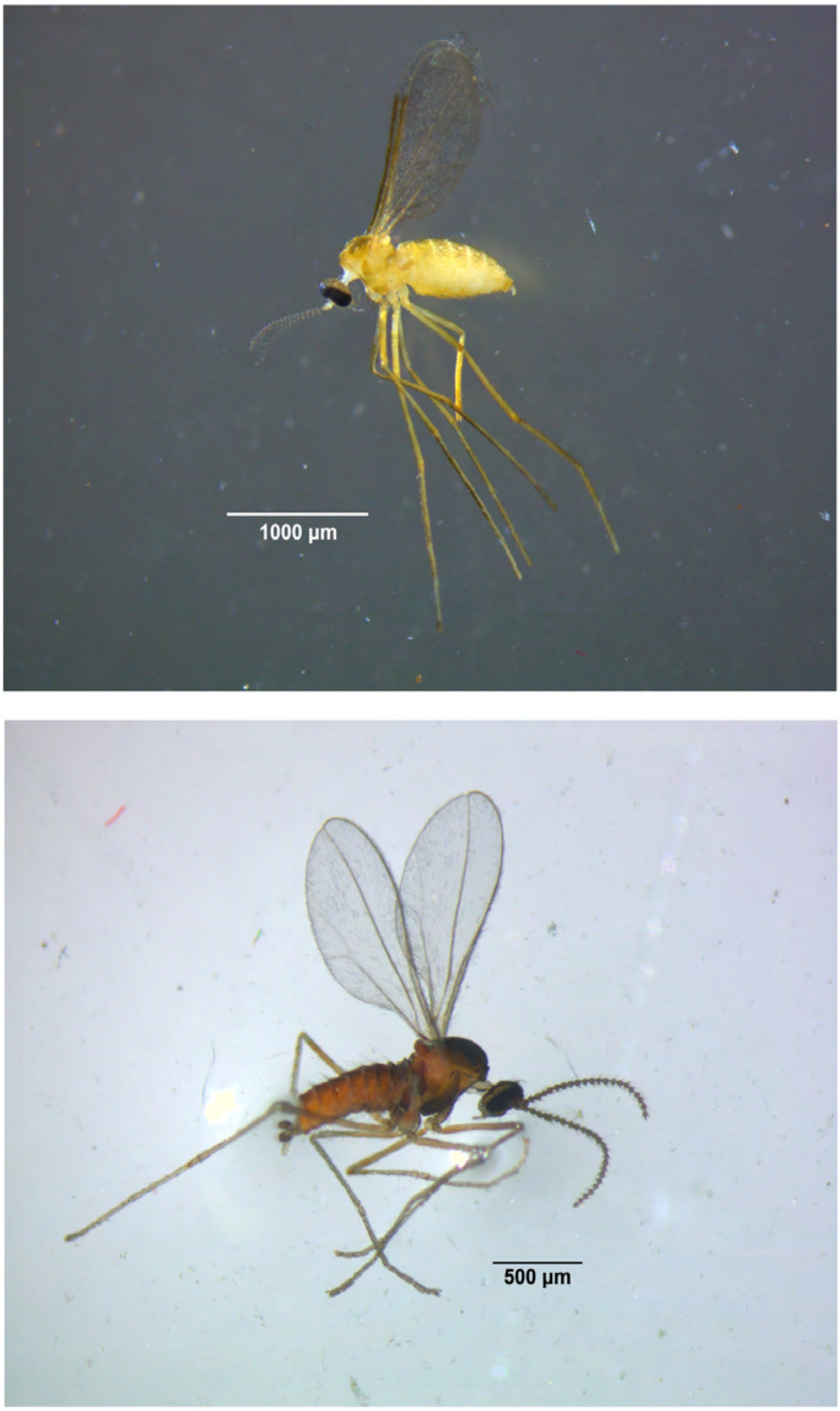

Figure 2.7. Common seed midge morphospecies on Guinea grass: seed midge msp. 1 “light morph” (top) and seed midge msp. 2 “dark morph” (bottom). Both specimens are female.

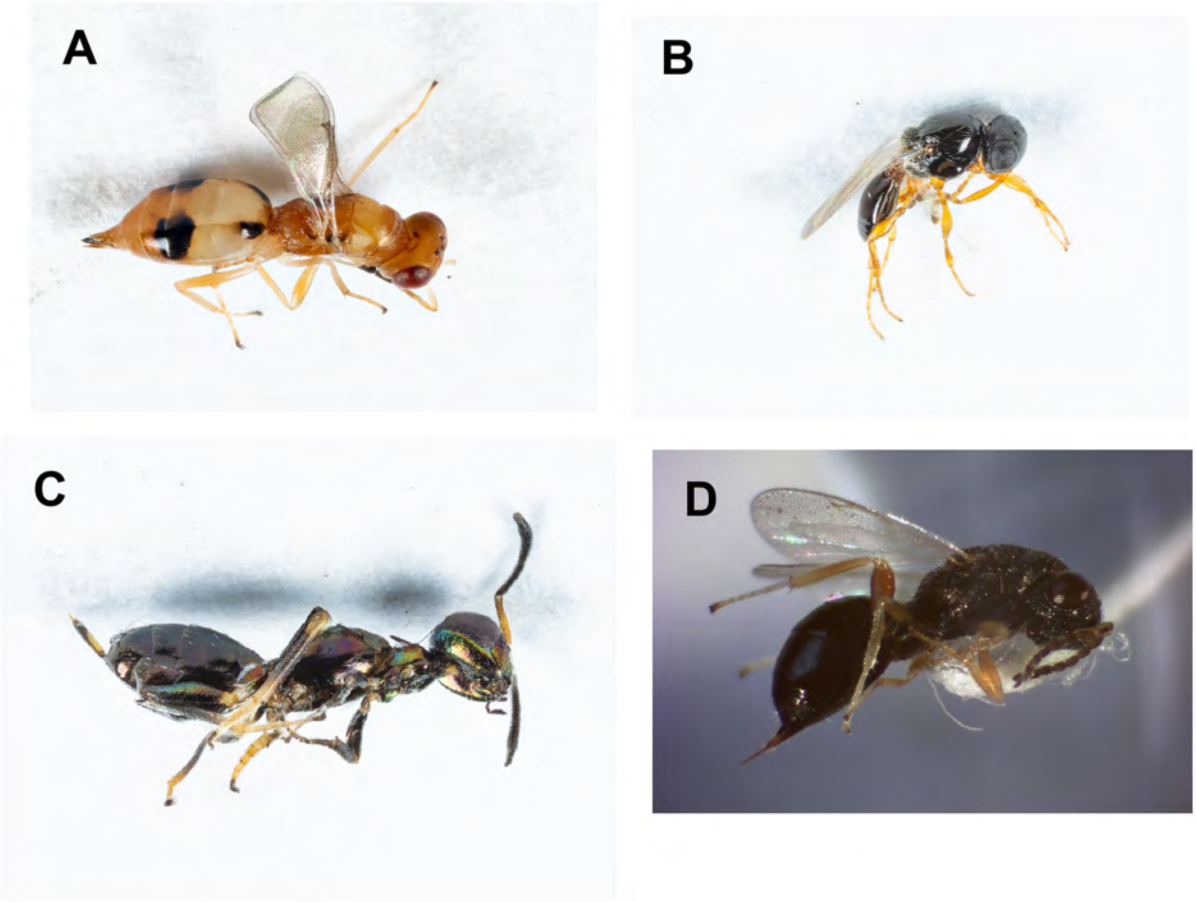

Figure 2.8. Common stem gall midge parasitoids. (A) *Neotrichoporoides* sp. (Eulophidae); (B) *Platygaster* sp. (Platygastridae); (C) *Eupelmus* sp. (Eupelmidae); (D) *Eurytoma* sp. (Eurytomidae). Not to scale. (Image A-C credit: David Taylor)

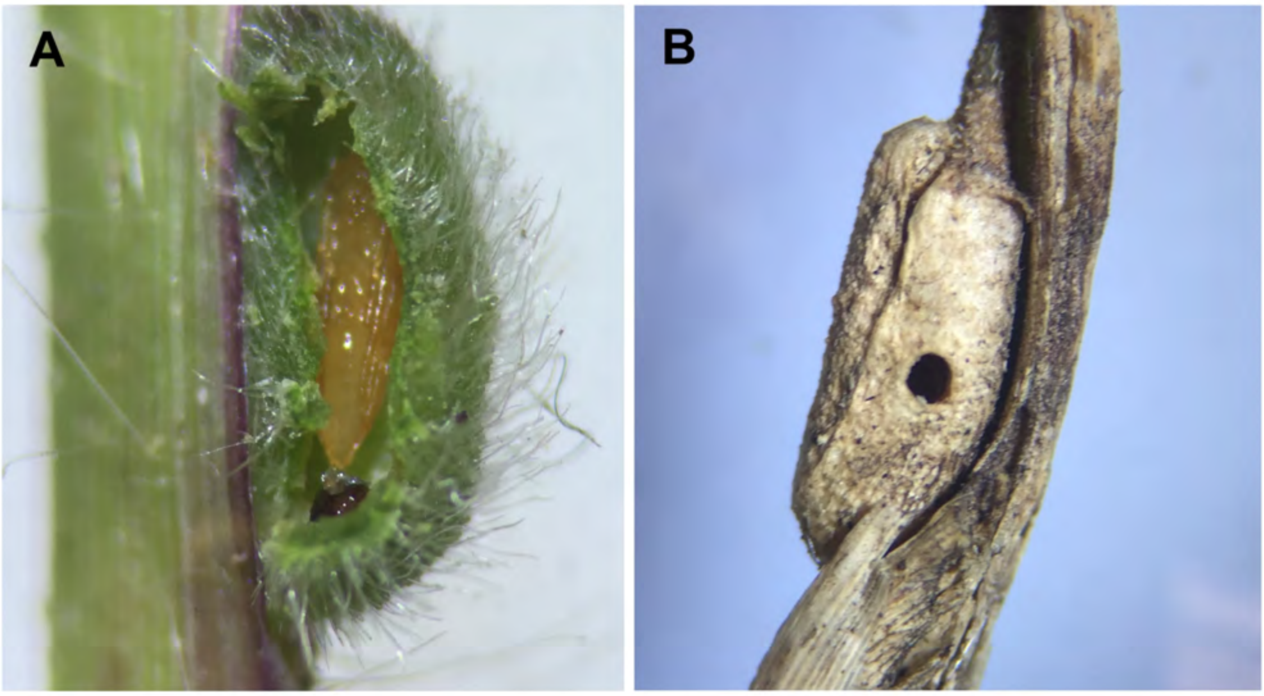

Figure 2.9. Indicators of gall a; A) dissected blister gall containing parasitoid pupa and meconium, B) parasitoid exit hole in old blister gall.

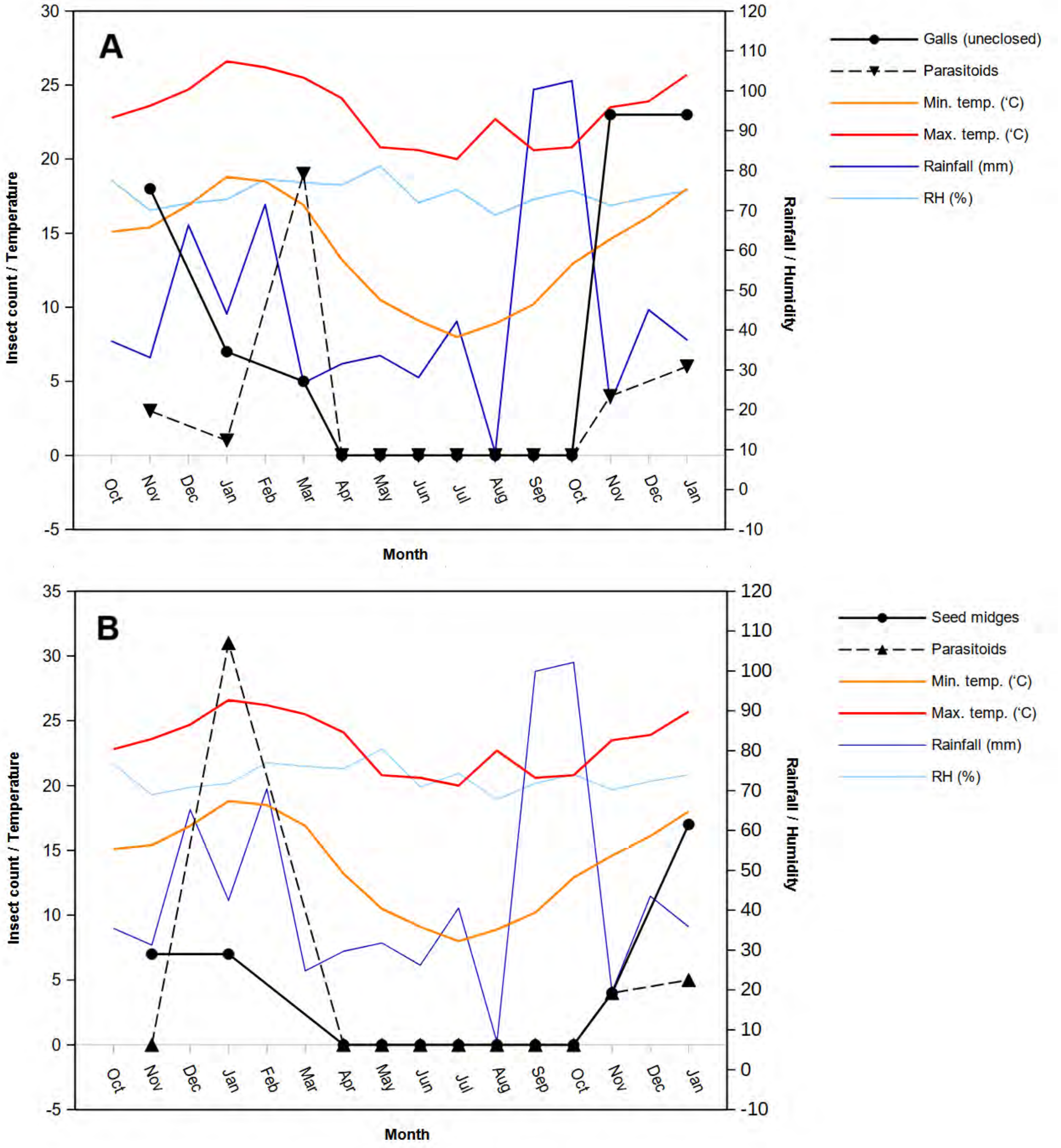

Figure 2.10. Monthly counts of uneclosed blister galls (A) and seed midge msp. 1 emergences (B) with parasitoid emergences, and monthly averages of minimum and maximum temperatures and relative humidity (Port Alfred repeat surveys and SAWS weather data).

2.4. Discussion

This survey is likely to have found most natural enemies associated with Guinea grass in South Africa, and represents the most diverse herbivorous arthropod assemblage so far recorded on this plant. Mercadier et al. (2009) and Rhodes et al. (2022a) each found 27 herbivorous arthropod taxa in Cameroon and Kenya, from 15 and 17 families, respectively. Assuming more or less equal sampling effort for surveys done so far (e.g. Mercadier et al. (2009) surveyed 21 sites, versus 20 sites surveyed in this study) this indicates that southern Africa, rather than east or west Africa, may be the centre of natural enemy diversity associated with this grass species. The diversity of specialized natural enemies associated with a plant species is expected to be highest in the plant's centre of diversification, since coevolution occurs on timescales of thousands to millions of years (Schroeder and Goeden, 1986; Pfennig and Pfennig, 2012). In Texas, where Guinea grass is thought to have been introduced less than a century ago, only 10 arthropod taxa (8 families) have been found, and none of these are in any way specialized on the plant (Rhodes et al., 2022a). Morrison et al. (2023b) found similar numbers of herbivorous arthropod morphospecies to this study on buffelgrass in a natural enemy survey in Kenya, and also found the Cecidomyiidae to be the most diverse arthropod family at four morphospecies, most of which are thought to be specialists.

Both the blister gall midge and seed midges follow the pattern of eruptive species, alternating seasonally between phases of low and high population density or abundance (Bernays, 1990). Using seasonal abundance as a proxy for damage, impacts on Guinea grass are predicted to be highest during during summer (Egli and Olckers, 2015). All cecidomyiid species studied here are multivoltine; although development times for the seed midges are not known, the blister gall midge may be capable of completing at least five generations during a single summer in their native range, assuming similar development times to those seen in rearing trials.

The fact that no new gall formation or seed emergence occurred in winter suggests that the adult phase is completely absent during this part of the year, at least in the Eastern Cape. The sudden resurgence of populations under favourable conditions is not likely to be the result of die-out and recolonization; cecidomyiids have a very short active dispersal range within their native host plant patch (several meters) with random wind dispersal to distant patches (several kilometers) (Briggs and Latto, 2000; Adair, 2005). The gall midge population must be maintained by immatures that survive the winter through diapause, either as a small number of galls or eggs undiscovered by parasitoids, or more likely as larvae in the leaf sheath (like the saddle gall midge *Haplodiplosis equestris*) or burrowed into grass stems without yet initiating galls (Nijveldt and Hulshoff, 1968). In KwaZulu-Natal, galls have been recorded overwintering in relatively warm refugia, where they may continue to drain plant resources at a low rate (Guy Sutton [CBC], personal communication). Seed midges presumably diapause as larvae in dropped seed in the leaf litter layer, as is the case for the sorghum midge *Stenodiplosis* (ex. *Contarinia*) *sorghicola* and wheat blossom midge *Sitodiplosis mosellana* (Jaquemin et al., 2014). These and other cecidomyiid pests of graminiferous crops in Africa, Europe and North America have a winter diapause period in their immature stages that is followed by *en masse* adult emergence in response to a combination of elevated temperatures and/or rainfall (Baxendale et al., 1984; Ogah et al., 2010; Jacquemin et al., 2014; Rowley et al., 2017). This also appears to be the case for the blister gall midge and seed gall midges in South Africa, suggesting that impacts on Guinea grass will be most apparent in summer, during the peak growing season of the plant, in both the native and introduced range (if released).

Seasonal abundances of the blister gall midge, its parasitoids, and the seed midges at the Port Alfred site were most correlated with monthly temperature and with rainfall in the current month, although peaks in abundance recorded in the summer of 2023 nonetheless occurred one to two months after a peak in rainfall. Research on stem gall midges on buffelgrass *Cenchrus ciliaris* L. in Kenya suggests that larvae remain dormant in the plant stem during the dry season (winter), then rapidly initiate galls with the onset of the wet season (summer); formation of fully-sized galls can take as little as four days in this case, but is probably dependent on the amount of rainfall (Aimee Gaitho [Mpala Research Station], personal communication). At summer-only survey sites in East London, however, the blister gall midge did not resurge with the onset of summer rains in 2023 (personal observation).

Like other cecidomyiids, gall midges and seed midges associated with Guinea grass are capable of rapid increases in population size, but suffer high rates of parasitism (Briggs and Latto, 2000). This implies that the insects' full population growth potential is not realized in the native range as long as this parasitoid pressure exists. There is evidence that gall-inducing herbivores can experience increased fitness in an introduced range as a result of escape from specialist parasitoids and inquilines (László et al., 2024). It is hoped that natural enemy release and under-utilization of African cecidomyiids by North American parasitoids will have this result, with accompanying benefits for their efficacy as biocontrol agents. Recruitment by native parasitoids is believed to a major factor in the poor performance of some cecidomyiid biocontrol agents, although recruitment does not always occur at levels that are significant for biocontrol, if at all (Hill and Hulley, 1995; Pratt et al., 2013). For example, there were no records of native parasitoids attacking *Lophodiplosis trifida* in Florida five years after release, and native parasitoid attack of *Zeuxodiplosis giardi* in South Africa was recorded at no more than 12 % more than twenty years after release (Hill and Hulley, 1995; Pratt et al., 2013). Recruitment can take several years (as with *Dasineura dielsi* after introduction from Australia to South Africa) and parasitism rates may be substantially lower than in the native range during the first few years after establishment (Adair, 2005). McFadyen (1985) reported parasitism rates of *Rhopalomyia californica* of under 10 % at 18 months after its release from California into Queensland, Australia, compared to almost 100 % in the native range, although Briggs and Latto (2000) recorded 80 % parasitism on average in the native range. Parasitism rates of Guinea grass gall midges and seed midges in South Africa also approach 100 % at some sites in the late summer, according to this study. North American cecidomyiid parasitoids are a similar assortment of taxa to those encountered in South Africa, dominated by the Platygastridae and Pteromalidae and including the Eulophidae and Eupelmidae (Calles Torrez et al., 2014).

Recruitment of biocontrol agents by parasitoids is related to the diversity of parasitoids in the agent’s native range and the presence of “ecological analogues” – related, native insects with similar feeding modes on the same or related plants (Paynter et al., 2010). The high diversity of parasitoids in the native range suggests that recruitment of introduced African cecidomyiids by some of these wasps is possible if they were released in Texas. The closest North American analogue to the African stem gall midges, however, is perhaps *Chilophaga virgati* Gagné, which is a leaf-sheath miner on *Panicum virgatum* L. and not a stem-galler (Calles Torrez et al., 2014); the risk of parasitoid recruitment may therefore be low. The risk may be higher for African seed midges, which have many analogues in the form of *Contarinia* species associated with North American grasses (https://www.gallformers.org/source/49), although none of these have been reported from Guinea grass in Texas (Colin Morrison [Utex], personal communication).

The formation of gall tissue by blister gall midge larvae is expected to be a resource sink on the plant, as with other gall-inducers (Dorchin et al., 2006). This could have a number of effects, including reduced panicle (seedhead) production or reduced number and viability of seed produced by panicles, leading to a direct reduction of reproductive output (Rhodes et al. 2022a), or reduced stem heights, leading to reduced seed dispersal range (Thomson et al., 2011; Mashau et al., 2021) and reduced flammable biomass contributing to wildfires (Rhodes et al., 2021). The primary impact of natural enemy loss on Guinea grass in Texas is increased panicle production, rather than increased vegetative spread, suggesting that reduction of reproductive output through biocontrol would be more easily achieved than reduction of biomass (Rhodes et al. 2022a). However, the blister gall midge may also form clusters of as many as ten galls on a single internode, and in these cases tillers have not produced a panicle, implying that impact on seed production may be dependent on agent density, as seen in other agents such as the weevil *Larinus latus* Herbst (Coleoptera: Curculionidae) on scotch thistle *Onopardum* spp. (Asteraceae) (Briese, 2000). Increased gall density and gall size of gall-inducers may increase the strength of the resource sink on the host plant sufficiently to have this effect on seed production, which may be possible under release from specialist parasitoids (Bagatto et al., 1996; László et al., 2024).

The impact of the seed midges is quantifiable in terms of seed destruction: complete seed midge development requires a presumably viable seed (one with a developing embyro and endocarp) and results in an inviable seed due to consumption of seed contents. Parasitism does not prevent seed destruction by the midges, since the host is apparently allowed to feed and develop to the pupal or pre-pupal stage before being consumed entirely. Therefore, the sum of adult midge and parasitoid emergences is a simple proxy for the number of viable seeds destroyed. Because each panicle produces several hundred seeds, the number of seeds destroyed per panicle may be very small – perhaps less than 1 %. Impacts may be much higher under conditions of release from parasitoids encountered by exported biocontrol agents, however. The seed midge *Spanolepis selloanae* Gagné on pampas grass *Cortaderia selloana* Asch. & Graebn., which is believed to have been introduced to Spain from South America along with its host plant, has acquired few parasitoids and is capable of destroying up to 87.5 % (on average three quarters) of the seeds produced by an individual plant (Fagúndez et al. 2021). For sorghum midge (an introduced African species) on Florida sorghum cultivars, percentage grain loss is almost directly proportional to the number of midges per panicle, and on Texas sorghum cultivars, as few as 2-15 midges per panicle may reach the threshold for economic damage (Wani, 1979; Prasad et al., 2021). Seed midge msp. 1 reaches this threshold even in its native range, where it is under heavy parasitism. These precedents suggest significant damage to Guinea grass seed output is possible. Even a relatively small order of reduction in seed output by a granivore may reduce eventual seedling recruitment below the threshold needed for establishment in new patches; this is because the majority of seeds will not land in a suitable microhabitat to germinate after dispersal, or will die because of harsh weather, disturbance or generalist granivores (Crawley, 1996). Guinea grass is likely to have a high threshold of seed output for recruitment because of low seed germination rates (Sukhchain and Sidhu, 1992) as well as a lack of directed seed dispersal and pre-dispersal seed destruction by several species of head smut fungi. This may make invasive Guinea grass populations very vulnerable to a granivorous biocontrol agent.

2.5. Conclusion

Guinea grass is associated with a diverse natural enemy assemblage in South Africa, including many herbivorous insects and their parasitoids, most of which appear to be undescribed species. The cecidomyiids, including several morphospecies of stem gall midges and seed midges, are the most diverse and abundant herbivorous insect group on the plant. Both gall and seed midges are endophagous and multivoltine, have an autumn-winter diapause followed by an eruptive emergence period triggered by spring-summer rains, and accumulate high parasitioid pressure by the late summer. Both candidate agents have the potential to be significantly damaging under reduced parasitoid pressure, which is likely given the strict biosanitary protocols employed in biocontrol and the lack of ecologically analagous cecidomyiids in North America.

Chapter 3: Field host range assessment of stem gall midges

3.1. Introduction

3.1.1. Risk evaluation in biocontrol

Biological control has a long history of effective implementation in the management of invasive weeds without significant negative economic or environmental consequences (Hinz et al., 2020). This is thanks to stringent risk evaluation and high standards of safety that must be met before candidate biological control agents agents are released (Hinz et al., 2020). Pre-release studies are essential to ensure that an agent is unlikely to cause damage to nontarget species in the country of introduction (Schaffner, 2001). Risk evaluation in biocontrol revolves mainly around an agent's host-specificity, usually defined as its tendency to utilize only one host or a narrow range of closely-related hosts (Marohasy, 1998).

Herbivorous insects may be monophagous (completing their lifecycle on only one plant species or subspecies) or oligophagous (utilizing multiple species within a wider taxonomic group such as a genus or subfamily) or polyphagous (utilizing multiple families of plants). Host specialization is the rule rather than the exception for herbivorous insects; it is thought that about half of all species only use host plants within a single genus, and that more than half use host plants within a single family (Jones et al., 2022). Although monophagy is less common than oligophagy, potentially hundreds of thousands of monophagous insect species exist (Novotny and Bassett, 2005; Jones et al., 2022). Polyphagy is rare in insects, possibly because the benefits of a wide host range are often outweighed by the high selective pressure on herbivores to escape competition by specializing on a host plant as an ecological niche (Jones et al., 2022). An insect need not be monophagous in its native range to be considered a suitably host-specific biocontrol agent, but must not use any valued nontarget species in its proposed range of introduction as a host (Schaffner, 2001; Hinz et al., 2014).

The selection of a list of plants that must be included in host-specificity testing typically follows the centrifugal phylogenetic method proposed by Wapshere (1974). Rather than testing all possible nontarget plant species of concern, the most closely related species are prioritized, followed by tests on fewer representatives of progressively more distantly related taxa (Lesieur et al., 2020). It is generally accepted that the closer the phylogenetic relationship of a nontarget species to the target species, the higher the risk of attack will be (Kelch and McClay, 2003). In the past, risk evaluation in biocontrol tended to focus only on ruling out risk to plants of economic importance, whereas modern risk evaluation is aimed instead at determining the host range of the agent and identifying native nontarget plant species that may be at greatest risk (Wapshere, 1989; McFadyen, 1998; Schaffner, 2001; Hinz et al., 2014).

3.1.2. Types of host-specificity tests

Herbivorous insects have a fundamental host range (the plant species they are physiologically capable of using for feeding or reproduction) and a realized host range (a smaller list of plant species they will actually develop on in the field) (Heard, 1999). Host selection in nature is often the result of a sequence of encounters with several potential hosts, which may range from high-ranked to low-ranked from the insect's perspective, mediated by sensory and chemical cues which may or may not be strongly linked to plant phylogeny (Marohasy, 1998; Jones et al., 2022). Host preference for an individual insect can vary depending on extrinsic factors such as weather conditions or the time since the last suitable host was encountered (Browne and Withers, 2002). Variation in host preference between conspecific individuals can also result from learned behaviour or intrinsic genetic variation (Heard, 1999; Haines et al., 2013).

Different kinds of host-specificity tests can provide more information about either the fundamental or the realized host range of an insect. No-choice tests, in which the candidate biocontrol agent is isolated on single plants of different species, are designed to assess the fundamental host range. If the insect is capable of using the offered host for feeding or reproduction, even if it is a low-ranked host, it is assumed that it will do so once it reaches a sufficient threshold of deprivation (Marohasy, 1998). The target plant species is used as a control. No-choice tests are the most conservative and most frequently used kind of host-specificity test (Hinz et al., 2014). Choice tests, in which several potential hosts are offered, are designed to provide information about the realized host range and host preference. The underlying assumption of choice tests is that a higher-ranked host should be more attractive to the insect than a lower-ranked host (Marohasy, 1998). Choice tests can vary widely in their design. Paired choice tests allow insects to choose between only two hosts, while multi-choice tests include three or more plant species. Sequential paired choice tests compare choice between successive pairs of hosts, with or without replacement of the individual insects tested (Marohasy, 1998). Insects exposed to the different test plants may be confined to small containers (Adair, 2005), caged plants (Paterson et al., 2019) or larger chambers that allow greater mobility, or allowed free reign in open-field tests (Briese, 1999). Test insects may be given access to the full range of sensory cues available from potential hosts, or a Y-tube may be used, allowing paired choice tests on the basis of olfactory cues alone (Heard, 1999; Cheng et al., 2020).

Laboratory tests have proven effective indicators of nontarget attack in the majority of modern biocontrol programs (Hinz et al., 2014). However, there are cases where laboratory tests may distort insect's natural responses by bypassing the typical sequence of cues involved in host finding, by introducing excitatory or repellent cues, or by introducing effects of associative learning, which may not be accounted for by the experimenters (Marohasy, 1998; Heard, 1999; Hinz et al., 2014). For example, a gravid female insect kept in a cage with a single plant species may initially refuse to oviposit, until it reaches a threshold of deprivation that forces it to release its eggs (Browne and Withers, 2002). Alternatively, the presence of attractive volatile chemicals from high-ranked host plants kept adjacent to the test plants in the laboratory may excite the female and cause indiscriminate oviposition. If the plant species are never utilized by the insect in the field, either result would be termed a *false positive* – suggesting risk when in fact there is none (Marohasy, 1998). Consider another case, in which cages from previous experiments are reused in subsequent experiments without being cleaned. If repellent volatiles from plants used in previous tests remain on the walls of the cages, this may inhibit feeding by an insect on some test plants. If these plants are in fact utilized by the insect in the field, this result would be termed a *false negative* – failing to identify an actual risk of nontarget attack (Marohasy, 1998). The results of poorly designed laboratory tests can lead to incorrect predictions of host use in the field (Hinz et al., 2014).

3.1.3. Field host range assessments

Field host range assessments involve surveys for the presence of the candidate biocontrol agent on plant species in its native distribution (Goolsby et al., 2006b). Although the environment is not experimentally manipulated in field assessments, they can be designed to parallel laboratory tests: the phylogenetic method can be used to prioritize the plant species to be surveyed; the presence of the target plant adjacent to nontarget plants can be used as a control; and the presence and relative abundance of the agent on different plants can be used to assess the realized host range and host preference (Yell et al., 2024). The benefit of field assessment is that the agent is allowed to express its natural host selection behaviour, arguably with more realistic results (in terms of realized host range) than laboratory tests, although field assessment alone may fail to reflect the fundamental host range (Marohasy, 1998; Goolsby et al., 2006b). Field assessment can also help assess whether limited nontarget attack is likely to occur in the presence of the target plant when the agent reaches high densities, an effect that is termed "spillover" (Hinz et al., 2020). Spillover is not typically cause for rejecting an agent as not being sufficiently host-specific, because the agent does not establish self-sustaining populations on the nontarget plant (Hinz et al., 2020). Since laboratory tests and field assessments both provide valuable information, it is good practice to integrate the two in pre-release studies (Goolsby et al., 2006b). Because field assessment does not involve the same logistical and financial difficulties of testing in quarantine and can eliminate unsuitable candidates early in the program, it is most effective when it precedes laboratory testing (Sutton et al., 2021b). However, in cases where the agent in question is difficult to test in a laboratory setting, the bulk of the information on host range must come from detailed field assessments (Sutton et al., 2021b).

