PHYLOGENETICS AND HISTORICAL BIOGEOGRAPHY OF THE TELOGANODIDAE (EPHEMEROPTERA)

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Abstract

The Teloganodidae are a mayfly family endemic to the southwestern Cape (South Africa), with relatives in Madagascar and Asia. Like many other aquatic invertebrates in Africa, they have been considerably understudied. Research into biodiversity and biogeography allows an understanding of the earth's biota, producing knowledge which can be used to develop strategies to preserve and monitor this biota. Mismanagement of water systems places biodiversity of river fauna under an ever-increasing extinction threat.

This investigation explores rivers in under-collected areas to determine how well teloganodids have been represented in the literature, with four genera and five species described at the onset of this study. A lectotype for *Lestagella penicillata* Barnard (1940) has been elected and described in detail, setting "benchmark" characters for future descriptions.

Standard DNA sequencing methods provide portions of three mitochondrial genes; cytochrome oxidase subunit I (COI), small subunit ribosomal 16S RNA (16S), 12S ribosomal DNA (12S) and two nuclear genes, Histone 3 (H3) and 28S ribosomal DNA (28S) for up to 255 specimens. Fore and hind wings of 79 teloganodid adults were used to examine phylogenetic signal and evolutionary divergence using geometric morphometrics.

A multi-faceted approach is used to investigate relationships between clades and the effects of deep-time climatic and landform changes which have influenced the diversity and distribution seen today. Tree (Bayesian Inference and Maximum Likelihood) and network (parsimony) phylogenies, ancestral reconstruction, historical biogeography and wing-evolution of the Teloganodidae are investigated.

Species tree analyses discovered 27 species and six genera. Distinct lineages are restricted to catchments, and strong phylogeographic structure was found within most genera. Southern African Teloganodidae are shown to have originated in the Cretaceous, with divergence and dispersal of lineages depended on their established locality at the time of tectonic events (uplift) and climatic changes (sea level regressions and transgressions). Geographic clines in wing-shape of *Lestagella* across its range imply evolutionary adaptations to specific catchment landscape and environment.

A detailed analysis of biodiversity has many valuable contributions, from directing future research, understanding adaptive processes, fine-tuning phylogeographical and evolutionary hypotheses, to improving management and conservation decisions in order to preserve endemic biodiversity hotspots.

Table of Contents

Abstract …… i
Table of Contents …… iii
List of Tables …… vii
List of Figures …… x
Preface …… xxiii
Acknowledgements …… xxv
Declaration …… xxviii

Chapter One: Introduction with a Review of Taxonomic History …… 1
1.1 Review of taxonomic history and distribution of family, Teloganodidae … 2
1.2 Species Conceptualisation and Delimitation, and Molecular Phylogenetics of Teloganodidae …… 9
1.3 Historical Biogeography and Ancestral Reconstruction …… 11
1.4 Geometric Morphometrics …… 13
1.5 Scope …… 14
1.6 General Aims and Overview …… 15

Chapter Two: Sampling Methods, Distributions and Biological Notes …… 16
2.1 Introduction …… 16
2.2 General Methods …… 18
2.2.1 Sampling …… 18
2.2.2 Preparation of samples …… 20
2.3 Distributions, Habitat Requirements and Life History …… 21
2.3.1 *Ephemerellina* Lestage …… 21
2.3.2 *Lestagella* Demoulin …… 21
2.3.3 *Lithogloea* Barnard …… 21
2.3.4 *Nadinetella* McCafferty and Wang …… 22
2.3.5 Previously unknown taxa …… 23
2.4 Conclusions …… 25

Chapter Three: Lectotype selection and Redescription of *Lestagella penicillata s.s* (Barnard) 1932 …… 26