Multiple species of stem-galling and seed-feeding Cecidomyiidae were found in native range surveys on Guinea grass *Megathyrsus maximus* in South Africa, and were selected as high-priority candidate biocontrol agents based on their expected host-specificity (Chapter 2). While the "short-form" biotype of Guinea grass is widely considered a noxious weed in Texas and much of the United States, the "tall-form" biotype is considered economically valuable by some stakeholders in the US and other countries, meaning that natural enemies specific to short-form Guinea grass should be prioritized (Rhodes et al., 2021; Gaskin et al., 2022). Collecting and rearing the stem-galling and seed-feeding cecidomyiids in sufficient numbers for laboratory host-specificity testing is a challenge because of their highly seasonal and short-lived adult phase, high rates of parasitism of larvae and pupae, and a lack of information on their larval development requirements (Chapter 2). Since both biotypes of Guinea grass and several of its closest relatives occur in sympatry in South Africa, field assessments for these taxa were determined to be the best option for an initial study of their host range. The results of these field host range assessments can be used to select a reduced list of candidate agents and test plants for future laboratory host-specificity testing.

3.1.4. Chapter aims

The aim of this chapter was to assess the realized host range of stem gall midges as candidate biocontrol agents for Guinea grass using records of host use in the field. Seed midges identified in natural enemy surveys (Chapter 2) are an unresolved species complex requiring genetic characterization for an adequate host range assessment, and are therefore treated separately in Chapter 4.

The host range of stem gall midges was assessed in surveys on Guinea grass and other native southern African grass species in the field, to to identify candidate agents with a realized host range that is limited to Guinea grass. On this basis, candidate agents were either rejected or recommended for further evaluation.

3.2. Methods

3.2.1. Field sites

Seven transect surveys were done in the two largest eastern coastal provinces of South Africa, the Eastern Cape (EC – three transects) and KwaZulu-Natal (KZN – four transects) from late March to late May 2023. Transects were roughly 100 km in length heading inland (generally north west) from the coast, along which an average of 5 sites (range 4 to 7) at least 20 km apart were selected for sampling. Transects were structured in this way to sample across a large area and a range of climatic gradients, reducing the risk of false negatives (failing to find non-target feeding due to environmental stochasticity). Additional targeted surveys at specific sites in 2023 and 2024 were included to improve spatial coverage, including a site in Mpumalanga province (MP), to a total of 61 Guinea grass sites (Fig. 3.1). The geographic coordinates and physical description (eg. roadside, plantation edge) of each site were recorded. Sites were selected on the criteria that each must contain a continuous patch of Guinea grass live growth at least 5 m^2 in size and at least 60 cm in height on average. These criteria were based on the assumption that large, dense, monospecific grass patches would be ideal habitat for cecidomyiids.

3.2.2. Host range surveys

Nontarget grass species were selected for sampling at field sites on the basis of the centrifugal phylogenetic method (Wapshere, 1989), prioritizing nontargets that are phylogenetically close to Guinea grass. Members of the Panicoideae subtribe Melinidinae, including the genera *Urochloa*, *Brachiaria* and *Melinis*, and *Batochloa deusta* Salariato & Zuloaga (ex. *Panicum deustum* Thunb.), were the first priority, followed by the genus *Panicum* (sensu stricto) in the Panicinae, the genera *Setaria* and *Cenchrus* in the Cenchrinae, and locally common representatives of more distant subtribes within and outside of the subfamily Panicoideae, such as *Adenochloa* (Panicoideae: Paniceae), *Digitiaria* (Panicoideae: Anthephorinae) and *Eragrostis* (Chloridoideae: Eragrostidinae) (Morrone et al., 2013; Delfini et al., 2023) (Fig. 3.2). Nontargets occurring adjacent to or within 500 m of a Guinea grass patch were considered suitable to assess for spillover. Some important nontargets could not be found in the same habitat as Guinea grass and were sampled separately at an additional 15 field sites, but could not be used to assess spillover.

To assess the field host range of stem-galling taxa, 200 tillers (individual, rooted reproductive units) of Guinea grass and the same number of tillers of one or more nontarget grasses (if both Guinea grass and nontargets were present and suitably abundant) were collected at each site. Tiller collections were separately bundled and labeled with a site code and the grass species

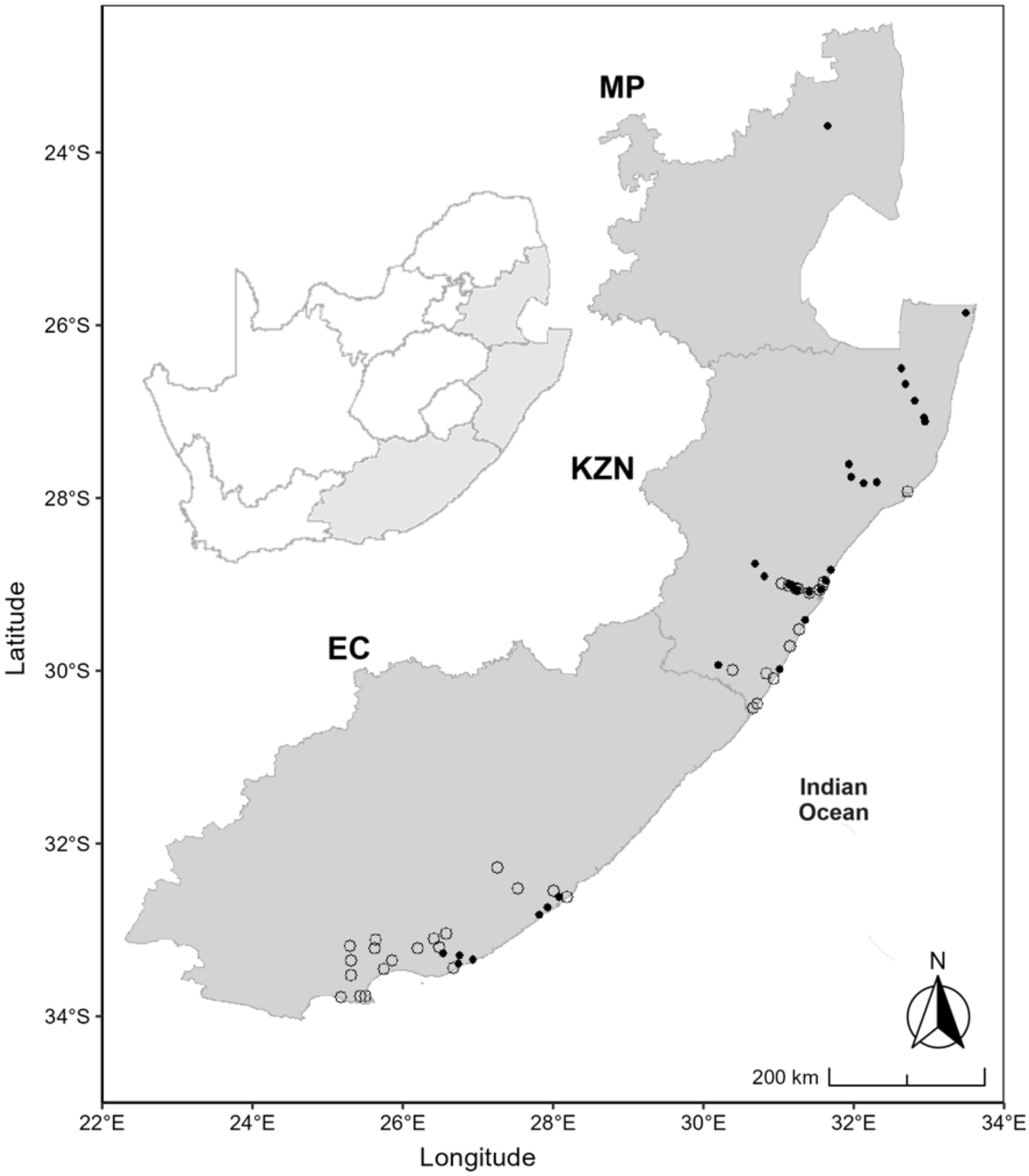

Figure 3.1. Map of field host range survey sites for stem gall midges. Sites where species were present are represented by filled circles, sites where absent by empty circles. Provinces sampled in South Africa (grey areas in country inset) were the Eastern Cape (EC), KwaZulu-Natal (KZN) and Mpumalanga (MP).

collected. If it was not possible to collect 200 tillers, as many suitably large tillers (at least 60 cm in length) as possible were collected and counted. Grass bundles collected in the field were examined within 12 hours.

The unique gall morphology of cecidomyiids on different host plants is a reliable indicator of the species of gall-inducer (Carneiro et al., 2009). Therefore, the distinct galls induced by each stem gall midge (Chapter 2) were used as an indicator of species presence and abundance. Both live galls (containing a larva or pupa) or old galls (wherein the gall-inducer had already pupated or been parasitized) were suitable, provided the galls were intact enough for a clear identification. Gall types that could not be assigned to a known species of gall-inducer were noted for further investigation.

Tillers were examined individually and the number of galls per tiller, if present, was counted. If no roots were collected, a stem with at least two nodes was counted as a tiller. Guinea grass tillers were separated from accidentally collected nontarget tillers, which were recorded by species and examined for galls but not included in the Guinea grass tiller count.

Galled stems were cut and placed in labelled plastic containers with wadded wet tissue paper to provide humidity, which is essential to encourage adult gall midge emergence (Impson et al., 2021). Emergences were monitored for 10-14 days after collection. Any emerging insects were separated by morphospecies and stored at 3-5 °C in vials of ethanol labelled by site code and grass species of origin.

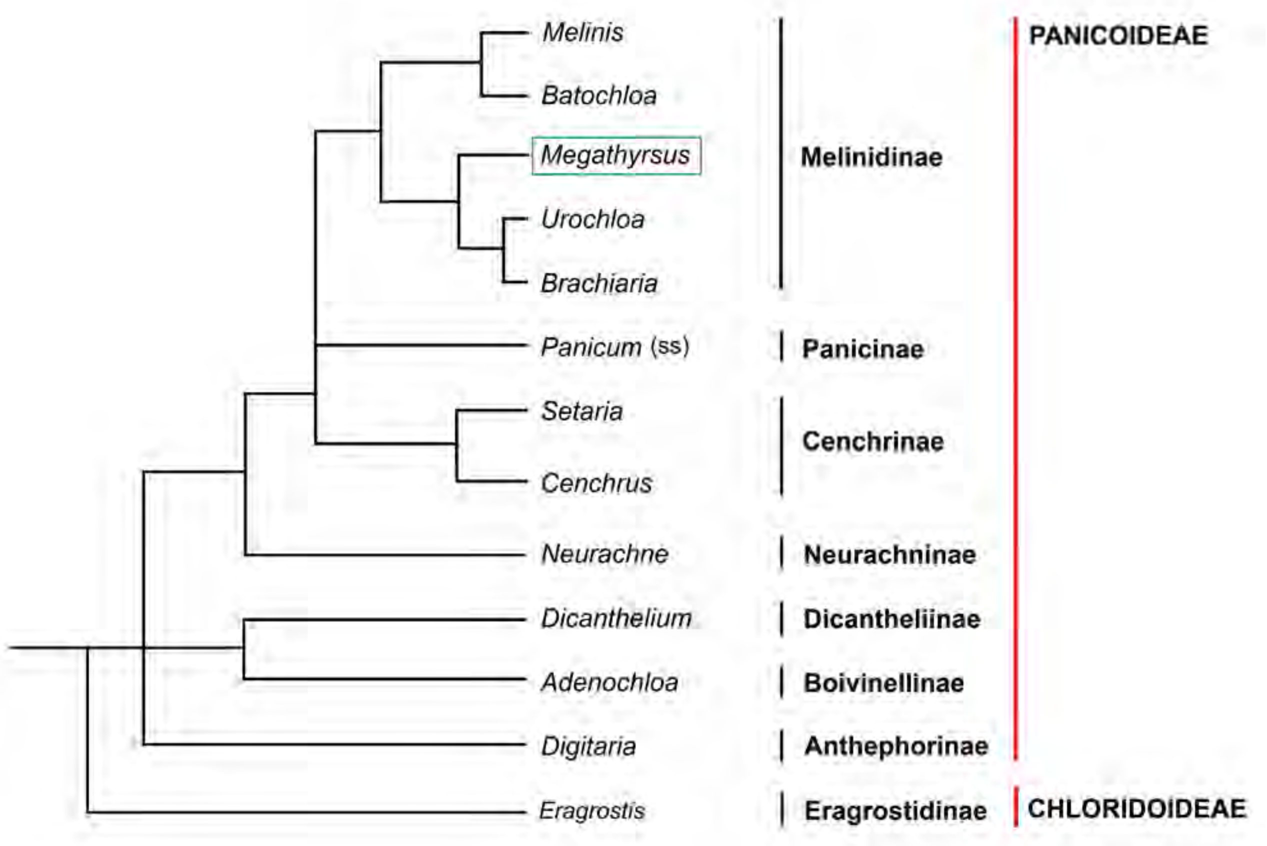

Figure 3.2. Phylogenetic scheme of some representative genera of subtribes in the grass subfamily Panicoideae and an outgroup subfamily (Chloridoideae), organized by relationship to the genus Megathyrsus (from Morrone et al., 2012 and Soreng et al., 2017). Branch lengths do not represent true phylogenetic distances.

3.3. Results

Over 12700 short-form Guinea grass tillers, 2500 tall-form Guinea grass tillers and over 25000 tillers from 29 nontarget grass species were examined (Table 3.1). Nontarget gall induction was recorded on three grass species (tall-form Guinea grass, *Batochloa deusta*, *Setaria sphacelata* Stapf & Hubb and *Hyparrhenia hirta* Stapf) at sites with short-form Guinea grass and on two grass species (tall-form Guinea grass and *B. deusta*) at sites without short-form Guinea grass (Table 3.2). Galls found on *H. hirta* (1 site) were determined to be an unknown species of gall-inducing wasp. Galls found on *S. sphacelata* (3 sites), the most-sampled nontarget after tall-form Guinea grass, were similar to “blister” galls but thicker-walled and more spherical. Like the majority of galls recorded in surveys, these were collected after the gall-inducer had already eclosed or been parasitized, and no stem gall midges have been reared from *S. sphacelata*. Gall morphology suggests that this is an unknown gall-inducing species, which is supported by cross-inoculations of the blister gall midge from Guinea grass on *Setaria* spp., which have not produced galls (Guy Sutton, unpublished data). Adult stem cecid emergences were only recorded from galls collected at three sites in the transect surveys (four and three female blister gall midges in KZN and EC respectively, and one female horn gall midge in KZN). The field host range findings for each stem gall midge candidate agent are described individually, below.

Arabukodiplosis vesicaria (blister gall midge)

The blister gall midge *A. vesicaria* was found on short-form Guinea grass, but not on tall-form Guinea grass or any nontarget species in the field, whether in sympatry or allopatry (Table 3.1, Table 3.2). At a mean 15.26 ± 0.75 galls per site, this was the most abundant gall-inducer species on any of the grasses sampled.

Horn gall midge

The horn gall midge utilizes both short-form and tall-form Guinea grass, and the related *Batochloa deusta*, in the field. Tall-form Guinea grass was the most heavily utilized host (6.53 ± 0.79 galls per site), followed by short-form Guinea grass (2.08 ± 0.15 galls per site) and *B. deusta* (1.6 ± 0.31 galls per site). Utilization of tall-form Guinea grass was similarly high at sites with sympatric short-form Guinea grass, whether with galls on the control (maximum 24 galls on the nontarget, at one site) or without (maximum 28 galls, mean 6.33 ± 1.16 galls per site) but was lowest at sites where short-form Guinea grass was absent (1.33 ± 0.77 galls per site). Utilization of *B. deusta* did not occur at sites where Guinea grass was absent (Table 3.1, Table 3.2).

Balloon gall midge

The balloon gall midge was recorded on short-form Guinea grass at four sites in KwaZulu-Natal. At a mean 0.23 ± 0.3 galls per site, this was the least abundant of the stem gall midges studied (Table 3.1, Table 3.2).

Table 3.1. Summary of field host range survey results for stem gall-inducing insects on biotypes of the target grass species *Megathyrsus maximus* and its phylogenetic relatives. Number of sites, tillers and mean and total gall abundances per insect species are indicated.

			Field host range of gall-inducing insects (mean abundance per site ± SE). Total abundances indicated in brackets.			
Plant species	**Total sites**	**No. tillers collected**	*A. vesicaria* (Blister gall midge)	Horn gall midge	Balloon gall midge	Other gall-inducers
Target species (Poaceae: Panicoideae: Melinidinae)						
Megathyrsus maximus (short form)	61	12720	15.3 ± 0.8 (931)	2.1 ± 0.2 (127)	0.23 ± 0.3 (14)	0
Biotypes / subspecies						
Megathyrsus maximus (tall form)	13	2500	0	6.5 ± 0.8 (85)	0	0
Congeneric species						
None found	0	0	NA	NA	NA	NA
Other species within subtribe Melinidinae						
Batochloa deusta	10	1760	0	1.6 ± 0.3 (16)	0	0
Brachiaria brizantha	1	200	0	0	0	0
Melinis repens	11	2200	0	0	0	0
Urochloa serrata (= *Brachiaria arrecta*)	5	1000	0	0	0	0
Urochloa mosambicensis (= *U. trichopus*)	10	2000	0	0	0	0
Urochloa panicoides	2	400	0	0	0	0
Species from other subtribes within subfamily Panicoideae						
Adenochloa ecklonii	2	400	0	0	0	0
Cenchrus ciliaris	9	1800	0	0	0	0
Cenchrus clandestinus	1	200	0	0	0	0
Panicum coloratum	3	600	0	0	0	0
Panicum schinzii	1	200	0	0	0	0
Panicum subalbidum	3	600	0	0	0	0
Paspalum urvillei	1	100	0	0	0	0
Setaria incrassata	5	1000	0	0	0	0
Setaria megaphylla	10	2000	0	0	0	0
Setaria sphacelata	17	3400	0	0	0	3 ± 0.3 (57)
Sorghum halepense	4	800	0	0	0	0
Species outside subfamily (non-Panicoideae)						
Andropogon gayanus	3	600	0	0	0	0
Chloris gayana	2	400	0	0	0	0
Cynodon dactylon	2	400	0	0	0	0
Digitaria natalensis	4	800	0	0	0	0
Digitaria sp.	1	200	0	0	0	0
Eragrostis curvula	5	1070	0	0	0	0
Eragrostis superba	2	400	0	0	0	0
Hyparrhenia hirta	3	340	0	0	0	0.05 ± 0.01 (3)
Hyperthelia dissoluta	2	400	0	0	0	0
Sporobolus africanus	2	420	0	0	0	0
Sporobolus pyramidalis	7	1200	0	0	0	0
Themeda triandra	2	400	0	0	0	0

Table 3.2 (A-C). Field host range survey results for three stem gall midge candidate agents on biotypes of the target grass species Megathyrsus maximus and its phylogenetic relatives. Per insect and plant species, the proportion of sites where galls were recorded and mean and total gall abundances are broken down by sites with conspecific galls recorded on sympatric short-form *M. maximus* (control sites), sites with conspecific galls not recorded on short-form *M. maximus*, and sites where short-form *M. maximus* was absent.

A. *Arabukodiplosis vesicaria* (Blister gall midge) field host range

Plant species	Total sites	Sites with galls on short *M. maximus*			Sites without galls on short *M. maximus*			Sites with short *M. maximus* absent		
		No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)
Target species (Poaceae: Panicoideae: Melinidinae)										
Megathyrsus maximus (short form)	61	32	0.52	15.26 ± 0.75 (931)	29	NA	NA	NA	NA	NA
Biotypes / subspecies										
Megathyrsus maximus (tall form)	13	1	0	0	9	0	0	3	0	0
Congeneric species										
None found	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae										
Batochloa deusta	10	5	0	0	4	0	0	1	0	0
Brachiaria brizantha	1	1	0	0	0	0	0	0	0	0
Melinis repens	11	3	0	0	7	0	0	1	0	0
Urochloa serrata (= *Brachiaria arrecta*)	5	1	0	0	1	0	0	3	0	0
Urochloa mosambicensis (= *U. trichopus*)	10	1	0	0	6	0	0	3	0	0
Urochloa panicoides	2	1	0	0	1	0	0	0	0	0
Species from other subtribes within subfamily Panicoideae										
Adenochloa ecklonii	2	0	0	0	0	0	0	2	0	0
Cenchrus ciliaris	9	0	0	0	1	0	0	8	0	0
Cenchrus clandestinus	1	1	0	0	0	0	0	0	0	0
Panicum coloratum	3	0	0	0	1	0	0	1	0	0
Panicum schinzii	1	0	0	0	0	0	0	1	0	0
Panicum subalbidum	3	1	0	0	1	0	0	1	0	0
Paspalum urvillei	1	0	0	0	1	0	0	0	0	0
Setaria incrassata	5	1	0	0	3	0	0	0	0	0
Setaria megaphylla	10	1	0	0	4	0	0	5	0	0
Setaria sphacelata	17	0	0	0	14	0	0	3	0	0
Sorghum halepense	4	1	0	0	3	0	0	0	0	0
Species outside subfamily (non-Panicoideae)										
Andropogon gayanus	3	0	0	0	3	0	0	0	0	0
Chloris gayana	2	0	0	0	2	0	0	0	0	0
Cynodon dactylon	2	1	0	0	1	0	0	0	0	0
Digitaria natalensis	4	2	0	0	2	0	0	0	0	0
Digitaria sp.	1	1	0	0	1	0	0	0	0	0
Eragrostis curvula	5	2	0	0	3	0	0	0	0	0
Eragrostis superba	2	1	0	0	1	0	0	0	0	0
Hyparrhenia hirta	3	1	0	0	2	0	0	0	0	0
Hyperthelia dissoluta	2	1	0	0	1	0	0	0	0	0
Sporobolus africanus	2	1	0	0	0	0	0	1	0	0
Sporobolus pyramidalis	7	2	0	0	3	0	0	2	0	0
Themeda triandra	2	2	0	0	0	0	0	0	0	0

B. Horn gall midge field host range

Plant species	Total sites	With galls on short *M. maximus*			Without galls on short *M. maximus*			Sites with short *M. maximus* absent		
		No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)
Target species (Poaceae: Panicoideae: Melinidinae)										
Megathyrsus maximus (short form)	61	32	0.25	2.08 ± 0.15 (127)	29	NA	NA	NA	NA	NA
Biotypes / subspecies										
Megathyrsus maximus (tall form)	13	1	1	24 ± 0 (24)	9	0.44	6.33 ± 1.16 (57)	3	0.33	1.33 ± 0.77 (4)
Congeneric species										
None found	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae										
Batochloa deusta	10	1	1	3 ± 0 (3)	8	0.38	1.63 ± 0.43 (13)	1	0	0
Brachiaria brizantha	1	0	0	0	1	0	0	0	0	0
Melinis repens	11	0	0	0	10	0	0	1	0	0
Urochloa serrata (= *Brachiaria arrecta*)	5	0	0	0	2	0	0	3	0	0
Urochloa mosambicensis (= *U. trichopus*)	10	0	0	0	7	0	0	3	0	0
Urochloa panicoides	2	0	0	0	2	0	0	0	0	0
Species from other subtribes within subfamily Panicoideae										
Adenochloa ecklonii	1	0	0	0	0	0	0	2	0	0
Cenchrus ciliaris	9	0	0	0	1	0	0	8	0	0
Cenchrus clandestinus	1	0	0	0	1	0	0	0	0	0
Panicum coloratum	3	0	0	0	1	0	0	1	0	0
Panicum schinzii	1	0	0	0	0	0	0	1	0	0
Panicum subalbidum	3	0	0	0	2	0	0	1	0	0
Paspalum urvillei	1	0	0	0	1	0	0	0	0	0
Setaria incrassata	5	1	0	0	3	0	0	0	0	0
Setaria megaphylla	10	0	0	0	5	0	0	5	0	0
Setaria sphacelata	17	0	0	0	14	0	0	3	0	0
Sorghum halepense	4	1	0	0	3	0	0	0	0	0
Species outside subfamily (non-Panicoideae)										
Andropogon gayanus	3	0	0	0	3	0	0	0	0	0
Chloris gayana	2	0	0	0	2	0	0	0	0	0
Cynodon dactylon	2	0	0	0	2	0	0	0	0	0
Digitaria natalensis	3	0	0	0	4	0	0	0	0	0
Digitaria sp.	1	0	0	0	2	0	0	0	0	0
Eragrostis curvula	5	0	0	0	5	0	0	0	0	0
Eragrostis superba	2	0	0	0	2	0	0	0	0	0
Hyparrhenia hirta	3	1	0	0	2	0	0	0	0	0
Hyperthelia dissoluta	2	0	0	0	2	0	0	0	0	0
Sporobolus africanus	2	0	0	0	1	0	0	1	0	0
Sporobolus pyramidalis	7	0	0	0	5	0	0	2	0	0
Themeda triandra	2	1	0	0	1	0	0	0	0	0

C. Balloon gall midge field host range

Plant species	Total sites	With galls on short M. maximus			Without galls on short M. maximus			Sites with short M. maximus absent		
		No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)	No. sites	Prop. sites galls recorded	Mean gall abundance ± SE (total)
Target species (Poaceae: Panicoideae: Melinidinae)										
Megathyrsus maximus (short form)	61	32	0.13	0.23 ± 0.3 (14)	29	NA	NA	NA	NA	NA
Biotypes / subspecies										
Megathyrsus maximus (tall form)	13	0	0	0	10	0	0	3	0	0

Congeneric species										
None found	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae										
Batochloa deusta	10	0	0	0	9	0	0	1	0	0
Brachiaria brizantha	1	0	0	0	0	0	0	0	0	0
Melinis repens	11	0	0	0	10	0	0	1	0	0
Urochloa serrata (= *Brachiaria arrecta*)	5	0	0	0	2	0	0	3	0	0
Urochloa mosambicensis (= *U. trichopus*)	10	0	0	0	7	0	0	3	0	0
Urochloa panicoides	2	0	0	0	2	0	0	0	0	0
Species from other subtribes within subfamily Panicoideae										
Adenochloa ecklonii	1	0	0	0	0	0	0	2	0	0
Cenchrus ciliaris	9	0	0	0	1	0	0	8	0	0
Cenchrus clandestinus	1	0	0	0	1	0	0	0	0	0
Panicum coloratum	3	0	0	0	1	0	0	1	0	0
Panicum schinzii	1	0	0	0	0	0	0	1	0	0
Panicum subalbidum	3	0	0	0	2	0	0	1	0	0
Paspalum urvillei	1	0	0	0	1	0	0	0	0	0
Setaria incrassata	4	1	0	0	3	0	0	0	0	0
Setaria megaphylla	4	0	0	0	5	0	0	5	0	0
Setaria sphacelata	7	0	0	0	14	0	0	3	0	0
Sorghum halepense	4	1	0	0	3	0	0	0	0	0
Species outside subfamily (non-Panicoideae)										
Andropogon gayanus	3	0	0	0	3	0	0	0	0	0
Chloris gayana	2	0	0	0	2	0	0	0	0	0
Cynodon dactylon	2	0	0	0	2	0	0	0	0	0
Digitaria natalensis	3	0	0	0	4	0	0	0	0	0
Digitaria sp.	2	0	0	0	2	0	0	0	0	0
Eragrostis curvula	5	0	0	0	5	0	0	0	0	0
Eragrostis superba	2	0	0	0	2	0	0	0	0	0
Hyparrhenia hirta	3	1	0	0	2	0	0	0	0	0
Hyperthelia dissoluta	2	0	0	0	2	0	0	0	0	0
Sporobolus africanus	2	0	0	0	1	0	0	1	0	0
Sporobolus pyramidalis	7	0	0	0	5	0	0	2	0	0
Themeda triandra	2	0	0	0	2	0	0	0	0	0

3.4. Discussion

This field host range assessment resulted in the prioritization of one candidate agent (the blister gall midge *A. vesicaria*), and the rejection of another candidate agent (the horn gall midge) due to nontarget gall induction. Field host range data suggest that the blister gall midge utilizes only short-form Guinea grass (*Megathyrsus maximus*) in the field. No non-target gall induction was recorded on tall-form Guinea grass or on members of the genera *Urochloa* or *Brachiaria* (the closest relatives to genus *Megathyrsus*) such as *Urochloa mosambicensis* Dandy, *Urochloa panicoides* P. Beauv or *Brachiaria brizantha* (syn. *Urochloa brizantha*) P. Beauv (Salariato et al., 2010). The blister gall midge was the most widespread of the gall-inducers, occurring at more than half of all sites and in all three provinces sampled. It is also the most abundant, with 200-300 galls recorded at some sites in the Eastern Cape. This may indicate that this species, as well as being highly host-specific in the field, has a relatively robust climate tolerance and the ability to reach high numbers despite high parasitism pressure (Chapter 2). These are desirable traits for a biocontrol agent, which must establish in an introduced range and become sufficiently abundant to exert control on the target plant (Egli and Olckers, 2015; Griffith et al., 2019). The balloon gall midge, a relatively rare associate of Guinea grass, also appears to utilize the short-form biotype exclusively but may not be suitably abundant to warrant its investigation as a biocontrol agent.

It is not uncommon for gall-inducing insects to specialize on biotypes, subspecies or hybrids of their host plants (Carneiro et al., 2009). In Belgium, the common reed *Phragmites australis* Trin. ex. Steud is utilized by three species of native gall midges with slightly different feeding niches, while in North America, the native gall midge *Calamomyia phragmites* is specific to the regional subspecies *Phragmites australis americanus* (Baetens and De Bruyn, 2011; Saltonstall et al., 2014; Blossey and Casagrande, 2016). The blister gall midge should proceed to laboratory host-specificity testing once its larval development requirements are better understood, to confirm that it is suitable for introduction as a biocontrol agent in Texas. Laboratory testing is essential to rule out candidates that may have a wider host range than predicted based on field surveys (Dhileepan et al., 2015; Reid et al., 2024). Should no-choice tests confirm the field host range results found for the blister gall midge in this study, this would support the release of this insect as a biocontrol agent for Guinea grass, and help to establish a precedent for the use of host-specific cecidomyiids as biocontrol agents of invasive grasses in future programs. The only cecidomyiid currently employed as a biocontrol agent for an invasive grass is the arundo leaf miner *Lasioptera donacis*, one of three biological control agents used for the control of *Arundo donax* in the United States and Mexico (Marshall et al., 2018a).

The horn gall midge was rejected because of nontarget gall induction on *Batochloa deusta,* whose closest relatives include grasses in the genera *Eriochloa* and *Rupichloa*, which include several species native to the American continent (Salariatio et al., 2009; Morrone et al., 2012). According to the phylogeny of the Melininidae and given the broad host range of the horn gall midge, any native North American species in these genera and in other genera more closely related to *Megathyrsus* would be at risk from an introduction of this insect to Texas – a list that includes but, is not limited to, southwestern cupgrass *Eriochloa acuminata* Kunth and Texas signalgrass *Urochloa texana* R.D. Webster (Morrone et al., 2012; Flora of North America, 2024). The fact that no gall induction by the horn gall midge on *Urochloa* spp. in South Africa was recorded is incongruous and suggests that other susceptible nontarget species may be found at other survey sites. Alternatively, this insect may only utilize melininid grasses in certain habitats that meet its developmental requirements, and these may be shared with Guinea grass but not with other related species, either as a result of spillover from Guinea grass or a coincidence of sympatry. The fact that utilization of *B. deusta* was not recorded at sites where Guinea grass was absent may support a spillover effect, although this was based on a single site only. Both Guinea grass and *B. deusta* are common in the moist undercanopy of lightly wooded areas, and the latter is almost exclusive to this habitat (Pieterse et al., 1997; Van Oudtshoorn, 2012). The horn gall midge was most abundant on tall-form Guinea grass, and utilized this biotype at sites where the short-form biotype was not present, suggesting that this is the preferred host.