3.1 Introduction and taxonomic background …… 26

3.2 Methods and Materials …… 27

3.3 Results ……..... 29

3.4 Discussion ……..... 43

3.5 Perspectives …… 45

Chapter Four: Molecular Phylogenetics and Species Delimitation ……..….. 46

4.1 Introduction ……. 47

4.2 Methods and Materials …… 49

4.2.1 Field work and laboratory protocols ……..…… 49

4.2.2 Phylogenetic analyses …… 52

4.2.2.1 Gene Tree analyses ……..….. 52

4.2.2.2 Splits Networks – Data summary and Reticulation …... 54

4.2.2.3 Species Tree Estimation . ……. 56

4.2.2.4 Landscape genetics ……..…….. 57

4.3 Results ……. 59

4.3.1 Data characteristics ……...……. 59

4.3.2 Phylogenetic and Data-summary Analyses ……... 62

4.3.2.1 Neighbour Network for COI dataset ……. 63

4.3.2.2 African/South American/Asian Group ("Afr/SAm Group") …….. 66

4.3.2.3 African/Madagascan Group ……..….. 71

4.3.2.4 *Ephemerellina*/Species A/SpeciesB/Species C Group ("Ephem Clade") …….. 75

4.3.2.5 *Lestagella* Clade ("Lest Clade") ……..……. 80

4.3.2.6 *Nadinetella*/*Lithogloea Clade* ("Nad/Lith Clade") …... 85

4.3.3 Species Tree Estimation and Rosenberg's Probability …….. 90

4.3.4 Landscape genetics ……..….. 95

4.4 Discussion …… 106

4.5 Conclusions ……..……... 110

Chapter Five: Ancestral Reconstruction and Historical Biogeography ……..…… 112

5.1 Introduction ……. 113

5.2 Methods and Materials …… 120

5.2.1 Molecular dating using BEAST …… 120

5.2.2 Ancestral Area Reconstruction ……..……. 121

5.3 Results ……..…… 124

5.3.1 Deeper time reconstruction …… 124

5.3.2 "Ephem Clade" ……..…… 129

5.3.3 "Lest Clade" ……..……... 132

5.3.4 "Nad/Lith Clade" ……..… 135

5.3.5 Dispersal, Vicariance and Extinction Time-Events …….…. 139

5.4 Discussion ……. 141

5.4.1 Deep time reconstruction ……..…. 143

5.4.2 Southern African Teloganodidae Reconstruction ……. 148

5.5 Conclusions ……..…… 159

Chapter Six: Geometric Morphometrics: Wing Shape Variation, Evolutionary Allometry and Integration ……..…… 161

6.1 Introduction ……. 162

6.2 Methods ……... 164

6.2.1 Specimen Preparation …… 164

6.2.2 Morphometric Data Features ……..…… 162

6.2.3 Exploring shape space with PCA, BG-PCA and CVA ……..... 168

6.2.4 Phylogeny and comparative approach ……... 170

6.2.5 Patterns of Evolutionary Diversification in shape space ……... 173

6.2.6 Evolutionary Allometry and Size Correction …… 174

6.2.7 Patterns of variation in evolutionary shape changes …… 175

6.2.8 Evolutionary integration between fore and hind wing ……. 175

6.2.9 Angular Comparisons of results from PCA, PLS and Regression ……... 176

6.3 Results ……. 177

6.3.1 Exploring patterns in original shape space …… 177

6.3.2 Exploring patterns in shape space (PCA, CVA, BG-PCA) ….. 181
6.3.3 Phylogeny and Patterns of Evolutionary Diversification …… 191
6.3.4 Evolutionary Allometry ……. 197
6.3.5 Patterns of variation in evolutionary changes ……... 202
6.3.6 Evolutionary integration ……..….. 206
6.4 Discussion …… 210
6.5 Conclusions ……..…… 214

Chapter Seven: General Discussion and Conclusions ……..…… 216
7.1 A Multi-faceted Approach ……... 216
7.2 Future Directions ……. 219
7.3 Concluding Remarks ……..…….. 221