The insects studied in these field host range surveys have overlapping geographic and ecological niches. In one instance, the blister, horn and balloon galls were all found at the same site, and in two instances, blister and horn galls were found on the same grass tiller, implying that these species do not have mutually exclusively habitat requirements, nor do they competitively exclude each other. Multiple cecidomyiid species may persist on the same host plant by specializing on different plant tissues (Joy and Crespi, 2007; Baetens and De Bruyn, 2011); in the case of the blister, horn and balloon gallers, these tissues are the internodes, nodes and basal shoots, respectively (Chapter 2). This suggests that additive impacts of stem gall midges on Guinea grass may be observable in the native range. With the plant facing resource sinks from the feeding of several herbivore species at multiple tissues or growth points, growth rates are expected to be reduced more than would be expected than by the feeding of a single species (Mnqeta and Paterson, 2019). Effects on the plant may even be be additive or even multiplicative (synergistic, with the agents facilitating each other), provided that the direct or indirect competition between agents is limited (Marlin et al., 2013, Stephens et al., 2013).

Gall-inducers collected in surveys in South Africa were more abundant and diverse in certain provinces. For instance, the blister gall midge is the only species recorded in the Eastern Cape and Mpumalanga, whereas the horn gall midge was dominant at some sites in KwaZulu-Natal, likely because of climate differences between the provinces. The geographic coordinate data collected during this survey provide a good basis on which to build models to characterize the habitat or climate niche of candidate agents, and to predict their likelihood of establishing on invasive Guinea grass in Texas. This modelling exercise is undertaken in Chapter 5.

Surveys were structured so that nontarget grasses growing adjacent to or near a Guinea grass patch were sampled primarily. This approach was intended to produce easily interpretable comparisons of gall occurrence on Guinea grass and other grasses at each site, in case nontarget attack was a result of spillover. In practice, large monospecific patches of nontarget grasses near or adjacent to large Guinea grass patches were rare or difficult to access (e.g. on private property or along busy highways). Roadsides were over-represented as sampling sites because of their ease of access and the tendency for Guinea grass to establish well in moderately disturbed to highly disturbed sites. A bias towards roadside sampling is common in biocontrol programs for tussock grasses and presents certain challenges, in that sensitive endophagous insect taxa on grasses tend to decline in abundance with repeated human disturbances – such as mowing and burning – that are typical of these sites (Sutton et al., 2023). The data may therefore under-represent the presence and abundance of gall midges, depending on the degree of disturbance at field sites. The study would have benefited from coordinating with private landowners and national parks to access and survey a greater number of sites within large, relatively undisturbed habitat as well as more disturbed sites.

This study used a field-based method for pre-release evaluation of candidate biocontrol agents that minimizes time consumption by prioritizing test plants based on phylogenetic similarity to target and basing candidate agent lists on host-specificity and plant damage in the field (Sutton et al., 2021b). This method assessed the risk of nontarget attack in the field for the most abundant insect taxa across a wide range of potential hosts~~,~~ using relatively low sampling effort, and could be applied to rapid field host range assessments of other endophagous insects on grasses in the future, especially if there are obstacles to laboratory testing.

3.5. Conclusion

The blister gall midge utilizes only short-form Guinea grass in the field and is recommended for further laboratory testing as a high-priority candidate agent.The horn gall midge utilizes both tall-form Guinea grass and *Batochloa deusta* and is therefore rejected as a candidate agent. Other stem-galling taxa were not widespread or abundant enough for an evaluation of their host range, implying that they would be of limited value as biocontrol agents. Laboratory no-choice host-specificity testing of the blister gall midge with southern African grasses (including many included in this study) and North American grasses are underway. The results of these laboratory tests can be compared to the field host range results of this study and used to optimize future field-based studies.

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Chapter 4: Field host range assessment and phylogenetic analysis of seed midges

4.1. Introduction

4.1.1. Importance of taxonomy in classical biocontrol

The unambiguous identification of an invasive species and its natural enemies is central to biological control, given the absolute requirement for biocontrol agents whose host plant associations are known and phylogenetically constrained (Smith et al., 2018). This requires the application of integrative taxonomy across multiple disciplines of biology. Integrative taxonomy uses molecular, morphological and ecological data to lay the boundaries that define living organisms as biologically distinct species (Padial et al., 2010). In the classical biocontrol of invasive plants, host-specificity assessments assume that the identities of the target plant and candidate biocontrol agents are well-defined prior to undertaking the study. This is not always the case, however, as a routine complication in biocontrol programs is the presence of cryptic species – entities that appear to be a single species but that are in fact a complex of multiple species or subspecies (Bickford et al., 2007; Hinz et al., 2020). Cryptic invasions of invasive plants introduce additional complications to biocontrol programs, and natural enemies being evaluated as biocontrol agents may themselves be a cryptic species complex, with implications for evaluating their host-specificity (Canavan et al., 2020; Van Steenderen et al., 2023).

Failing to apply integrative taxonomy of agents adequately in a biological control program in such a case may, in the worst-case scenario, result in the introduction of species or subspecies that are not suitably host-specific along with those that are. Another problematic result may be the introduction of species or subspecies that are not adequately suited for the control of the target plant in its invaded range (Paterson et al., 2016; Smith et al., 2018).

The program to control *Linaria* spp. (toadflaxes) (Plantaginaceae) in North America using various introduced European weevil species, for example, was plagued by such problems. The weevil *Mecinus janthinis* Germar was effective on *Linaria dalmatica* but produced dissappointing outcomes for *Linaria vulgaris*, while the weevil *Rhinusa antirrhini* Paykull proved difficult to establish on *L. dalmatica* (Toševski et al., 2018, 2023). The inclusion of molecular evidence revealed that weevil consignments had contained the previously unknown species *Mecinus janthiniformis* Tosevski & Caldara, which was much more effective at controlling *L. vulgaris* than *L. dalmatica*, and incorrectly identified specimens of the species *Rhinusa dieckmanii* Behne, which was specialized for a Macedonian genotype of *L. dalmatica* that does not match the genotype dominant in North America (Toševski et al., 2018, 2023).

Similarly, populations of *Eccritotarsus* spp. (Hemiptera: Miridae) imported as biocontrol agents of water hyacinth in South Africa from two localities in South America under the presumed identity of *Eccritotarsus catarinensis* Carvalho were later found to include a second, cryptic species now known as *Eccritotarsus eichhorniae* Henry, which fortunately appears to have a similar host range (Paterson et al., 2016; Henry, 2017).

The presence of cryptic species is especially problematic for the biocontrol of invasive grasses because their associated arthropod taxa are often poorly defined taxonomically. The development of biocontrol programs for invasive African grasses such as *Sporobolus pyramidalis* in Australia, for example, has proceeded slowly because the candidate agents – a species complex of undescribed *Tetramesa* wasps with varying host ranges – are difficult to distinguish morphologically (Van Steenderen et al., 2023). In such cases, phylogenetic and morphological data and additional lines of biological data (mating trials) or ecological data (field host range) have been used to infer the existence of separate species (Dorchin et al., 2009; Paterson et al., 2016; Van Steenderen et al., 2023). In the case of the grass-feeding *Tetramesa*, Van Steenderen et al. (2023) used correlations between phylogeny and host range to infer the existence of nine host-associated species, six of which had potential biocontrol applications.

4.1.2. Coevolution and host-associated species

Classical biocontrol of invasive plants is not possible without host-associated species of insects or other arthropods that serve as biocontrol agents. Host-associated differentiation and speciation is the phenomenon of parasitic organisms becoming ecologically and genetically distinct populations by specializing on different hosts (Stireman III et al., 2005). This phenomenon is widespread in herbivorous insects, and is the reason that insects are so often used as biocontrol agents (Samways, 1981; Schwarzländer et al., 2018). Insect-plant coevolution is believed to be responsible for the high number of monophagous or oligophagous herbivorous insect species (Vosteen et al., 2016). There is ample evidence that coevolution and host phylogeny govern host selection behaviour in European moths (Pearse and Altermatt, 2013), and the long history of success in risk evaluation of biocontrol agents based on host phylogeny certainly suggests that this applies to most, if not all, insect groups (Hinz et al., 2020). In some systems, phylogeny does not fully explain host selection; for example, plant volatiles are a better predictor than phylogeny of oviposition by moths on *Lygodium* ferns (Schizaeaceae) and perhaps on other plants (Wheeler et al., 2021; Simmons and Blossey, 2023). Herbivorous insects do not, of course, refer to phylogenies when choosing their hosts – all rely on some combination of chemical, visual and tactile cues from the plants they encounter (Heard, 1999) – but, since these are mostly a product of genetics, distinguishing between phenotype-based and phylogeny-based host selection is in many cases a “chicken or the egg” question, with some exceptions at genus level for certain arthropod species that feed on certain plant groups (Wheeler et al., 2021). Therefore, with both phylogenetic data and field host range data on an insect group, it is possible not only to infer the number of species or subspecies present, but also to make a reasonable prediction of their fundamental host range (Van Steenderen et al., 2023).

Host-associated differentiation and speciation is particularly common in the Cecidomyiidae, as shown by the number of host-associated species in this family (Stireman III et al., 2005; Gagné and Jaschof, 2021). Adaptive radiation of cecidomyiid species that utilize different hosts or a small number of related hosts has occurred on plants in several families, including the diverse Asteraceae, Aizoaceae, Fabaceae and Chenopodiaceae (Kolesik and Veenstra-Quah, 2008; Dorchin et al., 2009; Dorchin et al., 2015; Impson et al., 2021; Dorchin et al., 2022). In some cases, radiation on the same plant species matches that normally seen on a plant family; on creosote *Larrea tridentata* Waring & Price (Zygophyllaceae), there are 14 species of *Asphondylia* that have diverged from a single ancestor to occupy unique gall-inducing niches on different structures of the plant in sympatry, in different parts of the plant’s distribution, or at different times of year (Joy and Crespi, 2007). This ecological partitioning of hosts as a resource is both a result of competition and a means of relieving competition between ecologically similar species or subpopulations of a single species (Pfennig and Pfennig, 2012). This can be mediated by character displacement – such as the evolution of fidelity to different host plants (host-associated differentiation) seen in *Dasyneuriola* spp. on *Suaeda* shrub species (Chenopodiaceae) and *Dasineura folliculi* Felt subpopulations on *Solidago* species (goldenrods) (Asteraceae), which may be mediated by sensitivity to particular ratios of plant volatiles (Dorchin et al., 2006; Pfennig and Pfennig, 2012; Danon et al., 2017; Molnár et al., 2018). In *Dasineura folliculi*, there is evidence that ecological partitioning has led to the formation of host-associated subspecies or ecotypes (prezygotic isolation) and to reproductive partitioning of these ecotypes (postzygotic isolation by assortative mating) (Dorchin et al., 2006). Reproductive partitioning is expected to follow character displacement because of the need to preserve characters that increase fitness within a subpopulation, and leads, eventually, to the formation of host-associated species (Pfennig and Pfennig, 2012). In the absence of mating data, reproductive partitioning can be inferred by genetic evidence of divergence (Dorchin et al., 2022; Van Steenderen et al., 2023).

4.1.3. Chapter aims

During field surveys for biocontrol agents for Guinea grass *Megathyrsus maximus*, an invasive species in the United States and other countries, several morphospecies of seed-feeding cecidomyiids were reared from grass panicles (Chapter 2). None of these are formally described species, and it is therefore not clear whether certain morphospecies represent one or more insect species, or whether these species each utilize multiple host grasses or a single host grass. Different seed midge species from different host grasses that may exist within each morphospecies are not currently distinguishable based on known morphogical characters (Fig. 4.1). Genetic characterization of the seed midge morphospecies is necessary to contextualize field host range results and to determine if any are suitably host-specific for use as biocontrol agents.

This chapter has the following components:

1) Field host range surveys were conducted for seed midges on Guinea grass and related grass species in South Africa.

2) To determine whether each seed midge morphospecies comprises one species with multiple host plants, or multiple specialist species, particularly on Guinea grass, phylogenies based on multiple genetic markers were compared with field host use records.

4.2. Methods

4.2.1. Field host range surveys

Field host range surveys for seed midges were conducted at identical sites along the same seven 100 km transects used to assess stem gall midge host range (Chapter 3). Additional targeted surveys were conducted using the same rationale for selection of field sites and nontarget grass species as the prior surveys, to a total of 50 Guinea grass sites (Fig. 4.2). Some important nontargets could not be found in the same habitat as Guinea grass and were sampled separately at additional field sites, but could not be used to assess spillover.

Bouquets of on average 15 panicles (range 10 to 20, depending on availability) from Guinea grass and one or more nontarget species were collected at each site, provided the grasses were in flower. These were cut and placed in two-litre plastic containers labelled by site code. Emergences were monitored for 10-14 days after collection. Any emerging insects were collected and stored at

3-5 °C in vials of ethanol labelled by site code and grass species of origin. Seed midges were identified to tribe (based on characters in Dorchin et al., 2017) and divided into morphospecies based on descriptions of the antennae, wings, genitalia and other morphological features, using males and females of each morphospecies where possible.

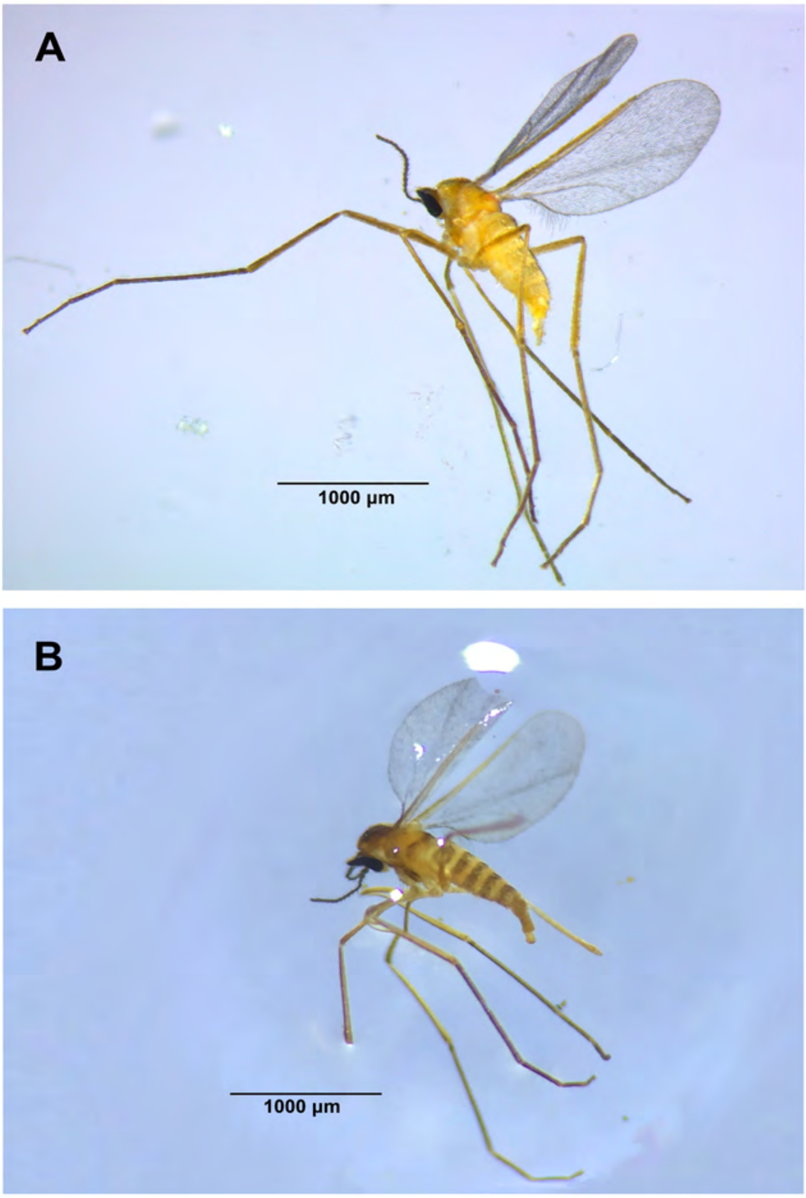

Figure 4.1. Seed midges of the same morphospecies (e.g. msp. 1, pictured) reared from seeds of *Megathyrsus maximus* (A) and *Setaria sphacelata* (B) are morphologically near-identical, but could be cryptic host-associated species. Both specimens are female.

4.2.2. Sequence collection and preparation

DNA was extracted from several individuals of each seed midge morphospecies (Msp. 1-4) from each host plant, and from several localities where possible, using the PureLink® DNA mini kit (Invitrogen®). Extraction began with manual and heat disruption of whole insect specimens (1 min mashing in 95 % ethanol followed by 8-10 hrs at 80 °C) and thereafter followed the kit manufacturer's protocol.

The genetic markers used in this study are the mitochondrial gene, cytochrome oxidase c subunit 1 (henceforth "Cox1") (Cameron, 2014) and a section of the 28S ribosomal RNA from the domains D2-D3 (henceforth "28S") (Hancock et al., 1988). Cox1 or CO1, the popular "barcoding gene", is a useful marker for measuring genetic divergence; it is generally not subject to meiotic recombination, and is highly conserved because it codes for an essential protein, but has a relatively high mutation rate over time frames relevant for speciation (Cameron, 2014; Folmer et al., 1994). 28S is a non-coding, functional RNA that forms part of the protein-synthesizing nuclear ribosome; it is subject to meiotic combination, and contains a combination of highly conserved and highly variable loci (Hancock et al., 1988). Cox1 is typically used for resolving species, while 28S is more useful for resolving genera and other taxonomic levels higher than species in several insect groups; accordingly, both markers have been used in large phylogenies of the Cecidomyiidae and other insects (Dorchin et al., 2019a; Van Steenderen et al., 2023).

Polymerase chain reaction (PCR) was used to amplify Cox1 using the "universal" invertebrate primers developed by Folmer et al. (1994) (LCO1490 [5'] GGT CA A CAA ATC ATA AAG ATA TTGG, HCO2198 [3']: TAA ACT TCA GGG TGA CCA AA A AAT CA) and 28S using the primers developed by Belshaw et al. (2001) (D2(3686) [5'] AGA GAG AGT TCA AGA GTA CGT G, D3R(4280) [3'] TAG TTC ACC ATC TTT CGG GTC). The PCR mixture consisted of 4 µL DNA extract, 2 µL of each of the forward and reverse primers, 12.5 µL Taq polymerase (Bio-Rad® iTaq SYBR green supermix) and 4.5 µL distilled water. The Cox1 protocol consisted of an initial denaturation stage at 95 °C (10 min), a 38-cycle denaturation-annealing-extension stage alternating between 95 °C (30 sec), 50 °C (1 min) and 72 °C (1 min), and a final extension stage at 72 °C (10 min) (Dorchin et al., 2009). The 28S protocol consisted of an initial denaturation stage at 94 °C (5 min), a 38-cycle denaturation-annealing-extension stage alternating between 94 °C (1 min) 55 °C (1 min) and 72 °C (1 min), and a final extension stage at 72 °C (10 min) (Van Steenderen et al., 2023). Both PCR protocols were automated with a Veriti thermal cycler (Fisher Scientific Applied Biosystems).

Sanger sequencing was done by Macrogen Europe. Chromatograms were inspected in Chromas 2.6.6. and compared site by site. Duplicate or misread nucleotides resulting from long or overlapping peaks were deleted or corrected where appropriate. Ambiguous nucleotides at the ends of the sequences were deleted. Sequences with fewer than 200 unambiguous nucleotides were discarded, which led to Msp. 4 being excluded from the analysis; this morphospecies is known from only a single collection, and the specimens may have degraded in storage. MEGA 11.0.13. (Tamura et al., 2021) was used to align sequences using the ClustalW algorithm, determine the most appropriate models of molecular evolution by maximum parsimony (AIC), estimate gamma parameters (used to describe the slope that approximates the variation in the rate of nucleotide substitutions across sites), calculate pairwise distances (p-distances), and to calculate within-group and between-group distances for each morphospecies and representatives from the two major divisions of the Cecidomyiinae (Lasiopteridi and Cecidomyiidi) and outgroups.

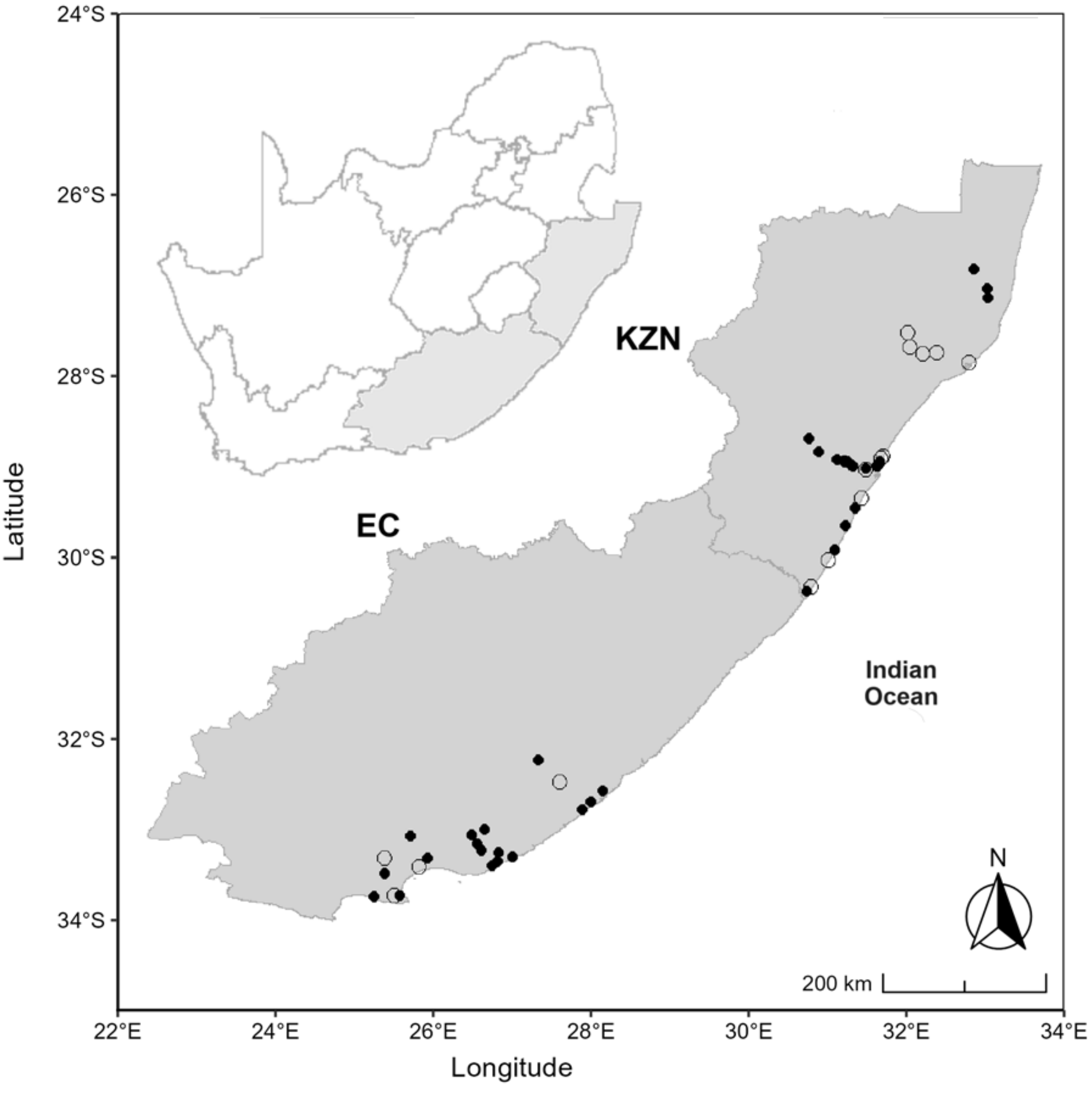

Figure 4.2. Map of field host range survey sites for seed midges. Sites where morphospecies were present are represented by filled circles, sites where absent by empty circles. Provinces sampled in South Africa (grey areas in country inset) were the Eastern Cape (EC) and KwaZulu-Natal (KZN).

4.2.3. Phylogenies

Phylogenies, or evolutionary trees, present the relationships between genetic sequences as a network of interconnected branches, where branch length represents divergence (Catanzaro, 2009). They are based on molecular evolution models that use assumptions about nucleotide substitution (e.g. the rate of transitions between the purine nucleotides, adenine and guanine, versus the rate of tranversions from purine to pyrimidine, e.g. adenine to thymine) to estimate when and where the sequences diverged with reference to a hypothetical common ancestor (Tamura and Nei, 1993). An information-theoretic approach such as AIC can be taken to select the most appropriate model, although this should also be informed by prior knowledge about a given gene, such as its average mutation rate and pyrimidine-purine ratios (Buckley et al., 2002; Cameron, 2014). A popular method for building phylogenies is Bayesian inference (Buckley et al., 2002, Dorchin et al., 2019a). Using this method, an initial tree is built (either randomly or using a neighbour-joining algorithm based on p-distances) and a Markov Chain Monte Carlo (MCMC) process is followed, whereby the tree is randomly modified and modifications that improve Bayesian posterior probabilities (using a prior specified molecular evolution model) are retained, until a maximum posterior probability is reached (Rzhetsky and Nei, 1993, Yang and Rannala, 1997; Huelsenbeck and Ronquist, 2001). This process is normally run with multiple interchangeable Markov chains for several million generations, with a fraction of error-prone early-generation trees excluded from sampling (called "burn-in"), to ensure the tree converges on the optimal phylogeny (Huelsenbeck and Ronquist, 2001; Dorchin et al., 2019a; Van Steenderen et al., 2023).

Preliminary pairwise distance comparisons of the undescribed morphospecies with other cecidomyiids were done using sequences sourced from the phylogeny by Dorchin et al. (2019a) and other authors on Genbank (www.ncbi.nlm.nih.gov/genbank; Table S2). The Cox1 phylogeny included representatives of closely-related genera to each morphospecies (mainly those likely to be in the same subfamily, judging by p-distances and morphology) to resolve species-level and genus-level placements. Grass-feeding taxa were prioritized, and included *Lasioptera arundinis* Schiner and *Calamomyia phragmites* Felt (both from *Phragmites australis*), *Stenodiplosis bromicola* Marikovskij & Agafonova (from *Bromus* spp.), *S. sorghicola* (from *Sorghum* spp.), *Contarinia* spp. (genus contains several grass feeders, but represented by *C. loti* (De Greer) from *Lotus* spp. and *C. lycii* Debski from *Lycium* spp.), *Orseolia oryzivora* (from *Oryza* spp.), and the blister gall midge *Arabukodiplosis vesicaria* (from *Megathyrsus maximus*) (Gagné and Jaschof, 2021; Chapter 2) (Table S2). The tree was rooted with a non-Cecidomyiinae outgroup sequence (*Micromyini* sp., a fungivore; Dorchin et al., 2019a). The 28S phylogeny served partly to back up the results of the Cox1 phylogeny, but because of its better performance at higher taxonomic levels, its main purpose was to resolve the placement of the three morphospecies within the

Cecidomyiinae as a whole. For this purpose, the 28S tree had to be contextualized with representatives of the entire subfamily. Representative sequences were included for all major phytophagous subtribes (Alycaulini, Asphondylini, Cecidomyiini, Clinodiplosini, Dasineurini, and Lasiopterini), a carnivorous subtribe (Lestodiplosini), and non-Cecidomyiinae (Lemestriinae and Porricondylinae) as an outgroup.

Sequence alignments were used to create Bayesian inference phylogenies for each genetic marker in MrBayes (v3.2.7) (Ronquist et al., 2012). Both phylogenetic inferences were run with a standard "4by4" nucleotide substitution model. The models used were the General Time Reversible model (Yang, 1994) with gamma rate parameter of 0.53 and a proportion of invariable sites (GTR+G+I) for Cox1, and the Hasegawa-Kishino-Yano (1985) model with gamma rate parameter of 0.46 (HKY+G) for 28S, which was the closest available model to the model selected for 28S in MEGA (Tamura 3-parameter with gamma rates – Tamura, 1992). The Cox1 and 28S phylogenetic inferences were run with $4x10^7$ MCMC generations each, with two runs, four chains and 25 % burn-in. Phylogenetic inferences were run with and without the runtime-optimizing algorithm BEAGLE and results were inspected visually in case BEAGLE produced anomalous topologies (Ayres et al., 2019).

4.2.4. OTU-picking methods

OTU-picking methods, also called species delimitation analyses, apply statistical analyses to the information provided by phylogenies or pairwise distance matrices (numerical measures of divergence) to identify operational taxonomic units (OTUs) – the smallest possible genetic subdivisions supported by the evidence that, in theory, correspond to species (Luo et al., 2018). Popular OTU-picking methods include the Poisson Tree Process (PTP) models, which use branch lengths on a specified tree to pick OTUs based on Bayesian inference (BPTP) or maximum likelihood (PTPML), and the Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP) methods, which attempt to distinguish pairwise distances resulting from hypothetical intraspecific and interspecific processes to set a threshold distance for picking OTUs (Puillandre et al., 2012, 2021; Zhang et al., 2013). None of these methods rely on any prior assumption of taxonomic grouping. Threshold distances for picking OTUs can also be determined non-algorithmically, by quantifying the average pairwise distances between already-described, well-defined species and genera (Dorchin et al., 2022).

The ASAP, ABGD, BPTP and PTPML OTU-picking methods were applied by uploading Bayesian trees or distance matrices to their respective online servers (https://species.h-its.org/, https://bioinfo.mnhn.fr/abi/public/abgd/, https://bioinfo.mnhn.fr/abi/public/asap/). In the phylogeny of the cecidomyiid genus *Ruschiola*, the majority of species are separated by Cox1 distances of 5 %

or more (Dorchin et al., 2022); this was therefore chosen as a ground-truthed minimum threshold distance for this gene. Minimum threshold distances of 1.6 % to 2.2 % for the 28S gene were chosen by comparing untransformed pairwise distances within the genus *Stefaniola* from sequences included in the phylogeny by Dorchin et al. (2019a), with variance estimated by the bootstrap method with 1000 replicates.

4.3. Results

4.3.1. Field host range surveys

Over 600 short-form Guinea grass panicles, 40 tall-form Guinea grass panicles and roughly 400 panicles from 13 other nontarget grass species were collected. Flowering nontargets were found at 26 sites, 19 with short-form Guinea grass present. Seed midge morphospecies were reared from short-form *M. maximus* (Msp. 1-4), *Adenochloa ecklonii* Zuloaga et al. (2015) (ex. *Panicum ecklonii* Nees.) (Msp. 4), *Cenchrus ciliaris* L., *Melinis repens* Zizka (Msp. 1), *Batochloa deusta* (Msp. 1), *Panicum schinzii* Hack (Msp. unknown), *Setaria megaphylla* Dur. & Schinz (Msp. 1), *Setaria sphacelata* Stapf & Hubb. (Msp. 1) and *Urochloa mutica* T.Q. Nguyen (Msp. 2). Nontarget feeding of Msp. 1 on *S. sphacelata* was only recorded at sites where this morphospecies was also reared from sympatric Guinea grass, but was recorded both in and out of sympatry with the target plant for *S. megaphylla* – with similar results for Msp. 2 and Msp. 3 – and was only recorded out of sympatry for Msp. 1 on *B. deusta* (Table 4.1, Table 4.2). Most morphospecies could be identified as being either in supertribes Lasiopteridi (Msp. 1 and 4) or Cecidomyiidi (Msp. 2 and 3), the two major divisions of the subfamily Cecidomyiinae (Gagné and Jaschof, 2021) (Table 4.3). The small number of specimens reared from *P. schinzii* were too damaged to be assigned to morphospecies or to be sequenced. A described species, *Stenodiplosis* (ex. *Contarinia*) *sorghicola*, was also reared from *Sorghum halepense* (Baxendale et al., 1984).