References …… 222
Appendix ……..……. 243

List of Tables

Table 1.1. A summary of the current global members of the Teloganodidae (the South African taxa are the focus of this study). The current valid names of the species (indicated by ●) are followed by subordinate names when these exist (indicated by ○). The subordinate names are followed by an abbreviation in parentheses, indicating their status. All names, valid or subordinate, are followed by the actual author of that name and the date that name was published. The authors responsible for the latest names changes are indicated at the end of each list of synonyms. Author names are included in this table for first mention …….. 3

Table 2.1. Details of the three unknown Teloganodidae taxon groups, currently known distribution as a result of this study and a brief description of the most important diagnostic morphological characters and habitat preferences ……. 24

Table 4.1. Data characteristics showing model selection after identical sequences were removed from the various alignments …….. 60

Table 4.2. Partitions, congruent and incongruent datasets used for analyses. Code names for each dataset are listed below and are used in the manuscript from this point forward. Mitochondrial datasets and 5-gene datasets listed are used for splits and reticulation networks. Model selection for ML analyses calculated in PartitionFinder, and models according to Bayes Factor results (Bayes factors < 2 for all datasets, therefore only one model was necessary) . 61

Table 4.3. Delta and Q-residuals scores for each dataset used in SplitsTree Network analyses …… 62

Table 4.4. The contribution of isolation by distance (IBD) for described species of Teloganodidae. Using log (genetic similarity (M)) and log (geographic distance). Bold values indicate a significant effect (alpha < 0.05). Please note that there were too few specimens of Species C and *N.* 'Robertson' to 95

conduct an IBD. Sample numbers used for IBD analyses can be found in Appendix 4E …….

Table 5.1. Summary of key events and effects on Africa from the Late Jurassic until the Holocene, with focus on southern Africa …… 114

Table 5.2. Ancestral range areas used in the RASP analyses, each area is coded with an alphabetic letter, codes are shown for each of the RASP analyses performed on the various datasets ……. 123

Table 6.1. Individuals included in geometric morphometric analyses, with number of representatives at each taxon level presented for right-side fore and hind wings …… 164

Table 6.2. River catchment localities associated with the species numbers assigned to the *Lestagella* genus, higher clade group indicated in brackets (corresponding to Chapter Four analyses) ……... 165

Table 6.3. Procrustes ANOVA for Centroid size variation, for the left and right Fore wings of 25 individuals …… 180

Table 6.4. Procrustes ANOVA for Shape variation for the left and right Fore wings of 25 individuals …….. 180

Table 6.5. Procrustes ANOVA for Centroid size variation for the left and right Hind wings of 25 individuals …… 180

Table 6.6. Procrustes ANOVA for Shape variation for the left and right Hind wings of 25 individuals ……... 180

Table 6.7. Tree lengths and phylogenetic signal permutation test results for the generic and specific taxon levels for the fore and hind wings ……. 191

Table 6.8. Fore wing PCA of variation among the shapes of generic means, both for original and size-corrected shape data ……. 193

Table 6.9. Hind wing PCA of variation among the shapes of generic means, both for original and size-corrected shape data ……. 193

Table 6.10. Fore wing PCA of variation among the shapes of species means and independent contrasts, both for original and size-corrected shape data ……. 194

Table 6.11. Hind wings PCA of variation among the shapes of species means and independent contrasts, both for original and size-corrected shape data ….... 194

Table 6.12. Angular comparisons between PCA for species means for complete shape variation and PCA for independent contrasts for complete shape variation, to examine patterns of evolutionary variation in wing shape. Permutation test against the null hypothesis that the vectors have random directions ….……. 204

Table 6.13. Angular comparisons between PCA for independent contrasts for complete shape variation and size corrected PCA for independent contrasts, to characterise for patterns of evolution without allometric effects. Permutation test against the null hypothesis that the vectors have random directions ……. 204