4.3.2. Cox1 phylogeny

The Cox1 phylogeny resolved Msp. 1 into a clade with five subclades strictly associated with *M. maximus* (C5,7,35,36), *B. deusta* (C27,28), *S. megaphylla* (C4,12,13,15), *S. sphacelata* (C17,18,19,22,23,24) and *C. ciliaris* (C32,33,34), plus two additional subclades associated with *M. maximus* (C8) or *M. maximus* + *S. megaphylla* (C1,37) (Fig. 4.3). These seven subclades were supported as distinct units by all five OTU-picking methods.

Msp. 2 was resolved into a clade with at least five subclades, including strongly supported subclades on *M. maximus* (C9,10) and *S. megaphylla* (C40). One subclade formed a polytomy containing individuals from *U. mutica* (C29,30,31), *M. maximus* (C38) and *S. megaphylla* (C39), which species delimitation divided into distinct units on *U. mutica* and *M. maximus* + *S. megaphylla*, respectively. Msp. 2 also contained a possibly polyphyletic group consisting of individuals from *M. maximus* (C2,3) and *M. repens* (C26); the ASAP, ABGD and Pdist methods grouped these as a single unit, while the BPTP and PTPML methods divided each of the three individuals separately. C41 (*S. sphacelata*) was supported as a distinct unit by all methods except PTPML, which grouped it with C40 (*S. megaphylla*), and Pdist, which grouped it with three disparate subclades.

Msp. 3 was paraphyletic because of the inclusion of C42 (*M. maximus*), which was basal to the Msp. 2 clade. The remainder of Msp. 3 formed a single clade basal to the entire seed midge phylogeny and contained three strongly supported subclades, one on *S. megaphylla* (C43) and two on *S. sphacelata* (C21,25).

Average within-group versus between-group Cox1 p-distances were: 5.8 % vs. 10.9 % for Msp. 1 vs. other Lasiopteridi; 5.2 % vs. 12.6 % for Msp. 2 vs. other Cecidomyiidi; and 9.8 % vs. 11.7 % for Msp. 3 vs. other Cecidomyiidi (Table 4.4).

4.3.3. 28S phylogeny

The 28S phylogeny resolved Msp. 1 into single subclades within the Lasiopteridi associated with *M. maximus* (N1,6,7,8) and *B. deusta* (N27,28), but split individuals from *S. megaphylla*, *S. sphacelata* and *C. ciliaris* into separate subclades (Fig. 4.4). Msp. 2 and 3 were resolved into separate subclades in the Cecidomyiidi, and Msp. 2 was further resolved into subclades associated with each host plant. OTU-picking methods identified the three morphospecies as distinct (except Pdist 1.6 %, which did not split N25 and N29). Msp. 1 was generally identified as a single unit by all methods, except ASAP, ABGD and Pdist methods for N1, N6, N7 and N12. Msp 2 was identified as a single unit by ASAP and by ABGD (except N2 and N3, which were each identified as distinct units), and as separate units on *U. mutica* (N29-31) and other host grasses by other methods. Pdist 1.6 % identified ambiguous units for N1,2,6,25,26,29,30,31, whereas Pdist 2.2% performed similarly to algorithmic methods.

Average within-group versus between-group 28S p-distances were: 1.5 % vs. 8.6 % for Msp. 1 vs. other Lasiopteridi; 6.1 % vs. 7.8% for Msp. 2 vs. other Cecidomyiidi; and 2 % vs. 8.1 % for Msp. 3 vs. other Cecidomyiidi (Table 4.5).

Table 4.1. Summary of field host range survey results for seed-feeding midges on biotypes of the target grass species *Megathyrsus maximus* (short form) and its phylogenetic relatives. Proportion of sites where each morphospecies (Msp. 1,2,3,4) and uncategorized morphospecies (Other Msp.) were recorded are indicated.

Plant species	**Total sites**	**No. panicles collected**	**Field host range of seed midges (prop. sites recorded)**				
			Msp. 1	Msp. 2	Msp. 3	Msp. 4	Other Msp. (described / unidentifiable)
Target species (Poaceae: Panicoideae: Melinidinae)							
Megathyrsus maximus (short form)	50	> 600	0.7	0.08	0.04	0	0
Biotypes / subspecies							
Megathyrsus maximus (tall form)	2	40	0	0	0	0	0
Congeneric species							
None found	0	0	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae							
Batochloa deusta	3	> 50	0.33	0	0	0	0
Brachiaria brizantha	1	10	0	0	0	0	0
Melinis repens	2	> 50	0	1.0	0	0	0
Urochloa mosambicensis (= *U. trichopus*)	2	30	0	0	0	0	0
Urochloa mutica	1	15	0	1.0	0	0	0
Species from other subtribes within subfamily Panicoideae							
Adenochloa ecklonii	1	20	0	0	0	1.0	0
Cenchrus ciliaris	2	> 30	1.0	0	0	0	0
Panicum schinzii	1	12	0	0	0	0	1.0
Setaria incrassata	1	15	0	0	0	0	0
Setaria megaphylla	3	45	1.0	0.33	0.33	0	0
Setaria sphacelata	4	60	0.75	0.25	0	0	0
Sorghum halepense	2	45	0	0	0	0	0.5
Species outside subfamily (non-Panicoideae)							
Themeda triandra	1	10	0	0	0	0	0

Table 4.2 (A-D). Field host range survey results for four morphospecies of seed-feeding midges on biotypes of the target grass species *Megathyrsus maximus* and its phylogenetic relatives. Per insect morphospecies and plant species, results are broken down by sites with identical morphospecies recorded on sympatric short-form *M. maximus* (control sites), sites without identical morphospecies recorded on short-form *M. maximus*, and sites where short-form *M. maximus* was absent.

A. Seed midge morphospecies 1

Plant species	**Total sites**	**Sites with seed midges on short *M. maximus***		**Sites without seed midges on short *M. maximus***		**Sites with short *M. maximus* absent**	
		No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded
Target species (Poaceae: Panicoideae: Melinidinae)							
Megathyrsus maximus (short form)	50	35	0.7	NA	NA	NA	NA
Biotypes / subspecies							
Megathyrsus maximus (tall form)	2	0	0	2	0	0	0

Congeneric species							
None found	0	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae							
Batochloa deusta	3	0	0	2	0	1	1.0
Brachiaria brizantha	1	1	0	0	0	0	0
Melinis repens	2	0	0	0	0	0	0
Urochloa mosambicensis (= *U. trichopus*)	2	0	0	0	0	2	0
Urochloa mutica	1	0	0	0	0	1	0
Species from other subtribes within subfamily Panicoideae							
Adenochloa ecklonii	1	0	0	0	0	0	0
Cenchrus ciliaris	2	0	0	0	0	2	0.5
Panicum schinzii	1	0	0	0	0	0	0
Setaria incrassata	1	0	0	1	0	0	0
Setaria megaphylla	3	2	0.67	0	0	1	1.0
Setaria sphacelata	4	4	1.0	0	0	0	0
Sorghum halepense	2	0	0	0	0	0	0
Species outside subfamily (non-Panicoideae)							
Themeda triandra	1	0	0	0	0	1	0

B. Seed midge morphospecies 2

Plant species	**Total sites**	**Sites with seed midges on short *M. maximus***		**Sites without seed midges on short *M. maximus***		**Sites with short *M. maximus* absent**	
		No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded
Target species (Poaceae: Panicoideae: Melinidinae)							
Megathyrsus maximus (short form)	50	4	0.08	NA	NA	NA	NA
Biotypes / subspecies							
Megathyrsus maximus (tall form)	2	0	0	2	0	0	0
Congeneric species							
None found	0	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae							
Batochloa deusta	3	0	0	2	0	1	0
Brachiaria brizantha	1	1	0	0	0	0	0
Melinis repens	2	2	1.0	0	0	0	0
Urochloa mosambicensis (= *U. trichopus*)	2	0	0	0	0	2	0
Urochloa mutica	1	0	0	0	0	1	1.0
Species from other subtribes within subfamily Panicoideae							
Adenochloa ecklonii	1	0	0	0	0	0	0
Cenchrus ciliaris	2	0	0	0	0	2	0
Panicum schinzii	1	0	0	0	0	0	0
Setaria incrassata	1	0	0	1	0	0	0
Setaria megaphylla	3	2	0.5	0	0	1	0
Setaria sphacelata	4	4	0.33	0	0	0	0
Sorghum halepense	2	0	0	0	0	0	0
Species outside subfamily (non-Panicoideae)							
Themeda triandra	1	0	0	0	0	1	0

C. Seed midge morphospecies 3

Plant species	Total sites	Sites with seed midges on short ***M. maximus***		Sites without seed midges on short ***M. maximus***		Sites with short ***M. maximus*** absent	
		No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded
Target species (Poaceae: Panicoideae: Melinidinae)							
Megathyrsus maximus (short form)	50	2	0.04	NA	NA	NA	NA
Biotypes / subspecies							
Megathyrsus maximus (tall form)	2	0	0	2	0	0	0
Congeneric species							
None found	0	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae							
Batochloa deusta	3	0	0	2	0	1	0
Brachiaria brizantha	1	1	0	0	0	0	0
Melinis repens	2	0	0	0	0	0	0
Urochloa mosambicensis (= *U. trichopus*)	2	0	0	0	0	2	0
Urochloa mutica	1	0	0	0	0	1	0
Species from other subtribes within subfamily Panicoideae							
Adenochloa ecklonii	1	0	0	0	0	0	0
Cenchrus ciliaris	2	0	0	0	0	2	0.5
Panicum schinzii	1	0	0	0	0	0	0
Setaria incrassata	1	0	0	1	0	0	0
Setaria megaphylla	3	2	0.5	0	0	1	1.0
Setaria sphacelata	4	4	0.33	0	0	0	0
Sorghum halepense	2	0	0	0	0	0	0
Species outside subfamily (non-Panicoideae)							
Themeda triandra	1	0	0	0	0	1	0

D. Seed midge morphospecies 4

Plant species	Total sites	Sites with seed midges on short ***M. maximus***		Sites without seed midges on short ***M. maximus***		Sites with short ***M. maximus*** absent	
		No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded	No. sites	Prop. sites midges recorded
Target species (Poaceae: Panicoideae: Melinidinae)							
Megathyrsus maximus (short form)	50	0	0	NA	NA	NA	NA
Biotypes / subspecies							
Megathyrsus maximus (tall form)	2	0	0	2	0	0	0
Congeneric species							
None found	0	NA	NA	NA	NA	NA	NA
Other species within subtribe Melinidinae							
Batochloa deusta	3	0	0	2	0	1	0
Brachiaria brizantha	1	0	0	1	0.	0	0
Melinis repens	2	0	0	0	0.	0	0
Urochloa mosambicensis (= *U. trichopus*)	2	0	0	0	0	2	0
Urochloa mutica	1	0	0	0	0	1	0

Species from other subtribes within subfamily Panicoideae							
Adenochloa ecklonii	1	0	0	0	0	1	1
Cenchrus ciliaris	2	0	0	0	0	2	0
Panicum schinzii	1	0	0	0	0	0	0
Setaria incrassata	1	0	0	1	0	0	0
Setaria megaphylla	3	0	0	2	0	1	0
Setaria sphacelata	4	4	0	3	0	0	0
Sorghum halepense	2	0	0	0	0	0	0
Species outside subfamily (non-Panicoideae)							
Themeda triandra	1	0	0	0	0	1	0

Table 4.3. Summary of seed midge morphospecies, hosts, locality and other information.

Morphospecies	**Hosts**	**Localities**	**Comments**
	*Megathyrsus maximus* (short form + tall form), *Panicum deustum*, *Setaria megaphylla*, *Setaria sphacelata*, *Cenchrus ciliaris*	Various, incl. Port Alfred, Makhanda, East London (EC); Durban, Hluhluwe, Falaza, Mkuze (KZN); and Mpala, Kenya	Lasiopteridi; very abundant and widespread
	*Megathyrsus maximus* (short form), *Setaria megaphylla*, *Setaria sphacelata*, *Melinis repens*, *Urochloa mutica*	Various, incl. Port Alfred, Mount Pleasant, East London (EC); Durban, Port Shepstone, Hluhluwe, Mkuze (KZN)	Cecidomyiidi; abundant and widespread
	*Megathyrsus maximus* (short form), *Setaria megaphylla*, *Setaria sphacelata*	East London (EC); Durban, Hluhluwe (KZN)	Cecidomyiidi; rare
	*Adenochloa ecklonii*	Hluhluwe (KZN)	Lasiopteridi; known from one collection on host plant

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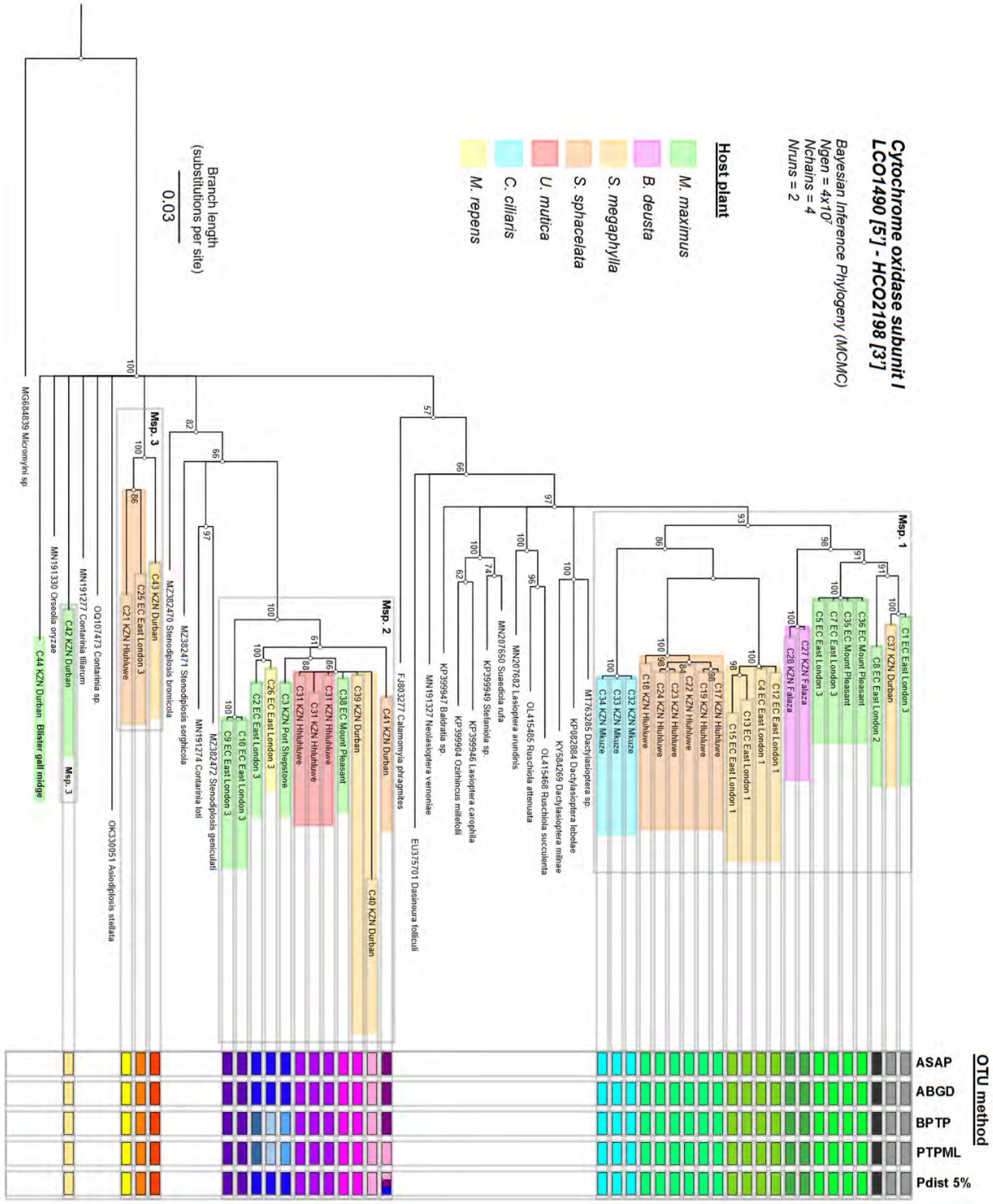


Figure 4.3. Cox1 Bayesian inference phylogeny for seed midge Msp. 1-3 with other representative taxa from the subfamily Cecidomyiinae and a non-Cecidomyiinae outgroup. Host range and results of OTU-picking methods for seed midge msp. are indicated by the corresponding legends (underlined), where colours correspond to host plant or OTU.

Table 4.4. Average within-group pairwise distances (A) and average between-group pairwise distances (B) for seed midge morphospecies 1-3, their respective tribes (Lasiopteridi and Cecidomyiidi) and the outgroup sequence (*Micromyini* sp.) on the Cox1 locus. Values are average p-distances with standard error (in brackets) estimated by 1000 bootstraps.

	A	**B**					
		1	**2**	**3**	**4**	**5**	**6**
1. Msp. 1	**0.058** (0.006)	0.0	(0.008)	(0.012)	(0.011)	(0.010)	(0.014)
2. Lasiopteridi	**0.127** (0.009)	**0.109**	0.0	(0.011)	(0.010)	(0.010)	(0.013)
3. Msp. 2	**0.052** (0.005)	**0.141**	**0.153**	0.0	(0.012)	(0.010)	(0.015)
4. Msp. 3	**0.098** (0.010)	**0.121**	**0.131**	**0.137**	0.0	(0.010)	(0.014)
5. Cecidomyiidi	**0.120** (0.009)	**0.133**	**0.145**	**0.126**	**0.117**	0.0	(0.014)
6. Outgroup	0.0	**0.188**	**0.198**	**0.200**	**0.171**	**0.196**	0.0

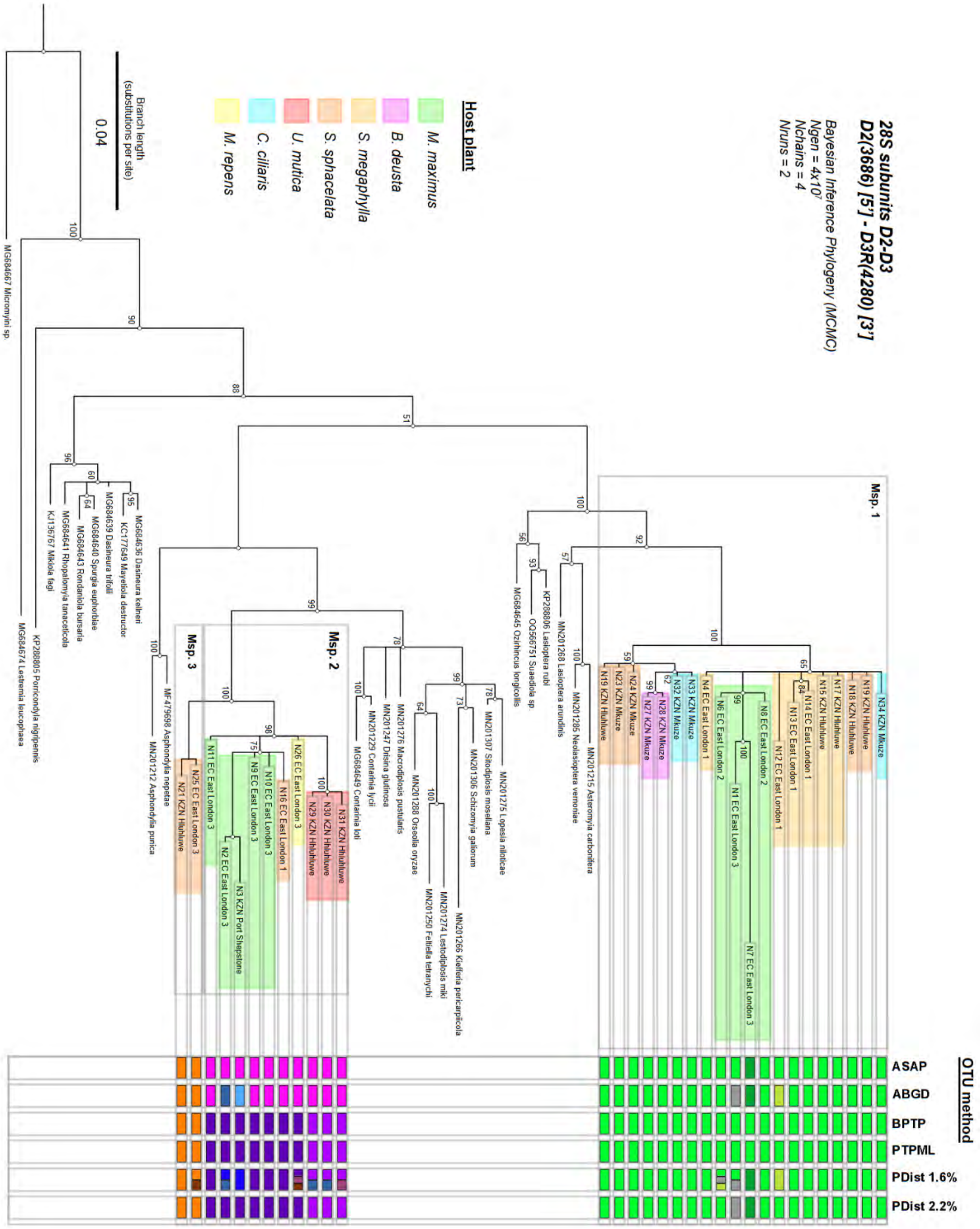


Figure 4.4. 28S Bayesian inference phylogeny for seed midge Msp. 1-3 with other representative taxa from the subfamily Cecidomyiinae and a non-Cecidomyiinae outgroup. Host range and results of OTU-picking methods for seed midge mspp. are indicated by the corresponding legends (underlined), where colours correspond to host plant or OTU.

Table 4.5. Average within-group pairwise distances (A) and average between-group pairwise distances (B) for seed midge morphospecies 1-3, their respective tribes (Lasiopteridi and Cecidomyiidi) and outgroup sequences on the 28S locus. Values are average p-distances with standard error (in brackets) estimated by 1000 bootstraps.

	A	**B**					
		1	**2**	**3**	**4**	**5**	**6**
1. Msp. 1	**0.0151** (0.013)	0.0	(0.0091)	(0.0093)	(0.0082)	(0.0155)	(0.0110)
2. Lasiopteridi	**0.076** (0.008)	**0.0857**	0.0	(0.0107)	(0.0114)	(0.0109)	(0.0108)
3. Msp. 2	**0.061** (0.007)	**0.0797**	**0.0955**	0.0	(0.0053)	(0.0100)	(0.0124)
4. Msp. 3	**0.020** (0.003)	**0.0667**	**0.0933**	**0.0305**	0.0	(0.0119)	(0.0123)
5. Cecidomyiidi	**0.015** (0.003)	**0.1517**	**0.1020**	**0.0782**	**0.0813**	0.0	(0.0139)
6. Outgroup	0.0	**0.1322**	**0.1424**	**0.1457**	**0.1351**	**0.1651**	0.0

4.4. Discussion

4.4.1. Phylogenies and OTUs

The Cox1 phylogeny and OTU-picking methods indicate that Msp. 1 midges on Guinea grass comprise at least two, perhaps three, subclades that may represent a species complex. One is only associated with *M. maximus*, a second is associated with both *M. maximus* and *S. megaphylla*, and a third is a singleton collected from *M. maximus*. The first two of these subclades show very low genetic divergence (< 1 %) between individuals separated by geographic distances of 150 km (*M. max.* subclade, Mt Pleasant - East London) to 450 km (*M. max.* / *S. meg.* subclade, East London - Durban), implying reproductive isolation within the subclade – i.e. biologically separate species, a hypothesis that is supported by OTU-picking methods. The high divergence of the singleton (collected in East London) from other *M. maximus* seed midges from the same area (> 5 %) also implies separation on the order of species rather than regional populations (Danon et al., 2017; Dorchin et al., 2022). The *B. deusta* subclade is a sibling to the *M. maximus* subclades and is also likely to be its own host-associated species, along with the subclades on *S. megaphylla* and *S. sphacelata*. There is evidence, albeit weaker, for other host-associated species within Msp. 2, particularly on *M. maximus* and *U. mutica*. The lack of Msp. 1 midges on *U. mutica*, one of the closest sampled relatives to Guinea grass, may be a result of undersampling.

As expected, the 28S phylogeny accurately represented the relationships between tribes, subtribes and genera for the Genbank sequences, but gave contradictory results to the Cox1 phylogeny regarding species-level relationships between the new seed midges. The broad OTUs identified with 28S do not correspond to species, but may be taken as evidence that each morphospecies makes up a single genus (Van Steenderen et al., 2023). The 28S phylogeny placed Msp. 1 in the Lasiopterini rather than the related Alycaulini, in agreement with morphology. Like many other Lasiopterini, the cerci on the ovipositor of Msp. 1 females are fused and bear hooked setae (Fig. 4.5 – Gagné and Jaschof, 2021), and there are no ventrolateral bulges above the palpal sheaths of the pupae, which were identified by Boe and Gagné (2011) as a common feature of grass-feeding Alycaulini. Phylogenetic evidence was still essential for accurate placement, given the sparse description of the Afrotropical representatives of this tribe (Dorchin et al., 2017). Phylogenies identified the nearest relatives to Msp. 2 and 3 as representatives of the genera *Contarinia and Stenodiplosis*, considered by some taxonomists to be synonymous, although *Contarinia* is a paraphyletic taxon (Dorchin et al., 2017; 2019). Shared morphological features and life history traits with species such as *Stenodiplosis* (ex. *Contarinia*) *sorghicola* and *Stenodiplosis spartinae* Gagné (both of which feed on the seeds of grasses as larvae) agree with this general placement. However, the paraphyly of *Contarinia* and the lack of 28S sequences for *Stenodiplosis* mean that the precise placement of Msp. 2 and 3 remains ambiguous.

Neither genetic marker suggests that Msp. 1 belongs to any previously sequenced genus, and specimens are not morphologically consistent with any described genus of Lasiopterini (Peter Kolesik [Cecidomyiidae taxonomist], personal communication). The sum of the evidence suggests that Msp. 1 belongs to a currently undescribed genus of Lasiopterini associated with the seeds of panicoid grasses. There are currently eight described species of Lasiopteridi that feed on grass seeds, but all are in tribe Dasineurini. These include eight *Dasineura* species from Europe and the recently described *Spanolepis selloanae* from *Cortaderia selloana* in Spain (Barnes 1939; Fagúndez et al. 2021). The undescribed genus in this study is the second genus of Lasiopteridi and the first of Lasiopterini known to feed on panicoid grasses (Gagné and Jaschof, 2021; Fagúndez et al., 2021). This genus and its constituent species will be formally described in a future publication.

4.4.2. Biocontrol implications

There is evidence for distinct lasiopterine seed midge host-associated species on Guinea grass and buffelgrass (*C. ciliaris*), both considered invasive plants in the United States (Rhodes et al., 2021). Lasiopterine seed midges reared from different Guinea grass and buffelgrass biotypes in Kenya may correspond to these putative species (Colin Morrison [UTex] and Aimee Gaitho [Mpala Research Station], personal communications). Seed midges (Msp. 1) have also been reared from tall-form Guinea grass in South Africa and Kenya outside of this study; although suitable specimens for genetic analysis were not available at the time this study was done, sequencing is planned (Guy Sutton [CBC] and Colin Morrison [UTex], personal communications). Given the existence of biotype-specific cecidomyiids such as *Calamomyia phragmites* (Baetens and De Bruyn, 2011), and evidence for biotype specificity of the blister gall midge in the field (Chapter 3), it is possible that seed midge species or ecotypes specific to the different biotypes of Guinea grass also exist. No-choice tests of the Guinea grass seed midges on tall-type Guinea grass and other alternative host grasses will, however, be needed to determine whether host association in the field translates to high host-specificity in the laboratory.

4.4.3. Evolutionary hypotheses

At least two separate radiations of seed midges on panicoid grasses appear to have occurred – one from a lasiopterine ancestor (Msp. 1) and one from a cecidomyiidid ancestor (Msp. 2 and 3). The pattern of radiation is most obvious within the Msp. 1 clade, where the genetic similarity of host-associated groups follows a gradient of genetic, structural and ecological similarity in the host plants (Fig. 4.6). The *B. deusta* host-associated group is the closest to the Guinea grass group, and *B. deusta* itself is the most similar structurally and genetically to Guinea grass of the sampled grasses that host lasiopterine seed midges (although cecidomyiidid seed midges do occur on the more closely related but structurally less similar *Urochloa mutica*, and lasiopterines may yet be found on this species). Both Guinea grass and *B. deusta* have wide, overlapping geographic ranges and often share the same habitat, under the canopies of trees in lightly wooded areas (Pieterse et al., 1997; Van Oudtshoorn, 2012). The racemous inflorescences of *S. megaphylla* and *U. mosambicensis* are the next most similar to that of *M. maximus*, while the spike-like inflorescences of *S. sphacelata* and *C. ciliaris* are the most dissimilar – although both *Setaria* species are shade-tolerant and therefore more likely to share habitat with *M. maximus*. The observation that the phylogeny of the Msp. 1 seed midges follows a gradient of phylogenetic, structural and habitat similarity in their host grasses suggests that the radiation of the ancestral seed midges on panicoid grasses has likely been driven by the evolution of host-finding ability. It is hypothesized here that different seed midge populations avoided competition for resources (seeds) by becoming progressively better at discriminating between sympatric host grasses. The need to preserve host-finding traits that conferred a fitness advantage (more offspring) would have led to the development of reproductive isolation mechanisms, whether behavioural, mechanical or pheromonal (there are no obvious visual display differences, such as wing markings, between the host-associated species of either sex). Resource limitation is implied in this hypothesis. This form of reproductive isolation is seen in several other species of cecidomyiids (Dorchin et al., 2009; Danon et al., 2017).

The percentage of seed set used by seed midges is relatively low at most sites at most times of the year, judging by phenology data on Guinea grass seed midges (Chapter 2). This argues against pure resource competition and suggests that another factor, the need to escape parasitoids, may be involved. The enemy-free space hypothesis states that when traits develop in a population that reduce predation on their offspring, and thereby increase fitness – such as the ability to use new habitats or new hosts that are relatively “enemy-free” – these traits are reinforced over time, leading to species divergence (Vosteen et al., 2016; Gratton and Walter, 2024). In this scenario, rates of parasitism on the ancestral seed midges were as high or higher than today, but most parasitoids used kairomones from a limited range of grasses for long-range host-finding (Degenhardt, 2009). Seed midge lineages able to overcome the fitness costs of shifting to a new host (in terms of host-finding, larval development and mate-finding challenges) were rewarded with reduced resource competition and predation, putting them at a net fitness advantage, and thus diverged quickly (Danon et al., 2017). Parasitoids would eventually evolve to exploit larvae on the new plant host, leading to an evolutionary arms race that would last as long as any relatively

Figure 4.5. Morphology of the adult female Guinea grass seed midge, Lasiopterini sp. "megathyrsae"; A) close-up of head and antennae; B) whole insect in profile, with body regions shown in close-up images indicated; C) close-up of last three abdominal tergites and ovipositor, partially eversed.

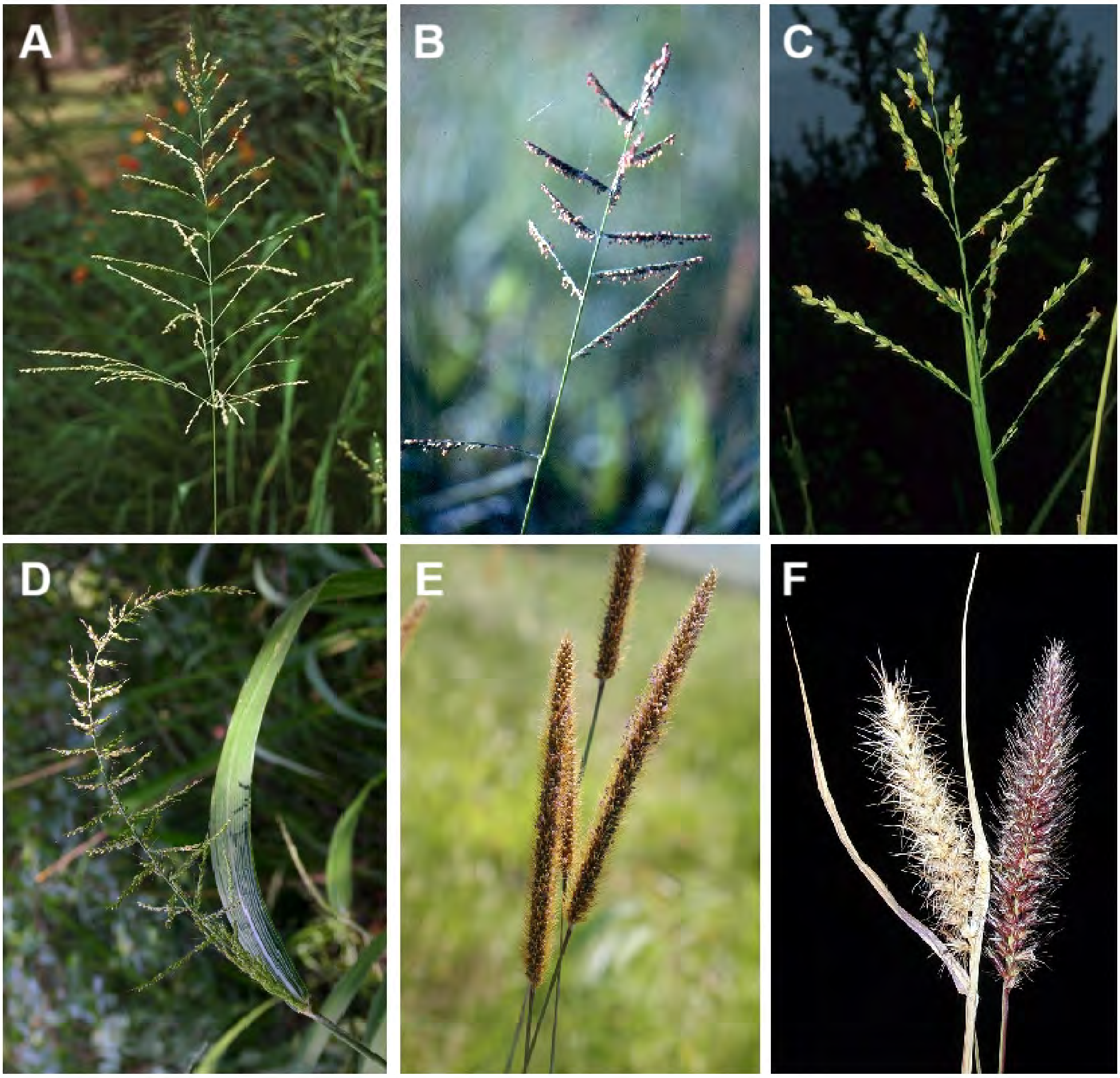


Figure 4.6. Inflorescences of the African panicoid grasses hosting seed midges, arranged in phylogenetic order relative to *Megathyrsus maximus*: A) *M. maximus*, B) *Urochloa mutica*, C) *Batochloa deusta (ex. Panicum deustum)*, D) *Setaria megaphylla*, E) *Setaria sphacelata*, F) *Cenchrus ciliaris*. (Credit: D. Sharp, Queensland Herbarium [A], Arthur Cameron, NTDPIR [B], biodiversityexplorer.info [C], B.T. Wursten, iNaturalist [D], Suvarna Parbhoo Mohan, iNaturalist [E], desertmuseum.org [F]).

unexploited host species remained. In this way, enemy escape could have been a co-driver or even the primary driver of seed midge radiation. There is evidence that this mechanism has played a role in the divergence of *Dasyneuriola* midges on parapatric species of *Suaeda* shrubs in Israel, and host-associated populations of the pea aphid *Acyrthosiphon sativum* (Vosteen et al., 2016; Danon et al., 2017).

Although co-evolution has produced a great diversity of host-specific insect species, there is no evidence that mutations have altered the fundamental host range of a biocontrol agent during the lifetime of any biocontrol program (Van Klinken and Edwards, 2002). Although unexpected feeding on nontarget hosts has been recorded in a small percentage of biocontrol programs, these have not been “host shifts”, but rather the result of a misestimation of the true host range or a misunderstanding of the phylogeny of the host species or the agent (Alonso-Zarazaga and Sánchez-Ruiz, 2002; Van Klinken and Edwards, 2002; Canavan et al., 2020; Marvaldi, 2024). Rapid evolution should therefore not be considered a risk to the safety of biocontrol; instead, an emphasis should be placed on the correct identification of the target plant and natural enemies and thorough host-specificity testing (Van Klinken and Edwards, 2002; Canavan et al., 2020; Hinz et al., 2024).

4.5. Conclusion

Phylogenetics and field host range data suggest that the lasiopterine midges reared from South African grass seeds are a complex of cryptic species. At least one species of seed midge – henceforth dubbed Lasiopterini sp. megathyrsae, the Guinea grass seed midge – appears to be specific to Guinea grass in the field, with a related cryptic species that also uses *Setaria megaphylla* where these grasses occur in sympatry. A final evaluation of Lasiopterini sp. megathyrsae requires laboratory host-specificity testing. A seed midge species that may be specific to buffelgrass (Lasiopterini sp. cenchrae) was also identified. Host-associated species of cecidomyiids on panicoid grasses have evolved multiple times, countering perceptions that grasses lack host-specific herbivores.

Chapter 5: Native range and invaded range ecological niche models for candidate agents

5.1. Introduction

5.1.1. Climate matching in classical biocontrol

Insects are ectothermic organisms with life cycles that are affected by temperature, rainfall and many other climate factors (Diaz et al., 2008; Hodkinson, 2005; Singh and Olckers, 2017). Different insect species vary widely in their climatic tolerances, which affects their geographic distributions. While some insects are climate generalists with broad distributions, others have specific climate requirements that lead to restricted distributons, which do not necessarily match the broad climate niche of their preferred hosts (Mukherjee et al., 2012; Cowie et al., 2023). This has implications for the biological control of invasive species. Biocontrol agents may range from effective to ineffective in different parts of the invaded range, or may fail to establish entirely, which has been attributed to climatic differences between the region of origin (the conditions under which the agents have evolved) and the region of introduction (the conditions to which they are forced to adapt) (Dennill and Gordon, 1990; Hill and Olckers, 2001; Goolsby et al., 2005; Cowie et al., 2023).

Climate mismatching is cited as the main factor in roughly a third of biocontrol programs where inconsistent outcomes or outright failure have been reported (Stiling, 1993; Harms et al., 2020). For example, the gall wasp *Trichilogaster acaciaelongifoliae* Froggatt (Hymenoptera: Pteromalidae), a biocontrol agent for *Acacia longifolia* Andrews (Fabaceae), established and performed well at cool coastal sites in South Africa that were similar to source regions in Australia, but either failed to establish or performed poorly at arid inland sites (Dennill and Gordon, 1990). For another set of wasps in the genera *Eretmocerus* and *Encarsia* (Hymenoptera: Aphelinidae) released as biocontrol agents of the silverleaf whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) in the western United States, one of the five *Eretmocerus* spp. and six of the seven *Encarsia* spp. introduced failed to establish because of similar climate incompatibility (Goolsby et al., 2005).

Testing, rearing and introducing insect agents that ultimately do not establish or are ineffective because of a predictable climate mismatch is a waste of time and resources (Ulrichs and Hopper, 2008). This can lead to biocontrol programs stalling, with severe consequences for ecosystems and people affected by invasive species. Ecological and climate modelling tools are increasingly being used to avoid such unfavourable outcomes in invasive species management by enabling biocontrol practitioners to make more informed decisions regarding where to collect and release agents (Robertson et al., 2008; Reid et al., 2020; Mukherjee et al., 2021).

Determining the climatic similarity between the distribution of a candidate agent and the distribution of the target species, and optimizing that similarity if possible, is called *climate matching*, and is an important step in predicting and maximizing the success of a biocontrol program before an agent is released (Robertson et al., 2008; Cowie et al., 2023). Climate matching is achieved with statistical models based on correlative data on the distribution and phenology of the species of interest in its native range or elsewhere, mechanistic data based on life history requirements as ascertained from laboratory rearing or field observations, or a combination of these (Kass et al., 2020; Sutton and Martin, 2022; Cowie et al., 2023).

5.1.2. Ecological niche models

Ecological niche models (ENMs), also called species distribution models (SDMs), are a broad class of models in ecological statistics that are designed to correlate the distribution of plant and animal species in space and time with abiotic or biotic determining factors (Elith and Leathwick, 2009; Elith et al., 2011; Kass et al., 2020). These correlations are used to generate testable hypotheses that may explain the known distributions of species and, increasingly, to predict the potential distributions of species in unsampled locations and times (Kéry, 2011). In ecological terms, the potential distribution of a species translates to its fundamental niche, which is the environmental space that a species should be able to occupy considering only factors such as climate suitability and the availability of habitat or food (Colwell and Rangel, 2009; Peterson and Soberón, 2012). ENMs have been used to predict the potential distributions of megafauna under various climate change scenarios in trophic rewilding projects (Jarvie and Svenning, 2018), of invasive plant species under eradication programmes (Cho et al., 2022), and of invasive vertebrates undergoing climate-induced range shifts (Elith et al., 2010; Istifanus et al., 2023). In the biological control of invasive plants, ENMs have been used as tools for identifying suitable parts of the native range to conduct surveys for climatically matched natural enemies (e.g. for Mexican waterlily *Nymphaea mexicana* Zuccarini – Reid et al., 2020), and predicting the establishment of candidate agents in their introduced range (e.g. the geometrid moths *Eueupithecia* spp. on *Parkinsonia aculeata* L. in Australia, and the cecidomyiid flower-galler *Dasineura rubiformis* on *Acacia mearnsii* in South Africa – Mukherjee et al., 2021; Sutton and Martin, 2022).

A wide range of methods are used in ENMs/SDMs, including regression-based methods such as generalized linear models (GLMs) although machine learning has rapidly become one of the most popular methods used today (Guisan et al., 2002; Phillips et al., 2006; Wisz et al., 2008; Elith et al., 2010; Phillips et al., 2017). Machine learning differs from regression-based methods in its use of algorithms that "learn" to recognize patterns in data by association, without necessarily testing data against presupposed random distributions to determine the weight of evidence for a non-random pattern (Bzdok et al., 2018). The method used depends on the study goal. Regression-based methods are usually designed for inference, while most machine learning applications are designed for prediction (Bzdok et al., 2018). Both machine learning and regression-based methods can, nevertheless, be used to construct predictive models. This is usually achieved by data partitioning and cross-validation – withholding a subset of the data to train the model to make predictions (training dataset) and testing the accuracy of those predictions against the remaining data (test dataset) (Elith et al., 2020).

MaxEnt is a popular machine learning algorithm used for ecological niche modelling (Phillips et al., 2006). MaxEnt uses presence-only records of a species to model current and potential distributions, whereas regression-based methods typically use both presence and absence records (Elith et al., 2011; Hallgren et al., 2019). The algorithm correlates presence with specified climate variables and estimates the species distribution that has "maximum entropy", i.e. is as close as possible to geographically uniform (Phillips et al., 2017). MaxEnt has previously been used as a climate matching tool for both grasses and insects: for example, to predict the potential invasive ranges of knotgrass *Paspalum distichum* var. *indutum* L. and the planthopper *Pochazia shangtungensis* Chou & Lu in South Korea (Byeon et al., 2018).

Presence-only methods such as MaxEnt are particularly useful when absence data for a species are unavailable or unreliable – which is often the case – and can be accurate with sufficient data and appropriate model calibration (Pearson et al., 2007; Sutton and Martin, 2022). Another advantage of MaxEnt models is that even relatively small datasets (e.g. 10-25 presences, typical of rare or undersampled species) can be useful for characterizing the climate in a known distribution (Pearson et al., 2007). Making use of models with both known presences and "known" absences (non-detections), on the other hand, reduces the effects of sampling bias and makes model evaluation more reliable (Kéry, 2011; Merow et al., 2013; Elith et al., 2020).

5.1.3. Modeled taxa

This study focuses on two South African cecidomyiids, the blister gall midge *Arabukodiplosis vesicaria* (Chapters 2-3) and the Guinea grass seed midge (Lasiopterini sp. megathyrsae – Chapter 4) that have been prioritized as candidate biological control agents for short-form Guinea grass (*Megathyrsus maximus*) in Texas.

Very little is known about the biology of the candidate agents and the factors that influence their presence and abundance, aside from what has been learned from rearing trials and seasonal abundance surveys in parts of their native range (Chapter 2). During preliminary surveys the blister gall midge was found on Guinea grass at several sites within 20 km of the coast or a coastal lake, whereas none were found at sites further inland, and appeared to be more abundant in the relatively tropical KwaZulu-Natal province than the relatively arid Eastern Cape province. On this basis, it was hypothesized that climate, particularly that associated with more humid regions and low-elevation areas nearer the coast, was more suitable for this species. From what is currently known of gall-inducing cecidomyiids, eggs and larvae require a moist microhabitat in order to hatch and establish galls, and adults have a very limited range of dispersal (Briggs and Latto, 2000; Samui et al., 2004; Dorchin et al., 2009). It was hypothesized that site-level chacteristics – particularly the height and density of Guinea grass patches – would also affect habitat suitability.

The seed midge appeared more widespread than stem-galling midges in preliminary surveys, but preference for a humid and coastal climate could not be ruled out. Emergences from standardized panicle collections are highly variable depending on the site and time of year, at some sites ranging from few to none in colder months to well over a hundred (Chapter 2), making accurate predictions of local abundance difficult.

5.1.5. Chapter aims and components

In this chapter, data collected during field host range surveys (Chapters 3-4) are used to predict the climate suitability for the blister gall midge and seed midge as biocontrol agents for Guinea grass in Texas, based on their climate preferences in their native range in South Africa. Climate data and distribution data for the target plant is also used to identify the most suitable part of the native range to collect climatically matched biocontrol agents for the invaded range. Different modelling approaches are taken for native range, invaded range and target plant models.

(1) *Native range models:* Climate and topographical data, measurements of grass patch structure, and data on the presence-absence and abundance of galls are collected and used to build GLMM-based native range ENMs. These models are used to determine the factors affecting the distribution of the insect, using an exploratory-generative approach (Tredennick et al., 2021). Native range ENMs are created for the seed midge based on presence-absence data and climate and topographical data only, using the same approach taken with the blister gall midge.

(2) *Invaded range models:* Biologically relevant climate variables identified in native range models are used to predict the prospects for establishment for each candidate agent in Texas, the target invaded range of Guinea grass, based on presence-only data in MaxEnt. Ideal sites for the establishment of each agent are identified on the basis of MaxEnt climate suitability scores.

(3) *Target plant models:* Presence data and climate data from the invaded range of Guinea grass in Texas are used to build MaxEnt models to identify climatically similar sites in South Africa to collect populations of biocontrol agents for export, on the basis of climate suitability scores.

5.2. Methods

5.2.1. Native range models

To determine the factors affecting the distributions of the blister gall midge and the seed midge in their native ranges in South Africa, GLMM-based ENMs were constructed using existing distribution data and topographical, climatic and grass patch structure data.

Distribution data

Data on the presence-absence and abundance of the blister gall midge and presence-absence of the seed midge were collected in field host range surveys, the methods of which are described in Chapter 3. This dataset contains 61 sites sampled for the blister gall midge (30 presences and 31 absences) and 50 sites sampled for the seed midge (35 presences and 15 absences) (Fig. 5.1). Data were only collected during October-April when the insects were not likely to be in diapause.

Model covariates

ENMS were based on two topographical variables (distance from coast and province sampled, viz. KwaZulu-Natal or Eastern Cape) and a set of 19 climate variables (variables BIO1 through BIO19 – Worldclim, 2024) (Table 5.1). Climate data were downloaded from Worldclim and extracted for the geographic coordinates of each sampling site at 2.5 minute (5 km grid) resolution, assuming 5 km to be sufficiently high spatial resolution for diverse habitats without being too computationally demanding for multi-model inference (terra::extract, Hijmans, 2023a).

In addition to the effects of climate and topography on the presence-absence and abundance of the blister gall midge, it was hypothesized that Guinea grass patches with greater height, tiller density and degree of monospecific growth would be more suitable due to the insect's limited dispersal ability and high host-specificity (Chapter 2). Guinea grass patch structure was measured within a subset of the distribution consisting of 36 sites (17 presences and 19 absences) using a quadrat method. Measurements were taken from five 0.25 m^2 quadrats thrown into a grass patch haphazardly at each site (Fig. 5.2). A visual estimate was made of the percentage total live plant cover (CoverT) and the percentage of which being Guinea grass cover (CoverGG) within the quadrat to the nearest 5 %, and all Guinea grass tillers rooted within the quadrat were collected by hand or using a sickle or pickaxe. Tillers were separately bundled and labelled with their site and quadrat information. Cover estimates for the first three transects were made using a 1 m^2 quadrat, with the 0.25 m^2 quadrat for tiller collection placed in the densest corner. Thereafter the use of the 1 m^2 quadrat was abandoned for the 0.25 m^2 quadrat alone, because estimates were effectively the same using the smaller quadrat. Tillers were examined within 12 hours of collection. The height of the longest tiller in the bundle (Hmax), the estimated average height of the bundle (Have), and the number of tillers were recorded (Till). If the number of non-target grass tillers was enough to affect the accuracy of the estimated percentage Guinea grass cover by at least 5 %, the estimate was amended to reflect this. The number of galls on tillers collected in quadrats was counted and added to the number of galls counted in field host range collections.

Unlike gall-inducing midges, seed midges do not leave easily quantifiable signs of presence that persist between seasons, making single-season abundance data (and to a lesser degree, single-season absence data) of questionable value for modelling purposes.

Table 5.1. Explanation of topographical and Worldclim climate variables.

Variable	**Type**	**Description**
Prov	*Topographical*	South African province sampled (Eastern Cape / KwaZulu-Natal)
Dist	*Topographical*	Straight-line distance from site to coast (km)
BIO1	*Climatic*	Annual Mean Temperature (°C)
BIO2	*Climatic*	Mean Diurnal Range (Mean monthly temperature range) (°C)
BIO3	*Climatic*	Isothermality (BIO2/BIO7) (×100)
BIO4	*Climatic*	Temperature Seasonality (standard deviation ×100)
BIO5	*Climatic*	Max Temperature of Warmest Month (°C)
BIO6	*Climatic*	Min Temperature of Coldest Month (°C)
BIO7	*Climatic*	Temperature Annual Range (BIO5-BIO6)
BIO8	*Climatic*	Mean Temperature of Wettest Quarter (°C)
BIO9	*Climatic*	Mean Temperature of Driest Quarter (°C)
BIO10	*Climatic*	Mean Temperature of Warmest Quarter (°C)
BIO11	*Climatic*	Mean Temperature of Coldest Quarter (°C)
BIO12	*Climatic*	Annual Precipitation (mm)
BIO13	*Climatic*	Precipitation of Wettest Month (mm)
BIO14	*Climatic*	Precipitation of Driest Month (mm)
BIO15	*Climatic*	Precipitation Seasonality (Coefficient of Variation)
BIO16	*Climatic*	Precipitation of Wettest Quarter (mm)
BIO17	*Climatic*	Precipitation of Driest Quarter (mm)
BIO18	*Climatic*	Precipitation of Warmest Quarter (mm)
BIO19	*Climatic*	Precipitation of Coldest Quarter (mm)

Model specification

Statistical analyses were done in R version 4.3.1 (R Core Team, 2023). Three GLMMs (glmmTMB::glmmTMB, Brooks et al., 2017) were built with a single response variable each – two models for presence of the blister gall midge and seed midge, and one for abundance (number of galls) of the blister gall midge. The appropriate distribution and log link function were used for each response variable (binomial distribution with logit link for presence, and negative binomial and log link for abundance).

The topographical variables distance in km from coast (Dist) and province sampled (Prov) for each site were included as fixed effects. An interaction term for distance and province (Dist:Prov) was included to account for possible differences in the effect of distance from the coast between provinces. The transect code was included as a random effect to account for the spatial clustering of sites in each transect, which was expected to make sites in the same transect more similar than sites in different transects.

Climate variables were passed through a principal components analysis (PCA, stats::prcomp, R Core Team, 2023) to avoid overfitting the model by including too many individual climate variables. PCA loadings were extracted using the 'factoextra' package (Kassambara and Mundt, 2020). The three principal component (PC) axes that explained most (> 85 %) of the variance in climate were included as fixed effects in the model, each containing different percentage contributions from each of the 19 temperature and rainfall variables. The top three contributing variables were BIO11 (mean temperature of coldest quarter) for PC1, BIO15 (precipitation seasonality) for PC2, and BIO5 (max. temperature of warmest month) for PC3, with varying contributions from other variables depending on the species being modeled. The fourth PC was not included because of problems with model fitting. Model fit was evaluated by examining scaled residuals plots (DHARMa::simulateResiduals, Hartig, 2022).

Model inference

Akaike's Information Criterion corrected for small sample sizes (AIC_C) was used to identify which explanatory variables and combinations thereof were the best predictors of presence and abundance (Burnham et al., 2011). AIC_C is a measure of parsimony based on model log-likelihood, penalized by model complexity (Burnham et al., 2011). Models with all possible combinations of variables were generated (MuMIn::dredge, Bartoń, 2023). Models with $\Delta AIC_C < 7$ (within 7 AIC_C points of the most parsimonious model) were considered plausible and used to calculate the sum of Akaike weights for each variable (Burnham et al., 2011). Higher summed Akaike weights indicate variables that are more likely to be included in the "best" models (Burnham et al., 2011).

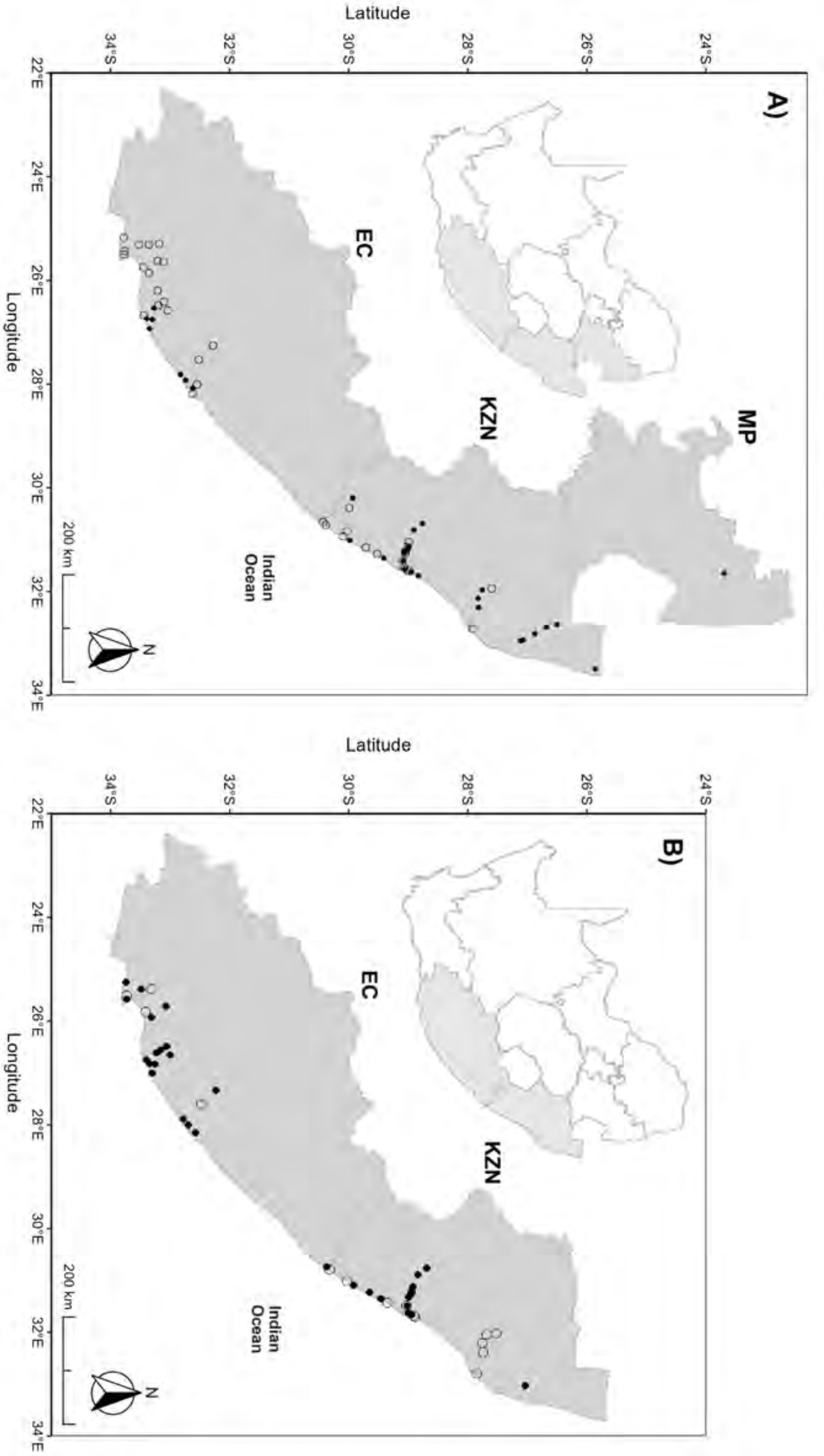


Figure 5.1. Map of field survey points used in habitat / climate models of A) the blister gall midge (*Arabukodiplosis vesicaria*) and B) the Guinea grass seed midge (Lasiopterini sp. megathyrsae). Presences are represented by filled circles, absences by empty circles. Provinces sampled in South Africa (grey areas in country inset) were the Eastern Cape (EC), KwaZulu-Natal (KZN) and Mpumalanga.

Figure 5.2. The quadrat sampling method demonstrated in a large Guinea grass patch in Hluhluwe, KwaZulu-Natal, South Africa.

Estimated slope coefficients (β) for fixed effects in binomial (presence) models (β_{binom}) and negative binomial (abundance) models (β_{nbinom}) were extracted (lme4::fixef, Bates et al., 2015) and transformed from the log-odds scale to the odds scale for ease of interpretation. Marginal effects plots (ggeffects::ggeffect, Lüdecke, 2018; ggplot2::ggplot, Wickham, 2016) were created to show the predicted relationships between the response and explanatory variables in each of the original models.

5.2.2. Invaded range models

To predict the potential distribution of the blister gall midge and the seed midge in Texas, presence-only records from South Africa and global climate data were used to specify MaxEnt models.

Distribution data

The distribution data consisted of 30 presences of the blister gall midge and 35 presences of the seed midge in South Africa from a combined survey dataset (Chapters 3-4). Spatial autocorrelation (clustering) presence points and variables due to sampling bias can cause overfitting by introducing bias towards the climate typical of areas with the greatest number of presences (Boria et al., 2013). The effects of sampling bias can be corrected for by removing spatially autocorrelated presence points and variables, sacrificing statistical power for accuracy (Kramer-Schadt et al., 2013). Presence points with significant spatial autocorrelation for the retained climate variables (< 2 km between points) were eliminated from the dataset using an algorithm based on minimum nearest neighbour distances (spThin::thin, Aiello-Lammens et al., 2015). Eliminating spatially autocorrelated presences produced final sample sizes of 28 presences for the blister gall midge and 34 presences for the seed midge.

Model covariates

Transferability – which refers to a model's ability to make valid predictions in novel conditions – can be improved by building models with variables that are ecologically relevant determinants of the fundamental niche, rather than variables that are merely correlated with the realized niche, as well by using appropriate model settings (Rodda et al., 2011; Sutton and Martin, 2022). Therefore, potentially relevant climate variables were selected from the top ten contributing variables to the principal component axes with the highest summed Akaike weights in native range models (factoextra::fviz_contrib, Kassambara and Mundt, 2020). Autocorrelated climate variables ($r > 0.7$) were eliminated (usdm::vifcor, Naimi et al., 2014). The retained variables for the blister gall midge were BIO12 (annual precipitation), BIO9 (mean temperature of driest quarter), BIO8 (mean temperature of wettest quarter) and BIO2 (mean monthly temperature range). The retained variables for the seed midge were BIO12 (annual precipitation), BIO2 (mean monthly temperature range) and BIO1 (mean annual temperature).

Model specification

Rather than comparing climate conditions at presence points and true absence points, MaxEnt uses a geographic background of assumed absences – called pseudoabsences or background points – to characterize the climate typical of the region sampled and distinguish this from the climate associated with species presence (Elith et al., 2011; Elith et al., 2020). Reasonable background points are physically accessible to the species being modeled (e.g. on land for a terrestrial species) and within the same general biogeographic region that the species is known to occur (VanDerWal et al., 2009). The size of the background is also important; specifying backgrounds that are much larger than the area in which presences are recorded (> 50 km between presence and background points) can lead to models that are overfit (VanDerWal et al., 2009). Overfitting refers to models that are overly specific to the range of conditions that they are trained on and poor at generalizing outside of this range, which is the opposite of transferability (Rodda et al., 2011). Background areas of 10 000 randomly generated points were generated (terra::spatSample, Hijmans, 2023a) using different background extents for the two species. The background for the seed midge was based on the Köppen-Geiger climate zones that contained at least one presence, following the method used by Sutton and Martin (2022). However, for the blister gall midge, the inclusive Köppen-Geiger zone extends far beyond the area actually sampled, into Mozambique. Instead, the background for this species was based on the South African provinces with at least one presence (i.e. Eastern Cape, KwaZulu-Natal and Mpumalanga).

MaxEnt can be specified with a range of feature classes to fit the shape of the relationship between presence and climate variables (linear, quadratic, hinge, threshold and product) and regularization multiplier values to balance model complexity with model fit (Muscarella et al., 2014). Optimal MaxEnt settings were determined by generating models with all possible combinations of feature classes (25 combinations) and regularization multipliers from one to five, and testing them by 4-fold spatial block cross-validation (ENMeval::ENMevaluate, Muscarella et al., 2014). In total, 100 models each for the blister gall midge and the seed midge were generated.

Model inference

The models generated in the previous step were evaluated according to the following criteria:

AIC$_C$ (Akaike's Information Criterion corrected for small sample sizes) is a measure of parsimony based on model log-likelihood, penalized by model complexity (Burnham et al., 2011). In information theory, models that make the most accurate predictions about reality based on the fewest assumptions are presumed to be the most correct (Burnham et al., 2011). For the same reasons, more parsimonious models are also less likely to be overfit and more likely to be transferable to unsampled localities (Warren et al., 2014). Because AIC$_C$ is based on the model parameters themselves and is therefore not cross-validated, it should not be used as the sole criterion for model selection (Kass et al., 2021).

AUC$_{test}$ (Area Under Curve test value) is the area under the curve obtained by plotting the ratio of true presence predictions against the ratio of false presence predictions (Hirzel et al., 2006). AUC values above 0.5 indicate a model that performs better than random at predicting presences in the test data based on the training data, i.e. a more discerning model, with values closer to 1 indicating increasing discernment. AUC_{test} accounts for both false negatives (i.e. predicting a species is not present / not recorded in a locality, when in fact it is present) and false positives (i.e. predicting a species is present in a locality, when in fact it is not present / not recorded). However, AUC is a poor metric for comparing model performance between species and regions or for identifying models that are overly complex or overfit (VanDerWal et al., 2009; Peterson et al., 2011).

AUC_{diff} (minimum difference in AUC values) is the average minimum difference in AUC values between the training and test datasets. Models that are well fit on the training data but relatively poor at predicting the test data will have higher AUC_{diff} values, which usually indicates overfitting and low transferability to unsampled localities (Warren and Seifert, 2011).

CBI (Continuous Boyce Index) is the correlation between classes of habitat suitability scores and the predicted-to-expected ratio (the frequency of correct suitability predictions over the expected frequency) corrected for over-sensitivity to the number of suitability classes. CBI values above 0 indicate a model that performs better than random at predicting habitat suitability (rather than species presence *per se*) with values closer to 1 indicating increasing discernment (Hirzel et al., 2006).

OR_{10} (10th percentile Omission Rate) is the average number of presences above the lowest 10 % likelihood threshold that the model fails to accurately predict. The model with the lowest omission rate is the one that is likely to generate the fewest false negatives (Boria et al., 2013). Models that omit a larger proportion of true presences will, as a matter of course, have lower predictive power and lower transferability.

Although each of the above criteria has its merits, the end goal of the modelling exercise is to find the best balance of predictive accuracy and transferability to unsampled localities. Therefore, a balanced model (**BAL**) was identified by ranking all models on an order of decreasing priorities – first parsimony (minimized AIC_C), then omissions (minimized OR10), then discrimination (maximized AUC_{test} and CBI), then overfitting (minimized AUC_{diff}).

The predictions of the optimal model for each evaluation criterion and the balanced model were computed as MaxEnt scores which represent climatic suitability on a continuous scale from 0.0 to 1.0, with 0.0 being totally unsuitable and 1.0 being totally suitable (‘terra::predict’, Hijmans et al., 2023). These predictions were plotted on maps of Texas and South Africa (‘ggplot2::ggplot’, Wickham, 2016). The predictions of the balanced model were thresholded to produce maps of binary suitable/unsuitable climate predictions for each agent in Texas, overlaid with presence records of Guinea grass from the Global Biodiversity Information Facility (GBIF, 2024). Methods that identify climate suitability thresholds by maximizing the sum of model sensitivity and specificity (the max SSS or “spec-sens” method) are better than many other methods when applied to presence-only data (Liu et al., 2013). However, because the apparent accuracy of a model may be lower than its true accuracy when sample sizes are low and no independent evaluation data are available, there is a chance of selecting oversensitive thresholds with any algorithmic method (Bean et al., 2012). Fixed threshold methods, in which climate suitability thresholds are defined by user confidence in a predefined value (such as 50 % climate suitability) are also widely used (Liu et al., 2005; Sutton and Martin, 2022). In the interest of balance, suitability thresholds for each agent were selected by taking the average of the spec-sens threshold (dismo::threshold, Hijmans et al., 2023) and the 50 % fixed threshold. The potential coverage of each agent in the invaded range of Guinea grass in Texas was calculated using the number of presences of the target plant within the predicted suitable range (raster::extract, Hijmans, 2023b).

MaxEnt, like other modelling methods, assumes that the reference conditions on which the model is trained are representative of the conditions to which it is transferred to make predictions (Elith et al., 2010). The greater the degree to which transfer conditions differ from the reference conditions, the more the model must extrapolate, which may lead to erroneous predictions. This can be accounted for by calculating environmental similarity between the two areas (Elith et al., 2010). Environmental similarity between the South African presence points (reference conditions) and Texas (transfer conditions) was computed and mapped for each species using the Multivariate Environmental Similarity Surfaces (MESS) method (dismo::mess, Hijmans et al., 2023). Negative MESS values indicate that the model is extrapolating outside of the reference conditions, while positive values indicate interpolation (more or less direct comparison between reference and transfer conditions). Regions with negative MESS values were interpreted with caution.

5.2.4. Target plant models

To identify suitable areas in South Africa for collecting climatically matched biocontrol agents, known distribution points of Guinea grass in Texas were used to build climate matching models in MaxEnt.

Presence records of Guinea grass in Texas were downloaded from the Global Biodiversity Information Facility (GBIF, 2024). Records that were duplicates or that had erroneous coordinates (well outside the borders of Texas) were removed. The majority of the remaining records were highly clustered. Presence points that were less than 5 km apart were removed to reduce autocorrelation (spThin::thin, Aiello-Lammens et al., 2015) (Kramer-Schadt et al., 2013). The remaining 171 presence points were used to specify ENMs for the invasive Guinea grass population in Texas using MaxEnt. The inclusive Köppen-Geiger zones were cropped within the extent of Texas (terra::crop, Hijmans et al., 2023a) (since the full climate zones, extending from Mexico to Florida, are much larger than the presence sampling area) and specified as a background with 10 000 randomly generated points. Climate data were downloaded from Worldclim (2023) (variables BIO1 through BIO19) and extracted for the geographic coordinates of each presence point at 2.5 minute resolution (terra::extract, Hijmans, 2023a). Initially, models were tested with variables selected only on the basis of non-autocorrelation, or the full list of 19 climate variables. These models were either extremely overfit, or produced highly disjunct distributions (violating the principle of maximum entropy). More plausible models were built instead with abiotic variables that are relevant to the broad ecological niche of most plants as well as insects: mean temperature (BIO1), rainfall (BIO12), and the temperature maximum (BIO5) and minimum (BIO6) (Kass et al., 2020). A total of 100 models were generated and evaluated using the criteria described previously.

5.3. Results

5.3.1. Native range models

Blister gall midge

The blister gall midge was recorded at a greater proportion of KwaZulu-Natal sites (14/20) than Eastern Cape sites (3/15), with gall abundances ranging from 1 to 100 per site when present. The two most north-easterly transects accounted for 47 % of blister gall midge presences and 20 % of blister gall collections. Sites within 25 km of the coast or a coastal lake accounted for 39 % of blister gall midge presences and 70.5 % of blister gall collections, although galls could still be found further than 80 km from the coast on some transects (Fig. 5.1).

The top three fixed effect variables in the blister gall midge presence models were climate PC2 (Sum Wt = 0.73, N = 162), province (Sum Wt = 0.53, N = 137) and climate PC1 (Sum Wt = 0.3, N = 107). Climate was the most important predictor of blister gall midge presence, containing the single highest-weighted variable (climate PC2) and the highest-weighted variables on average (Sum Wt = 0.39 ± 0.10, N = 107 ± 18), followed by patch structure (Table 5.2). The distance-province interaction was the lowest-weighted variable (Sum Wt = 0.03, N = 14). Higher probability of presence was correlated with (in order of summed Akaike weights): lower climate PC2 scores, i.e. lower precipitation seasonality (β = -0.668); KwaZulu-Natal sites (β = 2.002); higher climate PC1 scores, i.e. higher mean winter temperatures (β = 1.075); lower grass maximum height (β = -

1.045) and average height (β = -0.991); shorter distance from the coast (β = -2.007); lower tiller density (β = -0.98); higher Guinea grass cover (β = 0.991); lower total plant cover (β = -1.109); and higher climate PC3 scores, i.e. higher maximum summer temperatures (β = 0.668) (Fig. 5.3 A).

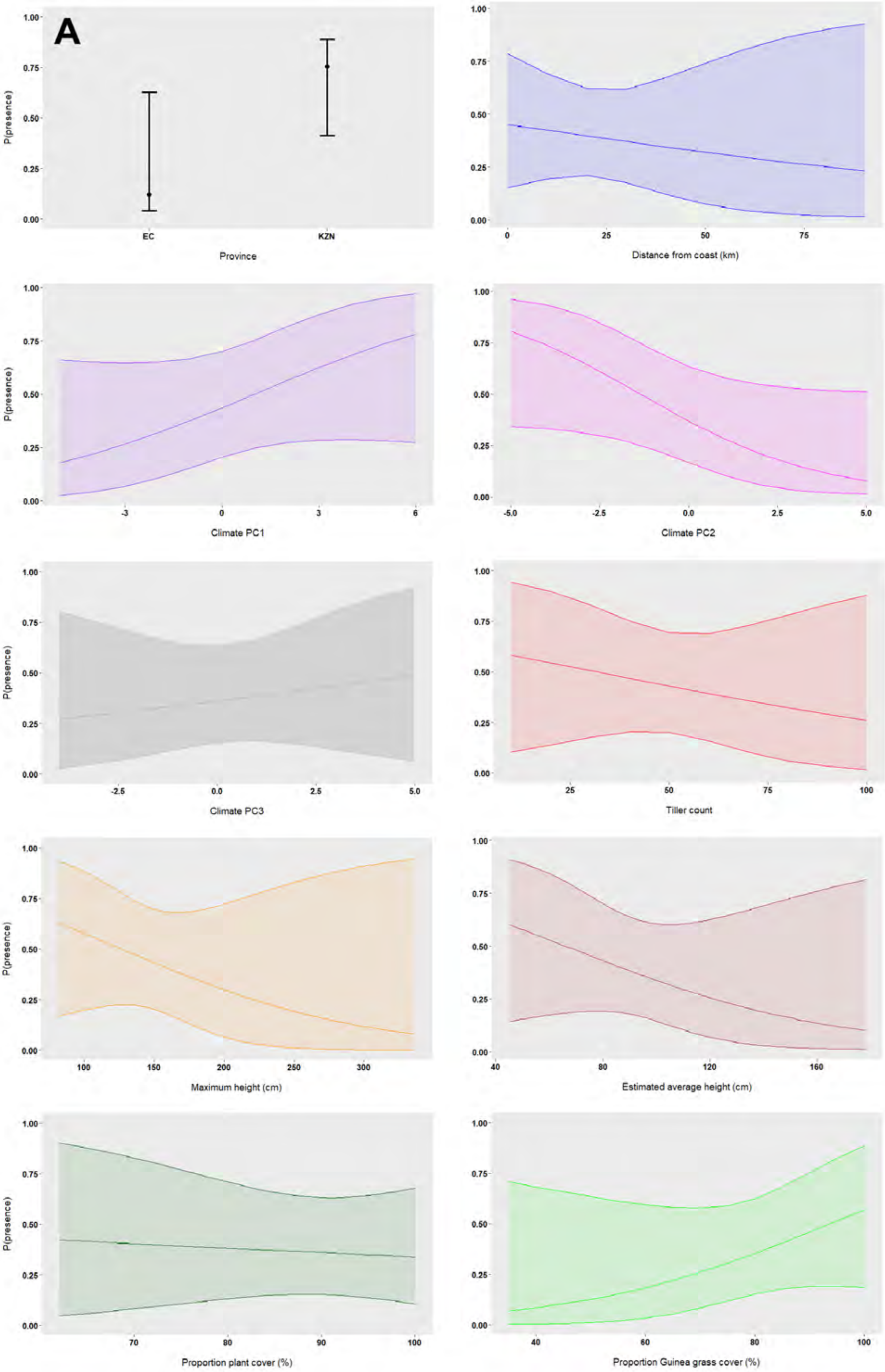
The top three fixed effect variables in the gall abundance models were province (Sum Wt = 0.78, N = 86), percentage Guinea grass cover (Sum Wt = 0.25, N = 40) and climate PC3 (Sum Wt = 0.23, N = 41). Topography was the most important predictor of blister gall midge abundance, containing the single highest-weighted variable (province) and the highest-weighted variables on average (Sum Wt = 0.48 ± 0.21, N = 26 ± 0.1), followed by patch structure (Table 5.1). The distance-province interaction was the lowest-weighted variable (Sum Wt = 0.04, N = 14). Higher predicted abundance was correlated with: KwaZulu-Natal sites (β = 2.245); greater Guinea grass cover (β = 1.032); lower climate PC3 scores, i.e. lower maximum summer temperatures (β = -0.843); higher climate PC1 scores, i.e. higher mean winter temperatures (β = 1.287); shorter distance from the coast (β = -0.969); lower total plant cover (β = -0.985); lower grass maximum height (β = -0.983); and lower tiller density (β = -0.980) (Fig. 5.3 B).

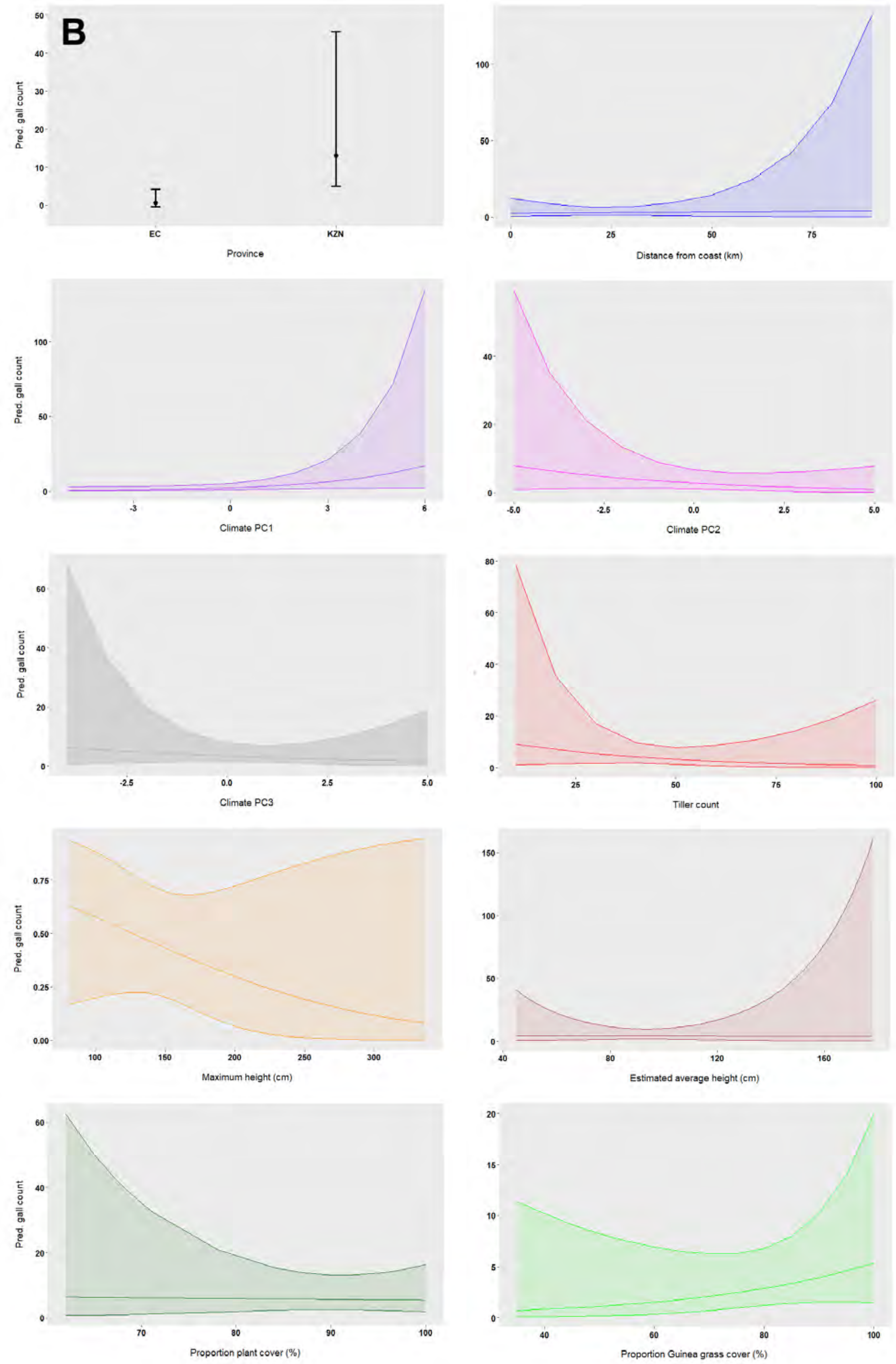
Seed midge

The seed midge was recorded at a greater proportion of Eastern Cape sites (18/21) than KwaZulu-Natal sites (18/29). The top three fixed effect variables in the seed midge presence models were climate PC1 (Sum Wt = 0.41, N = 27), province (Sum Wt = 0.40, N = 31) and distance from coast (Sum Wt = 0.30, N = 30). Climate was the most important predictor of seed midge presence, containing the single highest-weighted variable (climate PC1) and the highest-weighted variables on average (Sum Wt = 0.3 ± 0.02, N = 59 ± 19), followed by topography (Table 5.2). The distance-province interaction was the lowest-weighted variable (Sum Wt = 0.03, N = 5). Higher probability of presence was correlated with: higher climate PC1 scores, i.e. higher mean winter temperatures (β = 0.41); Eastern Cape sites (β = 0.40); shorter distance from the coast (β = -0.979); higher climate PC2 scores, i.e. lower precipitation seasonality (β = 2.262); and lower climate PC3 scores, i.e. lower maximum summer temperatures (β = -0.906) (Fig. 5.3 C).

Table 5.2. Sum of Akaike weights (Sum Wt), number of included models (N models) and GLMM beta coefficients (βbinom / βneg.binom) for each fixed effect variable in plausible models of species presence / abundance for the blister gall midge *Arabukodiplosis vesicaria* and the Guinea grass seed midge Lasiopterini sp. megathyrsae. [ClimPC1 = mainly BIO11, ClimPC2 = mainly BIO15, ClimPC3 = mainly BIO12]

	Prov	**Dist**	**Dist:Prov**	**ClimPC1**	**ClimPC2**	**ClimPC3**	**ClimPC4**	**CoverT**	**CoverGG**	**Hmax**	**Have**	**Till**
Presence blister gall midge												
Sum Wt	0.53	0.2	0.03	0.3	0.73	0.13	NA	0.17	0.16	0.23	0.21	0.19
N models	137	75	14	107	162	53	NA	62	64	77	77	69
β binom	2.002	-2.007	-0.95	1.075	-1.288	0.668	NA	-1.109	0.991	-1.045	-0.991	-0.98
Abundance blister gall midge												
Sum Wt	0.78	0.18	0.04	0.21	0.13	0.23	NA	0.16	0.25	0.17	0.13	0.14
N models	86	32	9	37	24	41	NA	27	40	28	24	26
β neg.binom	2.245	-0.969	1.069	1.287	-0.844	-0.843	NA	-0.985	1.032	-0.983	1	-0.98
Presence Guinea grass seed midge												
Sum Wt	0.40	0.30	0.03	0.41	0.29	0.23	0.28	NA	NA	NA	NA	NA
N models	31	30	5	27	26	26	26	NA	NA	NA	NA	NA
β binom	-0.001	-0.979	1.053	1.352	2.262	-0.906	0.564	NA	NA	NA	NA	NA


A
P(presence)
1.00
0.75
0.50
0.25
0.00
EC
KZN
Province
0
25
50
75
Distance from coast (km)
-3
0
3
6
Climate PC1
-5.0
-2.5
0.0
2.5
5.0
Climate PC2
-2.5
0.0
2.5
5.0
Climate PC3
25
50
75
100
Tiller count
100
150
200
250
300
Maximum height (cm)
40
80
120
160
Estimated average height (cm)
70
80
90
100
Proportion plant cover (%)
40
60
80
100
Proportion Guinea grass cover (%)


B
Pred. gall count
50
40
30
20
10
0
EC
KZN
Province
100
50
0
0
25
50
75
Distance from coast (km)
Pred. gall count
100
50
0
-3
0
3
6
Climate PC1
40
20
0
-5.0
-2.5
0.0
2.5
5.0
Climate PC2
Pred. gall count
60
40
20
0
-2.5
0.0
2.5
5.0
Climate PC3
80
60
40
20
0
25
50
75
100
Tiller count
Pred. gall count
0.75
0.50
0.25
0.00
100
150
200
250
300
Maximum height (cm)
150
100
50
0
40
80
120
160
Estimated average height (cm)
Pred. gall count
60
40
20
0
70
80
90
100
Proportion plant cover (%)
20
15
10
5
0
40
60
80
100
Proportion Guinea grass cover (%)

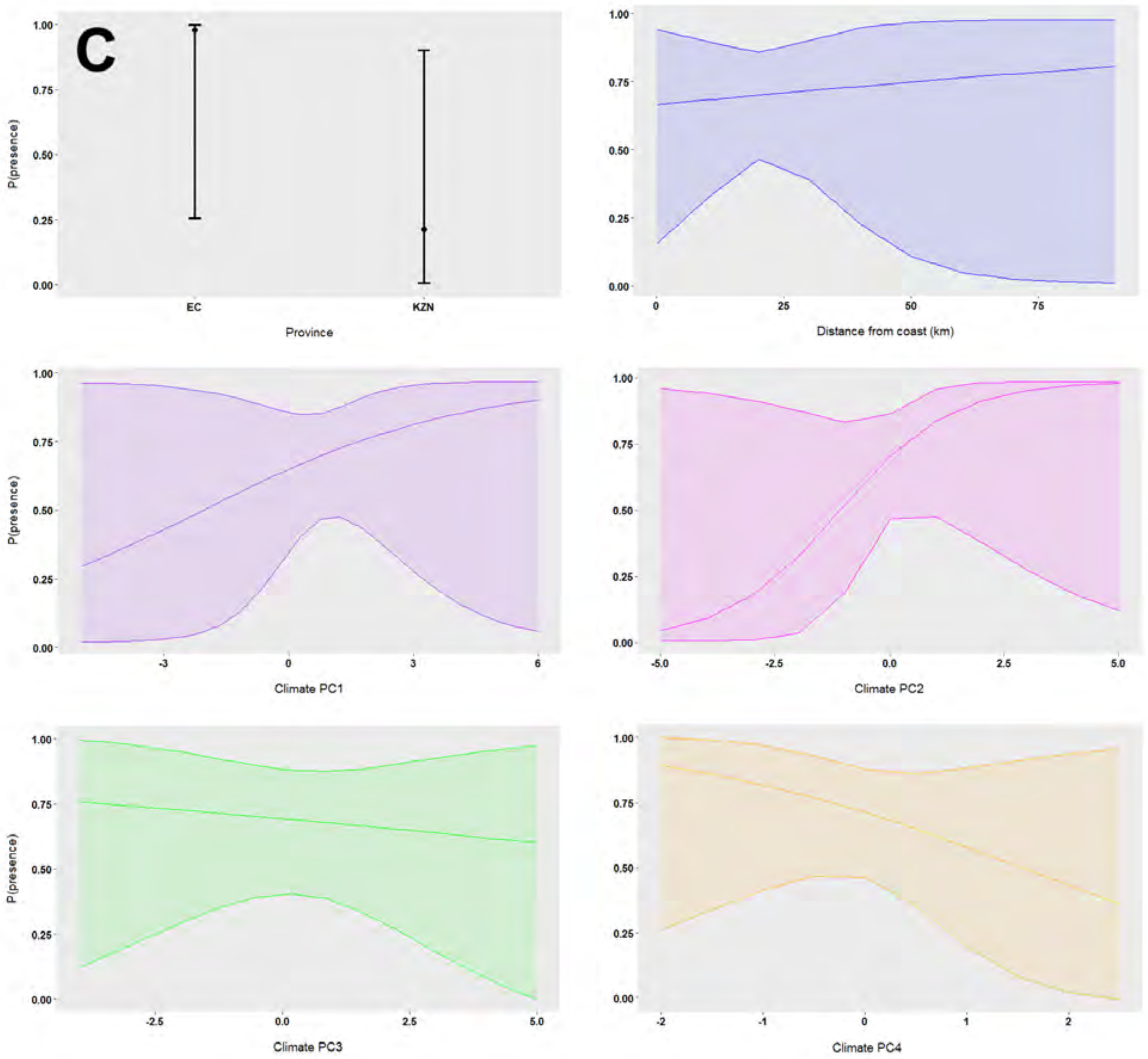


Figure 5.3. Marginal effects plots for ecological niche models of A) presence of the blister gall midge, B) abundance of the blister gall midge and C) presence of the Guinea grass seed midge, showing the response curve of predicted probability of presence / predicted gall abundance to each variable. Shaded areas indicate 95 % confidence intervals.

5.3.2. Invaded range models

In climate models of both cecidomyiid species and Texan Guinea grass, MESS values in the transfer area were positive or near zero in most areas of high to moderate predicted suitability, indicating that models are generally not extrapolating (Fig. 5.4).

Blister gall midge

Blister gall midge models generally predicted highest suitability in counties along the Gulf Coast of Texas. The BAL, AIC_C, AUC_{test} and AUC_{diff} models predicted high suitability (climate suitability scores ranging 0.8-1.0) in a more or less continuous band along the state's coast (Fig. 5.5). The AIC_C and AUC_{diff} models also predicted small areas of moderate suitability (scores ranging 0.6-0.8) inland in central Texas. The CBI model predicted high suitability in small pockets in coastal southern Texas and moderate suitability in some inland areas. High suitability for the blister gall midge was characterized by (in order of percent contribution to models) mean monthly temperature range (BIO2) not higher than 6.5 °C, temperature of driest quarter (BIO9) approaching 21.7 °C, annual rainfall (BIO12) approaching 1300 mm, and temperature of wettest quarter (BIO8) peaking at 26.6 °C (Table 5.3; Fig. 5.7).

Seed midge

Seed midge models, similarly, predicted highest suitability in coastal Texas. The BAL, AIC_C, OR10, CBI and AUC_{diff} models predicted areas of moderate to high suitability spanning most of southern Texas and parts of central Texas (Fig. 5.6). The AUC_{test} model predicted the most geographically restricted areas of high suitability, but also predicted almost the entirety of inland Texas to be moderately suitable. High suitability for the seed midge was characterized by (in order of percent contribution to models) mean monthly temperature range not higher than 7.2 °C, annual mean temperature peaking at 21.5 °C, and annual rainfall peaking at 1300 mm (Table 5.3; Fig. 5.7).

Average climate suitability thresholds corresponded to MaxEnt scores of 0.36 and 0.43 for the blister gall midge and seed midge, respectively. Of the known distribution of Guinea grass in Texas, over 68 % is predicted to be suitable for the blister gall midge, and over 87 % is predicted to be suitable for the seed midge (Fig. 5.8). The distribution that is climatically suitable only for the seed midge corresponds mainly to Guinea grass populations located > 100 km inland.

5.3.3. Target plant models

Texan Guinea grass models predicted the highest degree of climate similarity in northern KwaZulu-Natal and parts of the north east interior (eastern Mpumalanga and Limpopo provinces) (Fig. 5.9). The OR10 and CBI models predicted the highest similarity along almost the entirety of South Africa’s eastern coast. High similarity was characterized by minimum temperatures not lower than 11 °C, annual mean temperatures approaching 26.5 °C, annual rainfall peaking at 900 mm, and temperature maxima not higher than 29.5 °C (Fig. 5.7).

Table 5.3 Relative percent contributions of environmental variables to Maxent models (Chapter 5), calculated iteratively based on effect on regularized gain (percent contribution) or effect on AUC with random permutations of training presence and background data.

Species modeled	Variable	Percent contribution (%)	Permutation importance (%)
Blister midge	BIO2	87.9	97.1
	BIO9	6.8	1.1
	BIO8	4.7	1.8
	BIO12	0.6	0
Seed midge	BIO2	79.5	69.8
	BIO1	19	21.6
	BIO12	1.5	8.6

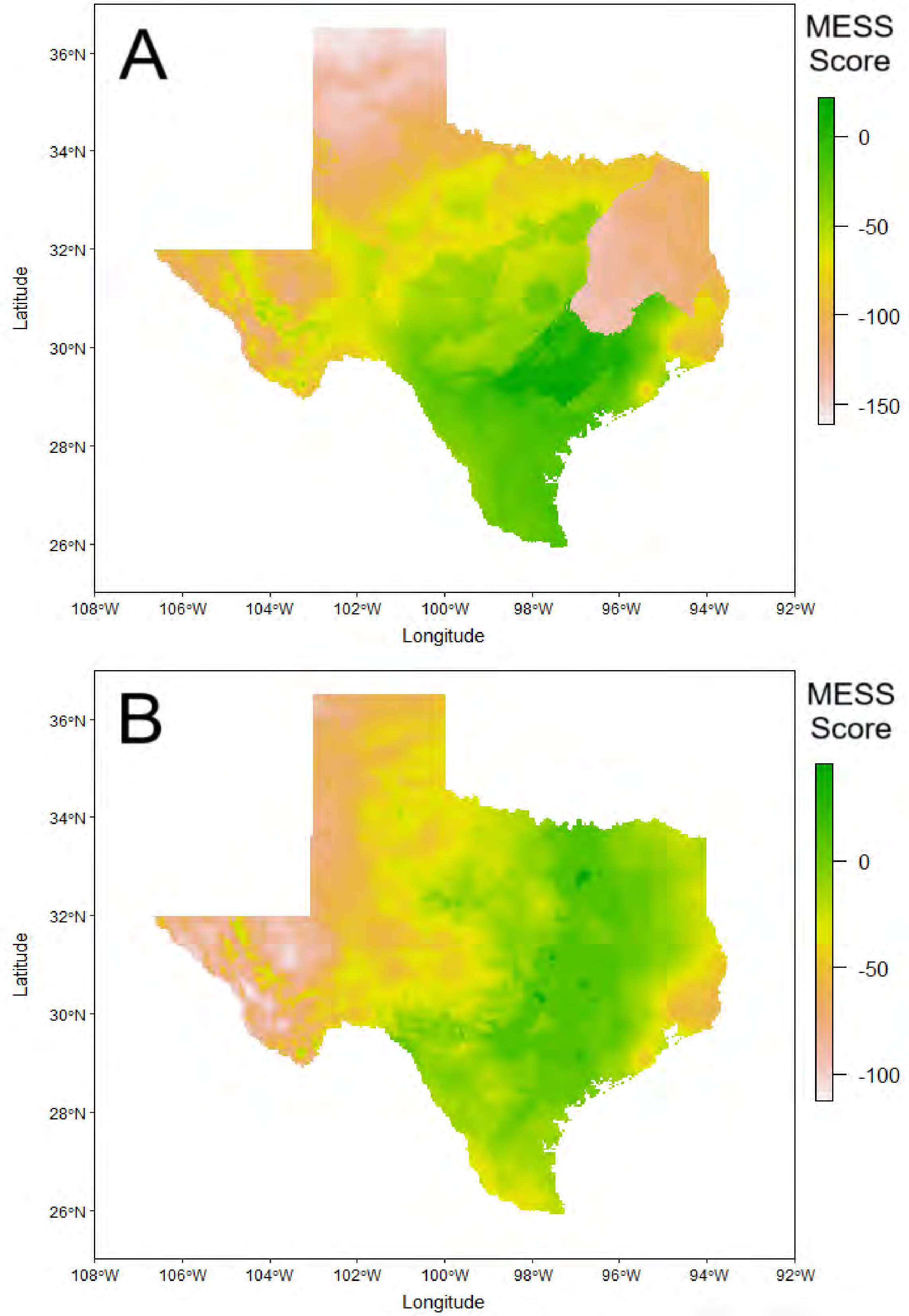


Figure 5.4. Multivariate environmental similarity surface (MESS) maps for Texas based on South African presence points of A) blister gall midge and B) seed midge. Colours indicate MESS scores.

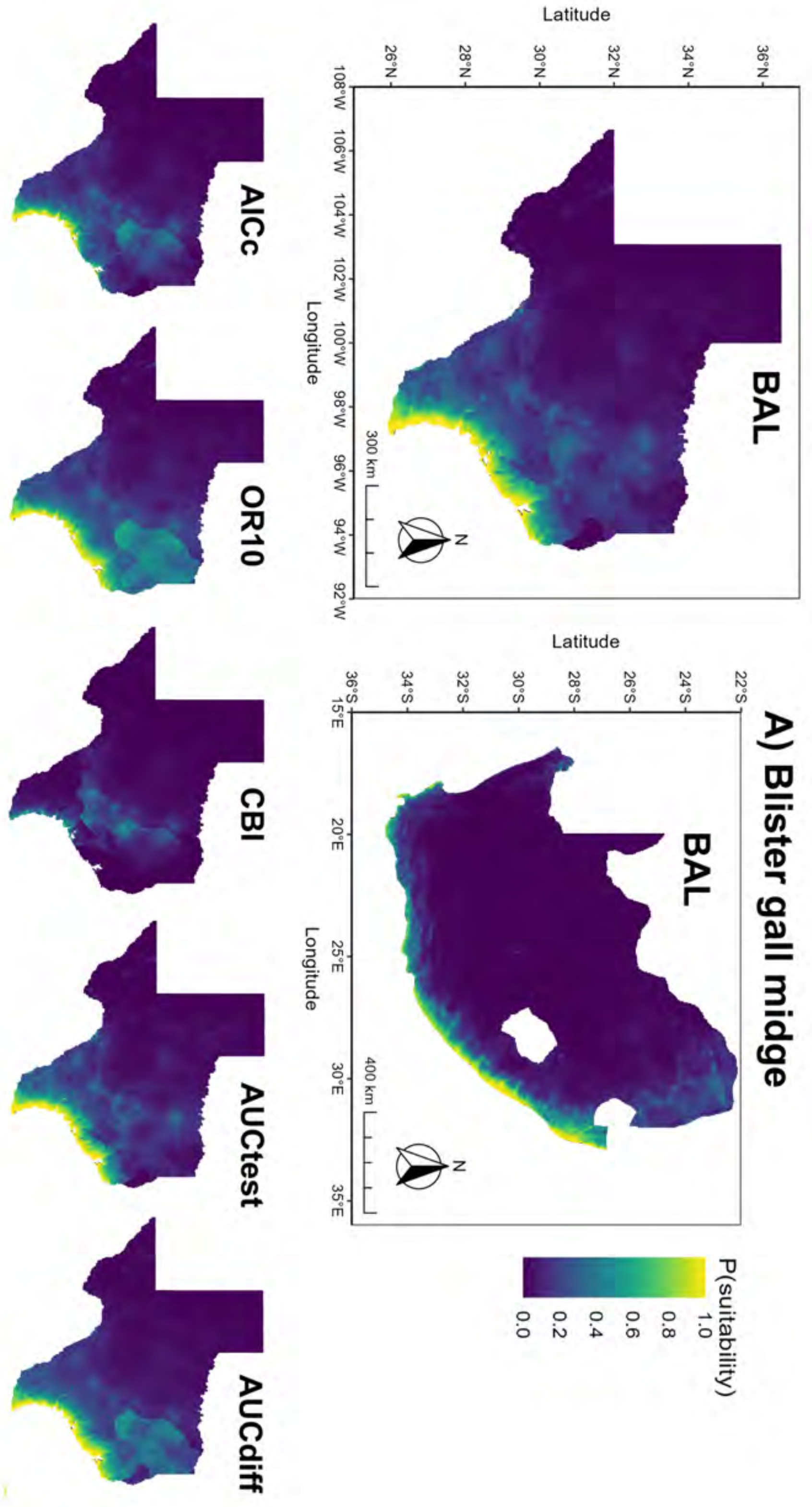


Figure 5.5. MaxEnt climate suitability predictions for the blister gall midge in Texas, optimized by different evaluation criteria (BAL, AICc, CBI, AUCtest, AUCdiff). BAL model predictions for South Africa are included for comparison.

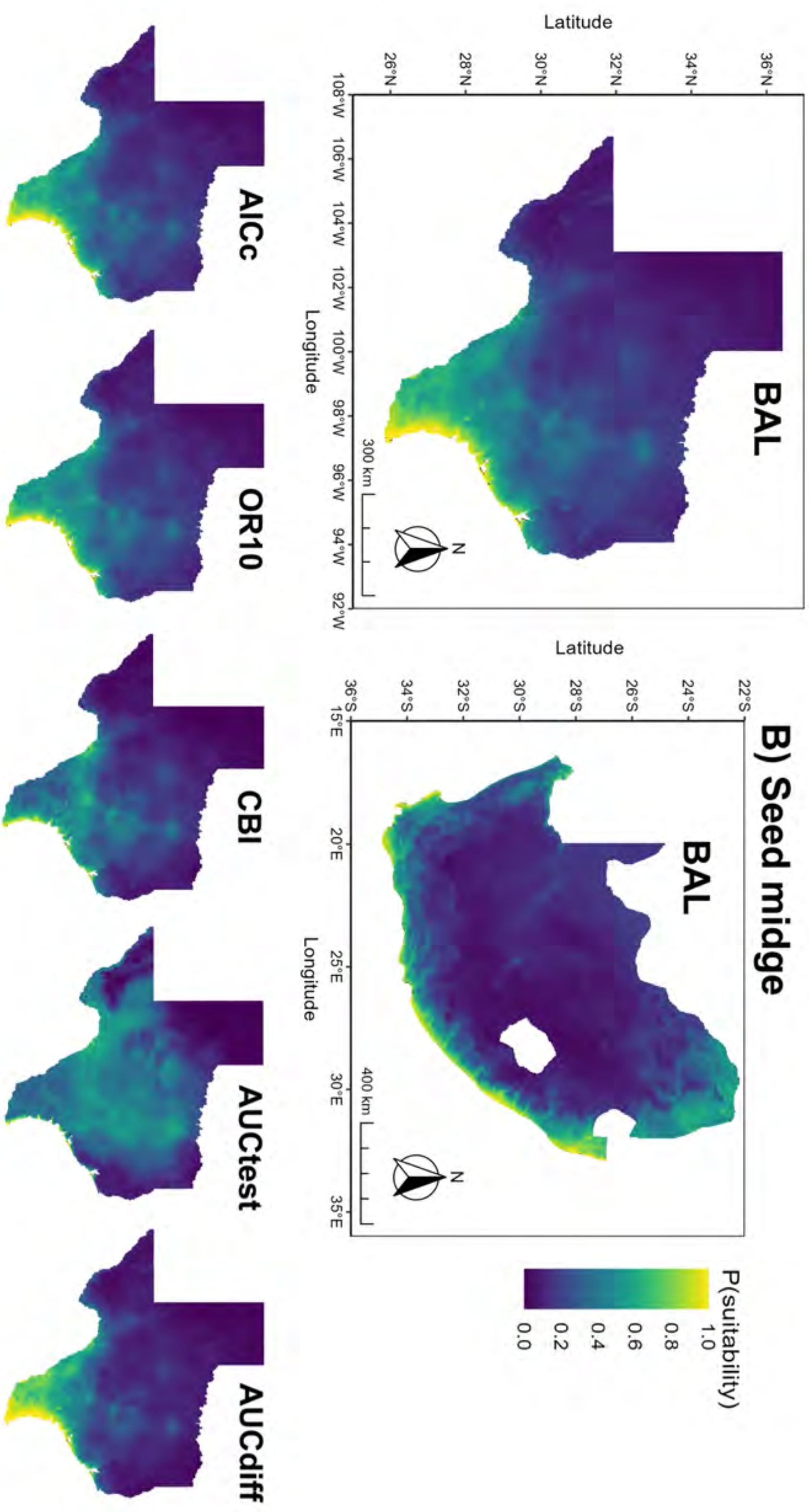


Figure 5.6. MaxEnt climate suitability predictions for the Guinea grass seed midge in Texas, optimized by different evaluation criteria (BAL, AICc, CBI, AUCtest, AUCdiff). BAL model predictions for South Africa are included for comparison.

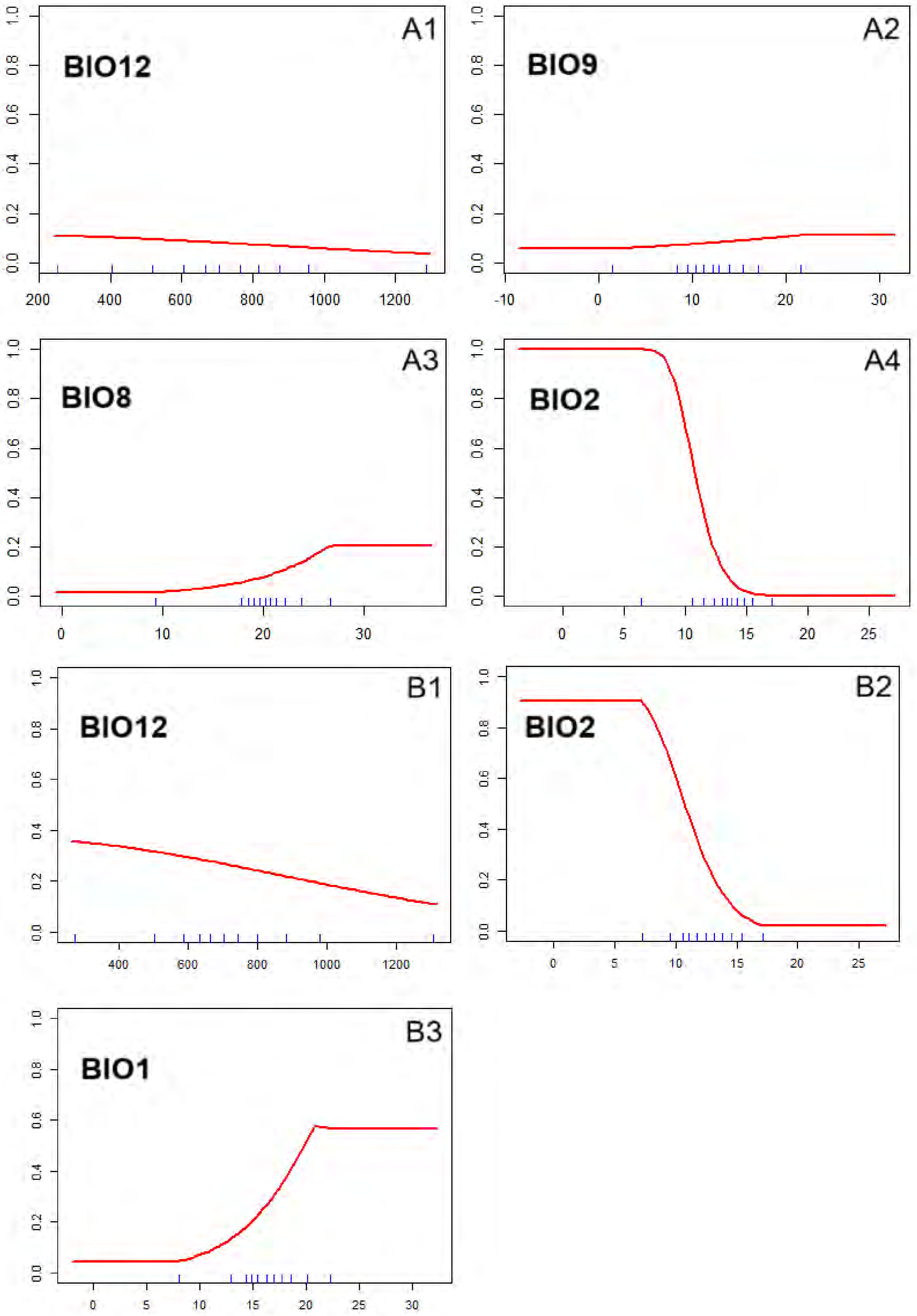


Figure 5.7. Predicted response curves for environmental variables used in MaxEnt models of A) blister gall midge (A1-4) and B) seed midge (B1-3).

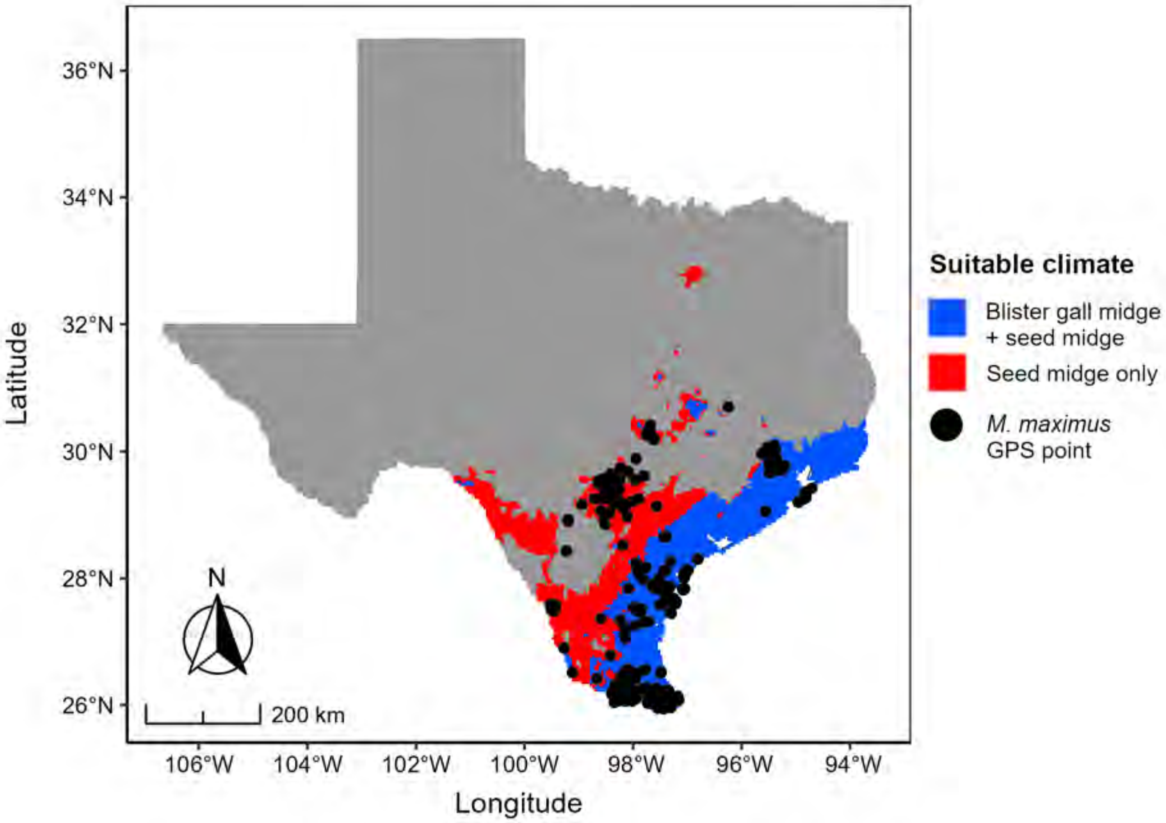


Figure 5.8. Thresholded MaxEnt climate suitability predictions for blister gall midge (blue) and Guinea grass seed midge (red) agents in Texas. Black circles are Guinea grass presence points from GBIF.

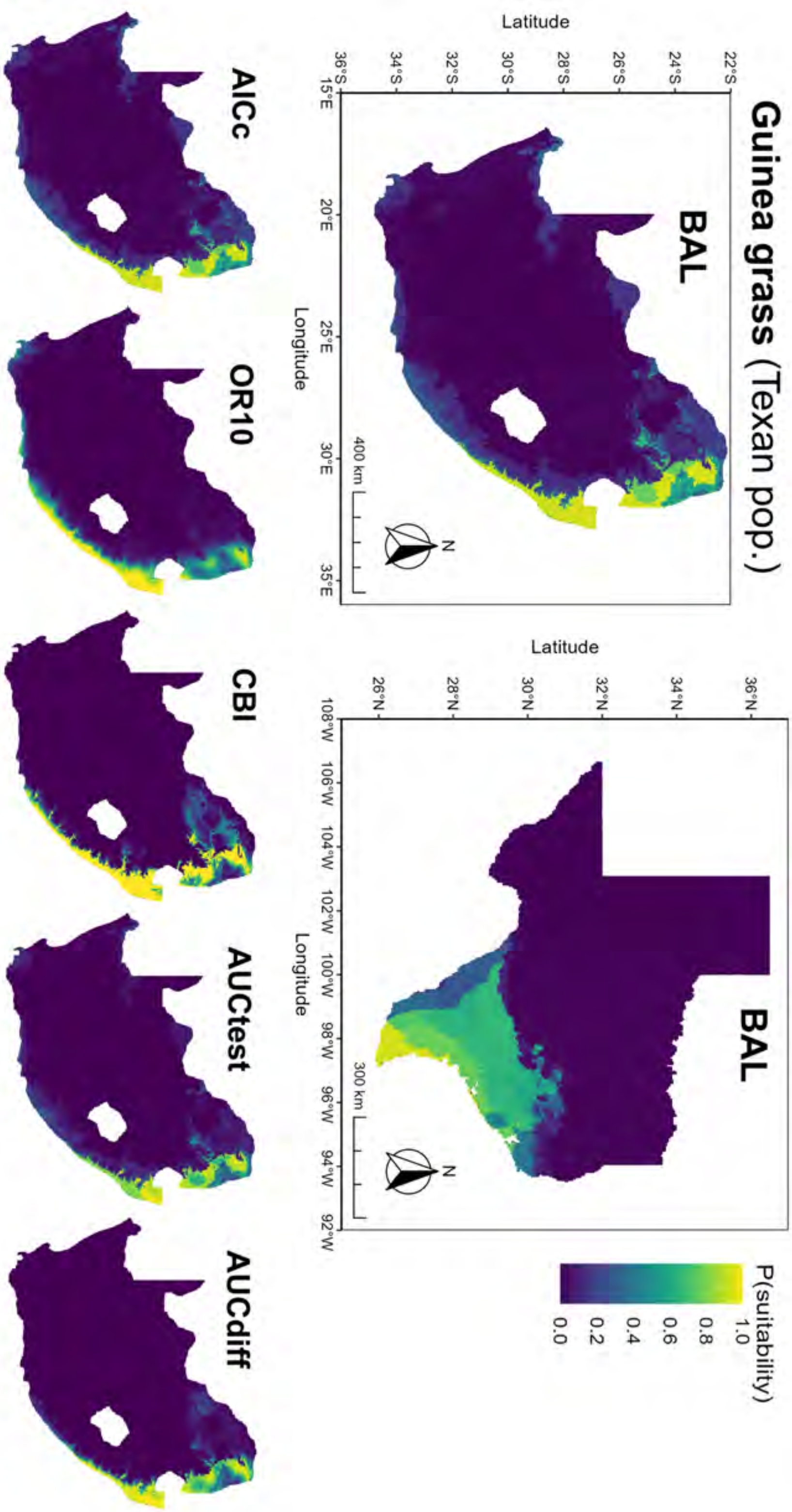


Figure 5.9. MaxEnt climate suitability predictions for Texan Guinea grass in South Africa, optimized by different evaluation criteria (BAL, AICc, CBI, AUCtest, AUCdiff). BAL model predictions for Texas are included for comparison.

5.4. Discussion

Native range models

The distributions of the blister gall midge and seed midge in South Africa were mainly characterized by climate, and more specifically by less seasonal rainfall and higher mean temperatures in both summer (wet season) and winter (dry season). This association with a warm temperate to tropical climate is typical of the global distribution of cecidomyiids (Dorchin et al., 2019a). Although secondary, topography also characterized distributions. The blister gall midge was more common in KwaZulu-Natal than the Eastern Cape, and vice versa for the seed midge. These differences between the provinces are largely explained by climate. Although both provinces receive most of their rainfall in the summer, KwaZulu-Natal receives higher rainfall and higher temperatures typical of more tropical latitudes. In terms of Köppen-Geiger zones, the Eastern Cape ranges from arid steppe to warm temperate and fully humid with warm summers, while KwaZulu-Natal ranges from warm temperate with dry winters and warm to hot summers, to warm temperate and fully humid with warm to hot summers (Peel et al., 2007). Accordingly, the blister gall midge was entirely absent from the relatively arid western regions of the Eastern Cape. The distributions of both species were associated with shorter distance from the coast, although the interaction between province and distance from coast was insignificant. This suggests that the coastal climate in general is also a contributing factor. In South Africa, there is a consistent gradient of increasing elevation with increasing distance from the coast (Singh and Olckers, 2017). Low elevation coastal areas are associated with higher humidity, less extreme temperatures, and higher temperatures in general, as well as other factors that can influence insect development either directly, or indirectly by affecting plant quality (Hodkinson, 2005; Singh and Olckers, 2017). There is also evidence that elevation is a significant dispersal barrier for cecidomyiids; Danon *et al.* (2017) showed that host-associated populations of *Dasineura* gall midges occurring on *Suaeda* shrubs in Israel are geographically separated by elevational differences.

The abundance of the blister gall midge was mainly characterized by topography and patch structure, with more galls being found at coastal sites in KwaZulu-Natal province than in the Eastern Cape province, and at sites with higher percentage Guinea grass cover. Higher abundance was also associated with sites where annual maximum temperatures were slightly lower. The latter point suggests the blister gall midge could be susceptible to heatwaves. Mortality could occur for the immature stages when unusually high transpiration rates due to combined heat and drought lead to xylem cavitation in stems and a resulting halt in fluid flow to galls (Carins-Murphy et al., 2023), or if temperatures inside the galls simply become too high, since the larvae or pupae of gall midges are unable to relocate.

Arthropod species richness is generally higher on grass species with a greater average height or shoot length, and is also higher on unmown (taller) grasses of the same species than mown grasses (although disturbance is a factor in the latter case) (Tscharntke and Greiler, 1995; Gillespie et al., 2022). It is therefore somewhat counterintuitive that blister gall midge presence was correlated with lower, not greater, maximum grass height and tiller density. A greater proportion of Guinea grass cover, however, was positively correlated with both presence and abundance. This could indicate that the blister gall midge prefers monospecific patches of early-stage Guinea grass growth, typical of sites recently colonized or disturbed – but since the majority of sites were roadsides (which by nature are likely to be recently colonized or disturbed) this could equally well be an artefact of sampling.

Invaded range models

MaxEnt models indicated high prospects for the establishment of both candidate agents in south Texas, particularly southern coastal areas and the eastern Rio Grande, where the oldest and densest populations of Guinea grass occur (GBIF, 2024). These areas should be prioritized for the release of both agents if they are approved. The seed midge is predicted to have much higher coverage of invaded areas (87 % total coverage) compared to the blister gall midge (68 % total coverage), particularly in inland areas extending to central Texas near Austin, which is presently the frontline of a gradual northward range expansion of Guinea grass in the state (Bannin, 2024). Considering that Guinea grass spreads mainly by seed, multiple introductions of the seed midge at the frontline should be considered to limit the rate of range expansion (Rhodes et al., 2022a).

Model predictions should be interpreted carefully, since MESS models indicated marginal climatic similarity between reference and transfer conditions. Although models were generally interpolating, particularly in areas with the highest predicted suitability, some models that predicted moderate to high suitability in parts of Texas with very low climatic similarity to South Africa (e.g. OR10 and AUCdiff blister gall midge models) were extrapolating far outside reference conditions. Balanced models performed the best at limiting predictions of higher suitability to areas of higher climatic similarity, and should therefore be considered the most reliable.

For both species, mean monthly temperature range was the largest contributor to climate suitability predictions; ranges above a certain threshold (around 7 °C) resulted in decreased suitability. Mean dry-season temperatures (blister gall midge) or annual mean temperatures (seed midge) slightly above 21 °C were ideal. This explains high suitability predictions being mostly restricted to coastal regions, in line with native range ENMs for these species. Due to a weather phenomenon known as the “climatic trumpet”, large parts of Texas are prone to sudden drops to near-zero or below-zero temperatures in the winter, which may produce snowfall (Goolsby et al., 2005) – a very rare event in South Africa. Extreme low temperatures are believed to have caused the failure of some wasp biocontrol agents for the crop pest *Bemisia tabaci* Gennadius that were introduced to Texas from countries with warmer winters (Goolsby et al., 2005). Similarly, frost has prevented the establishment of the *Melaleuca* gall midge, *Lophodiplosis trifida*, at some sites in Florida (Pratt et al., 2013). In coastal regions of Texas, the moderating effect of the ocean on temperature variation may negate the impact of colder winters on the blister gall midge and seed midge, if these species are able to acclimate. Rainfall was the smallest contributor to suitability predictions for both species, indicating that it is not likely to be a significant limiting factor in Texas.

Because insect life cycles and performance (e.g. rates of reproduction and feeding) are strongly affected by differences in climate and especially temperature, agents collected from areas that are climatically matched to the invaded range of the target plant tend to produce better biocontrol outcomes, and should therefore be prioritized (Griffith et al., 2019; Singh and Olckers, 2017). The best climatic match in South Africa for the Texan population of Guinea grass was northern KwaZulu-Natal, where the first biocontrol surveys in the country were done. Guinea grass populations in this region have already been shown to be the closest known genetic match in Africa to invasive Texan populations (Gaskin et al., 2022), and large numbers of the blister gall midge and the seed midge have already been recorded at several sites there, making these sites ideal for collecting biocontrol agents that are climatically matched to the target weed in its invasive range. The annual mean, minimum and maximum temperatures and the annual rainfall at invaded sites in south Texas are generally within the estimated climatic niche of the cecidomyiid agents, reinforcing the predictions of other models.

5.5 Conclusion

Northern KwaZulu-Natal is the most climatically similar region of South Africa to invaded areas in Texas, and any future collections of the blister gall midge and seed midge agents for biocontrol purposes should be sourced from this region. Coastal sites with a warm temperate climate are more suitable for both species in their native range, and sites with early-stage monospecific stands of Guinea grass are particularly suitable for the blister gall midge. Climate suitability for these species in Texas is predicted to allow for establishment and wide coverage of areas invaded by Guinea grass, especially the most densely invaded areas in coastal south Texas and the eastern Rio Grande. The blister gall midge is predicted to have the highest chance of establishment in coastal areas, while the seed midge has the potential to limit the northward range expansion of Guinea grass into central Texas because of its broader climate tolerance and ability to destroy seed propagules. Climate matching models such as those used in this study have high value for predicting biocontrol outcomes and informing if and how releases of agents should be conducted.

Chapter 6: General discussion

6.1. Biocontrol potential of cecidomyiid agents

This study found a larger assemblage of insect herbivores associated with Guinea grass in South Africa than previous published surveys in Cameroon and Kenya (Chapter 2; Mercadier et al., 2009; Rhodes et al., 2022b). Of these, the Cecidomyiidae are the most diverse in terms of feeding modes and number of species, comprising multiple stem gall-inducing and seed-feeding taxa, as found in surveys on other invasive grass species (Morrison et al., 2023b). The blister gall midge *Arabukodiplosis vesicaria* and the Guinea grass seed midge Lasiopterini sp. megathyrsae are the most promising candidate biocontrol agents for Guinea grass. The blister gall midge completes its lifecycle only on short-form Guinea grass according to field host range surveys, the results of which have so far been supported by cage-based host-specificity tests (Chapter 3; Guy Sutton, unpublished data). Lasiopterini sp. megathyrsae is likely to be a distinct species that only completes its lifecycle on Guinea grass in the field, according to phylogenetics and field host range data, but its ability to attack tall-form Guinea grass in the field and in cage-based host-specificity tests have not been evaluated yet (Chapter 4). The horn gall midge was rejected because of non-target attack on *Batochloa deusta,* whose closest relatives include *Rupichloa acuminata* Salariato & Morrone and its congeners, which are native to the American continent (Salariatio et al., 2009; Morrone et al., 2012).

Cecidomyiids are underutilized as biocontrol agents for invasive plants; only 16 species have been released, compared to the estimated millions of host-specific species available worldwide (Winston et al., 2024; Dorchin et al., 2019). Where cecidomyiids have been utilized, success has been limited by inefficient release strategies as well as inherent ecological challenges. More than 67 % of unique releases (i.e. of an agent on a plant in a distinct geographic region) have resulted in establishment, with failed establishment usually resulting from a combination of too few releases of too few insects, followed by extreme weather events and a subsequent failure to conduct follow-up releases (Winston et al., 2024). Although a small number of cecidomyiid agents have had heavy impacts, such as *Lophodiplosis trifida* on broad-leaved paperbark in Florida and *Zeuxodiplosis giardi* (together with the chrysomelid beetle *Chrysolina hyperici* Forster) on *Hypericum perforatum* in Hawaii, impacts are recorded as slight to variable in 75 % of releases that have led to establishment (Funasaki et al., 1988; Pratt et al., 2013; Winston et al., 2024). Agents not being abundant enough due to parasitoid accumulation is the most frequently cited cause of low impact (30 % of unique releases), followed by agents not being abundant enough for unspecified reasons (15 %) or due to an apparent climate mismatch (11 %) (Winston et al., 2024). The lack of parasitoid recruitment on *L. trifida* in Florida as well as a suitable climate may be the most important reasons why this agent has been so successful (Pratt et al., 2013). These potential limitations on cecidomyiid agents were anticipated in this study and steps have been taken to minimize them or rule them out where possible. The native parasitoids of the candidate stem gall midge and seed midge agents and the local seasonal abundances of each are known; with no known direct ecological analogues in the proposed region of introduction in Texas, the risk of low agent abundance due to parasitoid accumulation is predicted to be low (Chapter 2). The climatic factors determining the curent and potential distributions of the candidate agents are also known; the climate in the parts of Texas invaded by the target plant is largely suitable, and a source region for climatically matched agents has been identified (Chapter 5).

Stem gall midges are expected to drain the plant's carbon resources and potentially reduce stem height (thus reducing biomass and seed dispersal ability – Mashau et al., 2021; Thomson et al., 2011) and flowering rates (Rhodes et al., 2022b), while seed midges are expected to reduce the output of viable seeds into the environment (Wani, 1979; Prasad et al., 2021). A combination of reduced height, flowering and seed output could significantly reduce the spread of Guinea grass into new ranges and perhaps decrease its competitiveness within the already invaded range. The seasonal, eruptive lifecycle of the stem and seed midges means that most active damage to Guinea grass is done during warm, wet months, which is the peak time for the plant's growth and reproduction (Chapter 2). By removing specialized African parasitoids as a limiting factor, cecidomyiid biocontrol agents in Texas may be able to reach and sustain larger population sizes than are possible in the native range and actively damage Guinea grass for a longer period, as seen in other biocontrol programs (Adair, 2005; Pratt et al., 2013).

Native-range climate models suggest that the agents are dependent on moderate monthly average temperature ranges (around 7 °C) and, particularly for the blister gall midge, warm winters (above 21 °C on average) (Chapter 5). Given that parts of Texas are prone to high temperature variability and winter cold snaps, and that Australian stem gall midge agents introduced to similar latitudes in Florida have proven sensitive to frost, the potential distribution of the blister gall midge in inland Texas is not likely to be very wide (Goolsby et al., 2005; Pratt et al., 2013). The climate in Texas is however expected to be conducive to the establishment of the blister gall midge and Guinea grass seed midge in the temperate southern and coastal areas of south Texas, which are currently the most invaded parts of the state (GBIF, 2024). Chances of establishment in these areas are particularly high for agents sourced from KwaZulu-Natal province, which climate matching models identified as the ideal source of South African agents for Guinea grass in Texas based on the current invasive range of the plant. The seed midge has the advantage of a broader climate niche, and its distribution further inland in the native range suggests a greater tolerance to drought and frost (Chapter 5). This could be leveraged by releasing the seed midge near the vanguard of northward range expansion of Guinea grass in central Texas, where it could directly reduce seed propagule pressure (the primary means of spread) and the rate of range expansion (Rhodes et al., 2021; Bannin, 2023). Rearing populations of agents for cold acclimatization is an option for improving chances of establishment at the northern limits of the invasive range in Texas (Griffith et al., 2019). A tentative implementation plan would involve the export of agents from climatically suitable parts of South Africa to rearing facilities in Texas, followed by multiple introductions at densely-invaded sites to maximize the establishment and coverage of each agent in its respective, climatically suitable range (Fig. 6.1).

Most herbivorous cecidomyiids are thought to have obligate relationships with certain phytoparastic fungi which aid in gall induction and in some cases serve as a food source for larvae (Rohfritsch, 2008; Dorchin et al., 2019a). In a biocontrol context, this means that the introduction of a cecidomyiid agent may in fact involve the introduction of two agents – the insect and its fungal symbiont (Marshall et al., 2018b). Several cecidomyiid biocontrol agents have symbiotic relationships with phytopathogenic fungi, such as that between the fruit-and-ovule-attacking acacia midge *Asphondylia acaciae* Kolesik and the canker fungus *Botrysphaerida dothidea* Ces. & De Not. (Ascomycota: Botryosphaeriales: Botryosphaeriaceae), and between the leaf-mining arundo midge *Lasioptera donacis* (a biocontrol agent for *Arundo donax)* and the sac fungus *Arthrinium arundinis* (Ascomycota: Apiosporaceae) (Kolesik et al., 2010; Marshall et al., 2018). In some other grass-feeding species, such as *Lasioptera arundinis* on *Phragmites australis*, the fungal symbiont has been shown to be essential for feeding on – and therefore damage to – the host plant (Rohfritsch, 2008). There is similar, partial evidence of fungal symbioses in several other herbivorous cecidomyiids (Whaley et al., 2020; Dorchin et al., 2022). Fungal biocontrol agents, also referred to as mycoherbicides or bioherbicides, have been effective in the laboratory against widely invasive grasses such as Johnson grass *Sorghum halepense* Pers., itchgrass *Rotboellia cochinchinensis* Clayton, and Chilean needlegrass *Nassella neesiana* Barkworth (Del Serrone and Fornasari, 1992; Ellison and Evans, 1992; Sutton et al., 2019). Three species of sac fungi (Ascomycota: Pleosporaceae) isolated from native grasses in Florida have been tested as biological control agents of short form Guinea grass: *Dreschlera gigantea* Ito, *Exserohilum rostratum* Sivan and *E. longirorostratum* Leonard & Suggs. Guinea grass inoculated with emulsions of these fungi, alone or in combination, suffered severe leaf lesions and necrosis, while non-target crops were unaffected (Chandramohan et al., 2002). In its native range, the inflorescence of Guinea grass is affected by the fungi *Conidiosporomycetes ayresii* and *Claviceps* spp. which cause deformation of seeds or florets (while a *Balansia* / *Myriogenospora* sp. affects the stems) (Fig. 6.2 A-E). Seed midges presumably require normal seed development, although there is no evidence that these fungi are necessarily antagonistic to the seed midges. The females of certain species of *Lasioptera*, *Calamomyia* and *Asphondylia* carry fungal conidia on their ovipositors in what are hypothesized to be structures specially evolved for this purpose (Borkent and Bissett, 1985; Rohfritsch, 2008). Although *C. ayresii* has proven capable of spreading to the

United States without any documented association with seed midges (Rosskopf et al., 2019), as well as other fungi associated with Guinea grass such as *Claviceps maximensis* (USDA, 2024), it is not impossible that seed midges could facilitate the spread of such fungi on Guinea grass by transporting spores (e.g. Fig. 6.2 B), and therefore that the two organisms could have a synergistic seed-destroying effect. In the native range of Guinea grass, Lasiopterini sp. megathyrsae and *C. ayresii* or *C. maximensis* occur in sympatry at some sites, although the two are not always associated (personal observation). *Balansia / Myriogenospora* sp., on the other hand, is rare on Guinea grass in its native range (at least in a symptomatic stage) and is unlikely to be associated with the widespread stem gall midge species. Potential fungal symbionts of cecidomyiid candidate agents should be investigated prior to release as part of host-specificity testing, as the affinity of the symbiont (if present) for certain plant species could play a role in determining each insect's host range (Rohfritsch, 2008).

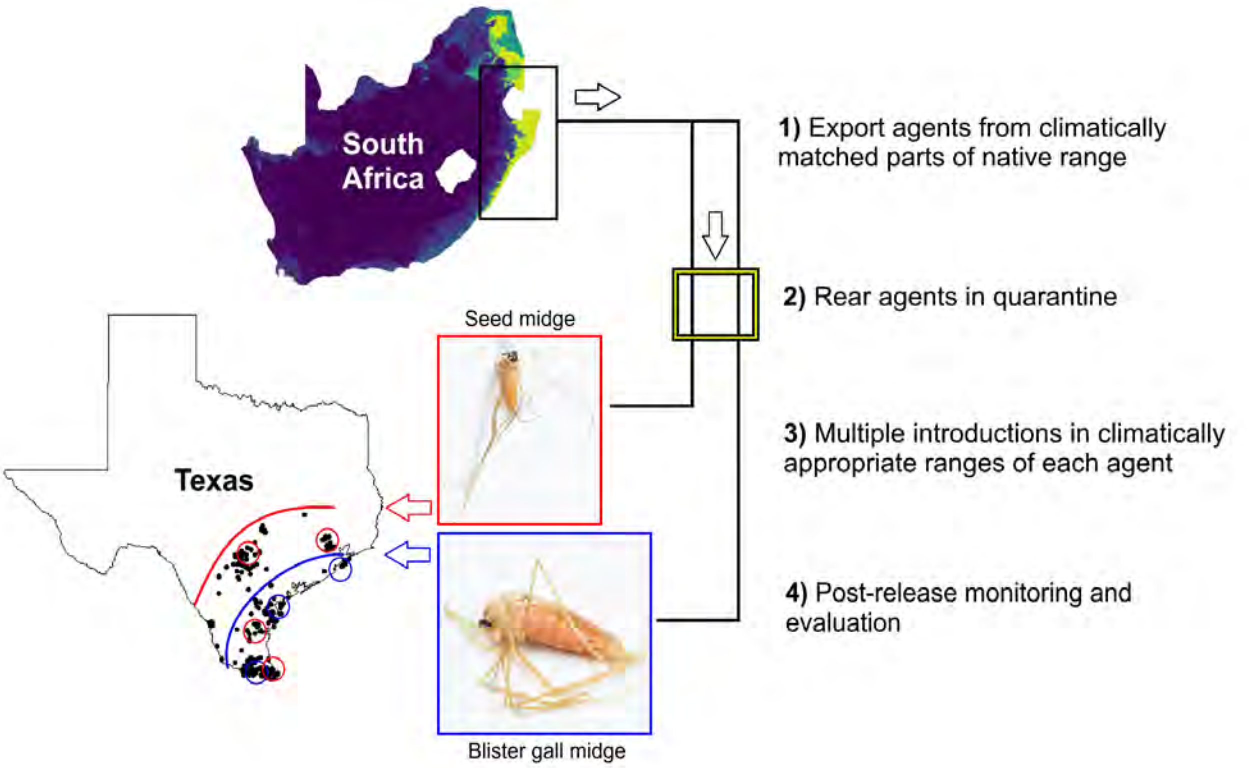


Figure 6.1. Implementation plan for candidate biocontrol agents prioritized in this study for controlling invasive short-form Guinea grass populations in Texas.

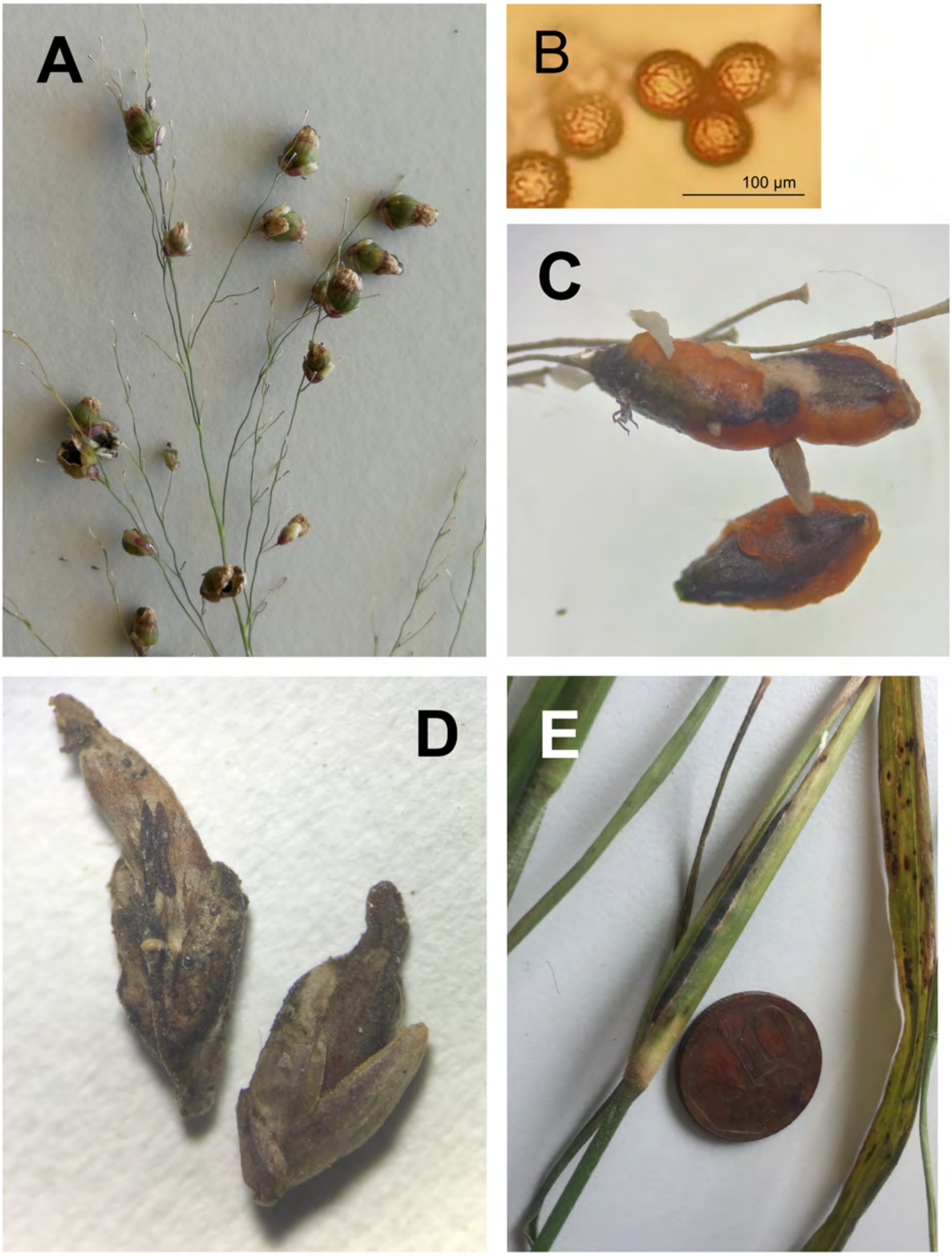


Figure 6.2. Fungi attacking florets of short-form Guinea grass. A) Inflorescence bearing sori or swollen ovaries containing spores of smut fungus *Conidiosporomyces ayresii*. B) Micrograph of the large urediospores of *C. ayresii* collected from sori. C) Florets affected by ergot *Claviceps* sp. 1. D) Sclerotia induced on florets by *Claviceps* sp. 2. E) Conidial growths of *Balansia* / *Myriogenospora* sp. on stems.

6.2. Value of native-range field surveys in invasive plant biocontrol programs

Field-based assessments of agent host range and impact are seen as some of the key methodologies for improving the safety and success of biocontrol programs in the future (Hinz et al., 2024). This study can be seen as further evidence for the effectiveness of the field-based method for rapid identification and pre-release evaluation of candidate biocontrol agents for invasive grasses. Using this method, field host range surveys are done in the native range of the target plant based on the centrifugal phylogenetic method, the assemblage of natural enemies on the target plant is identified, and this list is narrowed down to the most promising candidates based on evidence of host-specificity and plant damage in the field (Sutton et al., 2021b). Several forms of sampling are used, including visual inspection, dissection and emergences from collected material (Sutton et al., 2021b; Yell et al., 2024). In this study, the inclusion of sweep-netting was necessary to catalog several ectophagous taxa, and the separation of plant reproductive and vegetative tissues in emergence collections was necessary for isolating feeding modes of endophagous taxa such as seed feeders (Chapter 2).

The benefit of this field-based method compared to typical quarantine-based methods is that fewer natural enemies are imported into quarantine, and additional time and resources are not spent on testing agents that are unlikely to be suitable (Sutton et al., 2021b). Combined with phylogenetic analysis to delineate undescribed and/or cryptic taxa, this technique can identify likely host-specific taxa on multiple invasive grasses in a relatively short timeframe. This has certainly been shown in this study, which identified different species of seed-feeding Cecidomyiidae on Guinea grass and on buffelgrass – both of which are invasive in the United States and elsewhere – and in recent studies by Sutton et al. (2021b) and Van Steenderen et al. (2023) that identified host-specific stem-boring *Tetramesa* spp. on African giant rat's tail grasses (*Sporobolus pyramidalis* and *Sporobolus natalensis*) that are invasive in Australia. Importantly, subsequent surveys and laboratory testing confirmed the findings of the former two studies that another *Tetramesa* sp. on *Eragrostis curvula* – also invasive in Australia – was not sufficiently host-specific, showing that field evaluations could accurately reject candidate agents that are not safe to use as well as identify those that are (Yell et al., 2024). This study, similarly, led to the rejection of another candidate agent, the horn gall midge, without the need to proceed to laboratory testing; focus can therefore be directed towards those candidates that are more likely to be successful (Chapter 3).

The studies mentioned above all relied on small teams of researchers (5-6 individuals) operating on a relatively small budget, using both traditional taxonomy and modern genetic tools available in several African universities and most international universities with a dedicated biology department (Sutton et al., 2021b; Van Steenderen et al., 2023; Yell et al., 2024; Iain Paterson

[CBC] and Colin Morrison [UTex], personal communications). Given the global burden of invasive African grasses and invasive grasses in general, and the urgent need to alleviate this burden through sustainable management methods (Canavan et al., 2019; Rossiter-Rachor et al., 2023), the approach taken in these studies is recommended as a model for future pre-release biocontrol programs for grasses and possibly other invasive plant groups. This approach will be most effective when the target plant has several sympatric close relatives in the native range – as was the case with African panicoid grasses in this study – so that suitably comprehensive field host range testing is possible (Sutton et al., 2021b; Yell et al., 2024).

6.3. The taxonomic impediment and implications for the biocontrol of invasive grasses

In this study, molecular and ecological evidence was found for possibly 17 new species and a new genus of seed-feeding Cecidomyiidae on southern African panicoid grasses. These include at least five species of a new genus of Lasiopterini – and perhaps two or more *Stenodiplosis* species – that are each associated with a single grass host in the field, including two widespread invasives plants (Guinea grass and buffelgrass) (Chapter 4). Furthermore, it is clear that host-associated groups of African grass-feeding cecidomyiids have not only evolved separately in different tribes, but have produced mutiple specialist feeders with different feeding modes (stem gall-inducers and seed feeders) on the same host plant, in the case of Guinea grass (Chapter 2). This implies that host-specificity in African grass-feeding cecidomyiids may be as common as in European taxa, where a third of grass-feeding cecidomyiids are specific to a single host plant species (Wapshere, 1990). With a similar combination of phylogenetic and field host range evidence, Van Steenderen et al. (2023) found up to 16 new species of *Tetramesa* wasps, eight of which have some evidence for high host-specificity, on other southern African grasses.

The startling amount of novel biodiversity identified in these recent surveys refute a notion, once widely advocated in biocontrol research, that grasses lack a diverse assortment of host-specific insects (Bernays, 1985; Wapshere, 1990). The “escape and radiate” model of coevolution has presumed that the diversification of insect herbivores with limited host ranges has mainly been a response to plant secondary chemicals, which are less diverse and less abundant in grasses compared to most dicotyledonous plants (McNaughton et al., 1985; Kariñho-Betancourt, 2019). Structural components such as silica, and those secondary compounds that grasses do produce – including phenolics, terpenoids and nitrogenous compounds such as alkaloids, cyanogenic glycosides and hydroxamic acids – are nonetheless important general herbivore defences in grasses (Vicari and Bazely, 1993; Massey et al., 2006, 2009; Degenhardt, 2009). Grasses also employ an indirect defence system of herbivore-induced volatile compound blends that attract specific parasitic “defenders” (such as parasitoid wasps and nematodes) to attack specific herbivores (Degenhardt, 2009). Furthermore, highly monophagous insect herbivores such as cecidomyiids are extremely sensitive to plant volatiles in general, using these with visual and tactile cues to detect oviposition sites on preferred hosts (Molnár et al., 2019; Cheng et al., 2020; Sadeghi et al., 2021). Responses to volatiles cluster based on host plant phylogeny rather than herbivore phylogeny, with grass-feeding cecidomyiids showing similar (but not identical) response profiles across different lineages (Molnár et al., 2019). Volatiles as indirect, as well as direct, defences and as indicators of host plant suitability should therefore be expected to be the most prominent drivers of insect herbivore diversification on grasses.

The results of this study also highlight the taxonomic gap faced in the fledgling sub-field of grass biocontrol research as well as the potential for new discoveries. The greatest obstacle encountered in grass biocontrol programs to date has been the current state of taxonomic research in entomology, which is marked by the “taxonomic impediment”: knowledge gaps resulting from the neglect of certain taxa in certain regions; a lack of taxonomic resources, including identification keys; and a lack of taxonomists (Petrović, 2022). The taxonomic impediment is worst in the southern hemisphere, with some large taxa having no described representatives – or taxonomists to describe them – in the entirety of the Afrotropics and Neotropics (Hopkins et al., 2019; Petrović, 2022). It is hoped that these issues can be alleviated by training programs such as those underway to develop young entomologists in identification and taxonomy, particularly of Diptera, in the Afrotropical region, where these skills are lacking in general and particularly among black and female scientists (Jordaens et al., 2024).

Knowledge gaps in taxonomy and host-plant relationships in general, however they have arisen, are a consistent stumbling block to applied research and to biocontrol in particular (Marvaldi, 2024). For example, the global biocontrol program for water hyacinth required – and resulted in – the discovery and description of *Neochetina eichhorniae* Warner, the recollection and redescription of both *Megamelus scutellaris* and *Eccritotarsus catarinensis*, and later the discovery and description of the latter’s cryptic congener, *E. eichhorniae* (Warner, 1970; Deloach, 1975; Sosa et al., 2004; Paterson et al., 2016; Henry, 2018). The stumbling block is even greater for biocontrol programs on grasses, where host associations are more poorly recorded and entire genera of insects are poorly characterized (e.g. *Tetramesa* spp. – Van Steenderen et al., 2023) or, in the case of the cecidomyiids in this study, never collected or described (even informally) prior to the biocontrol program.

Tscharntke and Greiler (1995) pointed out a widespread lack of sampling and taxonomy of the insect fauna on wild grasses as opposed to graminaceous crops, especially endophagous taxa such as cecidomyiids. Other families containing species of agricultural or biocontrol importance, such as the ectophagous Delphacidae, have been better studied in the past, but are currently neglected as well. The current definitive comparative study of phylogeny and host association in the Delphacidae by Urban et al. (2010) relies on a review of host associations that is more than 30 years old at the time of writing, based on records from a relatively small number of researchers (Wilson et al., 1994). Little work has been done on specific host associations within this family since this time, except for studies of a small number of highly damaging agricultural pest species, and aquatic weed biocontrol agents or candidate biocontrol agents in the genus *Megamelus*, namely *Megamelus scutellaris* and *Megamelus toddi* Beamer (Tsai et al., 1996; Li et al., 2009; Tipping et al., 2011; Muduli et al., 2021; Reid et al., 2024).

Taxonomic neglect of grass associates does not necessarily extend to all taxa and regions of the world. For example, in a sweep-net survey of grassland arthropod taxa in Germany by Simons et al. (2016), all adult insects collected in the orders Coleoptera, Hemiptera (Auchennorhyncha and Heteroptera) and Orthoptera were identifiable to species level – 756 species in total. Such a comprehensive biodiversity catalogue is only possible thanks to the investment of effort and time by researchers and institutions into the taxonomy of these orders in Europe over many decades. On the other hand, large genetic barcoding surveys in both Germany and Canada suggest that the Diptera, and particularly the Cecidomyiidae, have been heavily undersampled and contain a very high proportion of “dark taxa” – genetically unique accessions that have either not been identified to species level or are undescribed species (Hebert et al., 2016; Morinière et al., 2019). Species-rich taxa with a small body size and cryptic habits (such as cecidomyiids) are the most likely to go undescribed (Morinière et al., 2019). Based on the proportion of dark taxa, Hebert *et al.* (2016) have estimated that the true species richness of cecidomyiids may be greater even than that of beetles. The cecidomyiids of the Afrotropics in particular are sorely understudied according to world experts on this taxon (none of whom are based in Africa) (Dorchin et al., 2017). A mere four native grass-feeding species have been described from this region to date, which are either grain pests or relatives thereof (the African rice gall midge *Orseolia oryzivora* and the sorghum midges *Stenodiplosis sorghicola* and *Stenodiplosis sorghi* Harris) or potential biocontrol agents (the pypgrass midge *Mitodiplosis graminis* (Kieffer) Kolesik and Wood (2019) (Dorchin et al., 2017).

The taxonomic thoroughness of surveys for arthropod grass associates often depends on the research context. The diverse pests of agriculturally important cereals and turf and pasture grasses are often well-characterized, while the arthropod associates of wild grasses are usually not (Tscharntke and Greiler, 1995; Karpyn Esqueda et al., 2017). Surveys of all arthropod orders occurring on a single grass species are rare outside of a biocontrol or agriculture context, and species identifications of arthropods found in surveys are often not provided (Di Giulio et al., 2001; Nickel and Hildebrandt, 2003; Gillespie et al., 2022; Kinneen et al., 2024; Silva-Monteiro et al., 2022). This may even extend to agricultural research. For example, in a study of the symbiosis between wild and cultivated varieties of tall fescue *Schedonorus phoenix* Scop. Holub. and its fungal endosymbiont *Neophytodium coenophialum* Morgan-Jones & Gams in Baltic Europe, Vesterlund et al. (2011) identified all arthropods present to family, although not to genus or species.

In contrast, in a survey of arthropod natural enemies on buffelgrass in Kenya, Morrison et al. (2023b) identified a third of unique herbivores to genus and morphospecies, the remainder presumably being undescribed or poorly characterized genera.

While species identifications for grass arthropod associates are arguably not necessary for studies of biodiversity or plant-herbivore interactions on a broad scale, their unavailability speaks to either of two problems, one or both of which must be widespread in non-applied research. Either taxonomic expertise is not sufficient to describe and identify the sheer number of species of grass arthropod associates, or these species are rarely considered in the grander picture of biodiversity, and are neglected out of expediency or a lack of awareness. Small, nondescript or cryptic species may easily be neglected, especially Afrotropical species (Hopkins et al., 2019) and species associated with grasses and grasslands (Tscharntke and Greiler, 1995), which are generally afforded low importance outside of their agricultural value or the threat posed by invasive grasses. For example, grassland is the second largest biome in South Africa, but less than 5 % is formally protected compared to over 40 % of forest, the country’s smallest biome (Stats SA, 2021).

Field surveys for candidate biocontrol agents have a high potential to contribute to the taxonomy of otherwise neglected systems and taxa for several reasons. Foremost of these is that biocontrol attracts investment from stakeholders outside of academia. Invasive plants affect agriculture, infrastructure, and human health and livelihoods; they are a tangible threat to landowners, governments and ordinary people (Pyšek et al., 2020; Walsh et al., 2023). The money and will from other sectors is present to mobilize academic resources – including taxonomists – to address a practical problem (i.e. weed control) even if the popularity of biocontrol varies by region and the amount of positive or negative press the discipline receives at a particular time, or if biocontrol efforts are obstructed by conflicts of interest (Charudattan, 2005; Walsh et al., 2023). In non-applied or “blue skies” research, this level of outside investment may be less forthcoming. The nature of classical biocontrol programs also predisposes them to discoveries of new species and species interactions. Biocontrol programs have a narrow focus, investigating a single system with relatively few participants – mainly the target plant, its arthropod herbivores and their predators or parasitoids, and perhaps plant pathogens (Sutton et al., 2019; Morrison et al., 2023b). If field host range surveys are included, close relatives of the target plant will also be investigated (Sutton et al., 2021b; Yell et al., 2024). This is in contrast to many ecological studies that have a broader focus, often encompassing subsections of an entire biome within a certain region (Di Giulio et al., 2001; Nickel and Hildebrandt, 2003; Pawson et al., 2010; Barnett and Facey, 2016). Furthermore, biocontrol surveys cover a wide range of taxa and aim for the highest possible taxonomic resolution. Many orders and families of herbivorous arthropods are used as biocontrol agents; a failure to survey most of the herbivorous associates of a plant means that potentially useful agents may be missed (Reid et al., 2020; Winston et al., 2024). Subsequent host-specificity testing for a candidate agent and granting of import permits require a reliable identification – ideally a formally described species, although a unique morphospecies identification within a genus is also acceptable in some countries, given the slow pace at which new species can be described versus the relative urgency of implementing a biocontrol agent (Dhileepan et al., 2015; Cowie et al., 2023).

6.4. Future direction for the Guinea grass biocontrol program

Host-specificity testing of candidate agents in a controlled setting is essential to determine their fundamental host range and the risk of non-target attack (Briese, 1999; Paterson et al., 2019). The current priority in implementing biocontrol for Guinea grass, therefore, is laboratory-based host-specificity testing of the top-priority candidate agents, the blister gall midge and Lasiopterini sp. megathyrsae. This testing is already underway in the case of the blister gall midge, and methods for testing the seed midge are being developed (Guy Sutton, unpublished data). With field host range data already available from this study (Chapters 3-4), an abridged test plant list based mainly on host phylogeny can be applied to both agents, as has been practiced recently with other cecidomyiid agents (Lesieur et al., 2020). The knowledge gained from this study regarding the biology and rearing of Guinea grass cecidomyiids (Chapter 2) will help to optimize laboratory methods for host-specificity testing.

This study used field host range surveys and phylogenetic methods to identify two cecidomyiid flies, the blister gall midge *Arabukodiplosis vesicaria* and the Guinea grass seed midge Lasiopterini sp. megathyrsae, that are apparently host-specific feeders on Guinea grass and have high potential as biocontrol agents for this plant in its invaded range in Texas. The Guinea grass seed midge was found to be part of an undescribed genus of cecidomyiids associated with the seeds of panicoid grasses, and which appears to contain a number of mainly monophagous species. Both candidate agents are climatically well-matched to invasive Guinea grass populations in Texas, and will now proceed to a final phase of laboratory-based host-specificity testing. This study demonstrates the effectiveness of native-range field host range surveys for the evaluation of agent host-specificity and damage potential. It also highlights the potential of grasses as classical biocontrol targets and reservoirs of taxonomically neglected monophagous insect diversity.

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Supplementary Tables

Table S1: Information on sampling sites visited for natural enemy surveys (repeat survey dates in brackets) and monthly seasonal abundance surveys (indicated by an asterisk *) (Chapter 2).

Sampling site	Province	Coordinates	Date sampled	Sampling method
Rhodes University Zoology Department (Makhanda)	EC	-33.310295, 26.518898	(01,02,03,09)/ 03/2022	Sweep, tillers, panicles
Botanical Gardens (Makhanda)	EC	-33.317844, 26.522003	(12,18)/03/2022	Sweep, tillers
Lothians Road	EC	-33.384429, 26.422246	04/04/2022	Sweep, tillers
Provenance Farm (Salem)	EC	-33.514643, 26.487012	04/04/2022	Sweep, tillers
Kenton-on-Sea	EC	-33.685017, 26.671551	04/04/2022	Sweep, tillers
Kasouga	EC	-33.640612, 26.715494	04/04/2022	Sweep, tillers, panicles
Port Alfred Golf Course	EC	-33.602775, 26.884491	05/11/2022	Tillers, panicles
Port Alfred George Street *	EC	-33.597398, 26.882815	30/11/2022-30/01/2024	Tillers, panicles
Port Alfred Hards Street *	EC	-33.600481, 26.889899	30/11/2022-30/01/2024	Tillers, panicles
Port Alfred Albany Road *	EC	-33.589830, 26.901744	30/09/2023-30/01/2024	Tillers, panicles
Port Alfred Rest Stop *	EC	-33.615076, 26.799669	30/11/2022-30/01/2024	Tillers, panicles
Abbotsford (East London)	EC	-32.957545, 27.912030	23/01/2023	Tillers, panicles
Boesmansriviermond	EC	-33.681288, 26.652853	28/02/2023	Tillers, panicles
Westville (Durban)	KZN	-29.818750, 30.909400	16/03/2023	Tillers, panicles
Gonubie	EC	-32.952667, 28.010900	20/03/2023	Tillers, panicles
Southwell	EC	-33.557833, 26.729633	24/03/2023	Sweep, tillers, panicles
Mount Pleasant	EC	-33.528300, 26.527183	24/03/2023	Tillers, panicles
Port Elizabeth	EC	-33.978633, 25.536600	03/04/2023	Sweep, tillers
Zuurberg	EC	-33.391668, 25.721615	04/04/2023	Tillers, panicles
Richard’s Bay	KZN	-28.690164, 31.708161	11/04/2023	Tillers, panicles
Bedlane	KZN	-28.706444, 31.547778	11/04/2023	Tillers, panicles
Mkuze	KZN	-27.679300, 32.059500	10/05/2023	Tillers, panicles
Melmoth plantation	KZN	-30.568222, 29.817500	24/05/2023	Tillers, panicles
Kidd’s Beach	EC	-33.138146, 27.68162	29/01/2024	Tillers, panicles

Table S2: GenBank sequence accession information for seed midge phylogenies (Chapter 4).

Sequence ID	**Species / OTU**	**Marker**	**Reference / Authors**
OK330051.1	*Asiodiplosis stellata*	Cox1	Dorchin et al., 2021
KP399947.1	*Baldratia* sp.	Cox1	Dorchin et al., 2015
FJ803277.1	*Calamomyia phragmites*	Cox1	Stireman III et al., 2010
MN191274.1	*Contarinia loti*	Cox1	Dorchin et al., 2019a
OQ107473.1	*Contarinia* sp.	Cox1	Netta Dorchin, Tel Aviv University
MN191277.1	*Contarinia tiliarum*	Cox1	Dorchin et al., 2019a
KP082884.1	*Dactylasioptera lebelae*	Cox1	Veenstra et al., 2018
KY584269.1	*Dactylasioptera milnae*	Cox1	Veenstra et al., 2018
MT763285.1	Dactylasioptera sp. n. Rixon 1	Cox1	Rixon et al., 2021
EU375701.1	*Dasineura folliculi*	Cox1	Dorchin et al., 2009
MN207682.1	*Lasioptera arundinis*	Cox1	Dorchin et al., 2019b
KP399946.1	*Lasioptera carophila*	Cox1	Dorchin et al., 2015
MG684839.1	*Micromyini* sp.	Cox1	Sikora et al., 2019
MN191327.1	*Neolasioptera vernoniae*	Cox1	Dorchin et al., 2019a
MN191330.1	*Orseolia oryzae*	Cox1	Dorchin et al., 2019a
KP399904.1	*Ozirhincus millefolii*	Cox1	Dorchin et al., 2015
OL415485.1	*Ruschiola attenuata*	Cox1	Dorchin et al., 2022
OL415468.1	*Ruschiola succulenta*	Cox1	Dorchin et al., 2022
KP399949.1	*Stefaniola* sp.	Cox1	Dorchin et al., 2015
MZ382470.1	*Stenodiplosis bromicola*	Cox1	Rector et al., 2021
MZ382472.1	*Stenodiplosis geniculati*	Cox1	Rector et al., 2021
MZ382471.1	*Stenodiplosis sorghicola*	Cox1	Rector et al., 2021
MN207650.1	*Suaediola rufa*	Cox1	Dorchin et al., 2019b
MF479698.1	*Asphondylia nepetae*	28S	Bernardo et al., 2018
MN201212.1	*Asphondylia punica*	28S	Dorchin et al., 2019b
MN201215.1	*Asteromyia carbonifera*	28S	Dorchin et al., 2019b
MG684649.1	*Contarinia loti*	28S	Sikora et al., 2019
MN201229.1	*Contarinia lycii*	28S	Dorchin et al., 2019b
MG684636.1	*Dasineura kellneri*	28S	Sikora et al., 2019
MG684639.1	*Dasineura trifolii*	28S	Sikora et al., 2019
MN201247.1	*Drisina glutinosa*	28S	Dorchin et al., 2019b
MN201250.1	*Feltiella tetranychi*	28S	Dorchin et al., 2019b
MN201266.1	*Kiefferia pericarpiicola*	28S	Dorchin et al., 2019b
MN201268.1	*Lasioptera arundinis*	28S	Dorchin et al., 2019b
KP288806.1	*Lasioptera rubi*	28S	Ševčík et al., 2016
MN201274.1	*Lestodiplosis miki*	28S	Dorchin et al., 2019b
MG684674.1	*Lestremia leucophaea*	28S	Sikora et al., 2019
MN201275.1	*Lopesia niloticae*	28S	Dorchin et al., 2019b
MN201276.1	*Macrodiplosis pustularis*	28S	Dorchin et al., 2019b
KC177649.1	*Mayetiola destructor*	28S	Wiegmann et al., 2011
MG684667.1	*Micromyini* sp.	28S	Sikora et al., 2019
KJ136767.1	*Mikiola fagi*	28S	Ševčík et al., 2014
MN201285.1	*Neolasioptera vernoniae*	28S	Dorchin et al., 2019b
MN201288.1	*Orseolia oryzae*	28S	Dorchin et al., 2019b
MG684645.1	*Ozirhincus longicollis*	28S	Sikora et al., 2019
KP288805.1	*Porricondyla nigripennis*	28S	Ševčík et al., 2016
MG684641.1	*Rhopalomyia tanaceticola*	28S	Sikora et al., 2019
MG684643.1	*Rondaniola bursaria*	28S	Sikora et al., 2019
MN201306.1	*Schizomyia galiorum*	28S	Dorchin et al., 2019b
MN201307.1	*Sitodiplosis mosellana*	28S	Dorchin et al., 2019b
MG684640.1	*Spurgia euphorbiae*	28S	Sikora et al., 2019
OQ566751.1	*Suaediola* sp.	28S	Zhao et al., 2023