Table 6.14. Angular comparisons between PCA for independent contrasts for complete shape variation and Partial Least Squares (PLS) analysis of integration between the fore wing and hind wing. Permutation test against the null hypothesis that the vectors have random directions ……... 208

Table 6.15. Angular comparisons between uncorrected Partial Least Squares (PLS) analysis of integration between the fore wing and hind wing and size corrected PLS analysis. Permutation test against the null hypothesis that the vectors have random directions ……. 208

List of Figures

Figure 1.1. Current suggested dispersal/vicariance route for global Teloganodidae, with currently known global generic distribution (synthesized from literature Edmunds, 1972; 1975; McCafferty and Wang; 1997; McCafferty, 1999; Monaghan et al. 2005; Sartori et al., 2008) 13

Figure 2.1. Reference map to the regions, boundaries and mountain ranges of the general study area, (a) Africa divided into three main sub regions referred to throughout this thesis, (b) the Greater Cape Floristic Region (GCFR) and Cape Floristic Regions defined in South Africa, and (c) map of the Cape Floristic Region where southern African Teloganodidae are recorded, showing the Cape Fold Belt and escarpment boundaries. Mountain ranges are labelled where main river catchments are located. Note that the Afromontane Forest Rivers include all coastal rivers in the Outeniqua and Tsitsikamma areas. Gt. Winterhoek = Groot Winterhoek Mountains, South African Provinces: WC – Western Cape, EC – Eastern Cape, KZN – KwaZulu Natal, FS – Free State, NW – North West, G – Gauteng, MP – Mpumalanga, LM – Limpopo …… 17

Figure 2.2. Map of Albany Museum Teloganodidae locality records (triangle shapes) obtained from the Albany Museum (AMGS) database at onset of study; all localities sampled throughout the study (red “X” shapes); and from these, the localities where Teloganodidae were found (blue diamond shapes). Sampling sites with only a red cross (“X”) show sites sampled where Teloganodidae were absent …….. 20

Figure 2.3. General habitats arrowed where southern African Teloganodidae have been collected, (a) *Isolepis digitata* in swift current about the waterfall, and in the waterfall, *Ephemerellina*, Species B and Species C are found mostly in this vegetation type, (b) *Lestagella* and *Lithogloea* are found in shallow (ca. 30 cm depth) stones in current, (c) Species A is found in marginal vegetation in moderate to no current, and (d) *Nadinetella* is strongly associated with *Wardia hygrometrica* moss that grows in swift currents and waterfalls …… 22

Figure 3.1. Map of recorded and sampled localities of the genus *Lestagella*. Diamonds represent sampled localities, triangles are previously recorded localities obtained from the Albany Museum (AMGS) database, and the cross ("X") denotes the lectotype locality from Table Mountain slopes …….. 28

Figure 3.2. Paralectotypes of *Lestagella penicillata*. (a) Lateral view of adult female; (b) lateral view of adult male. Dorsal views of (c) female nymph and (d) male nymph, arrow showing foreleg regeneration where one foreleg is noticeably smaller than the other and without various types of setae. (e) Ventral view of male nymph. Scale bars = 1 mm ……..…….. 31

Figure 3.3. Lectotype male imago of *Lestagella penicillata*. (a) Forewing with dotted line showing basally faint RS vein, dashed lines show variations in intercalaries seen in other specimens, intercalaries always absent between RA and RSa; (b) hindwing ovoid in shape with costal elevation and slight medial depression as depicted, variations in venation are shown as dashed lines; (c) Ventral margins of both fore- and hindwings showing jagged edge possibly as a result of shedding of subimaginal falcate microtrichia …….. 32

Figure 3.4. Paralectotype male imago of *Lestagella penicillata*. (a) Foreleg showing details of (b) femora; (c) tarsus and (d) tarsal claw. (e) Hindleg showing (f) details of the tarsi and claw and (g) spine-like processes of tarsi (female imago fore-, mid-and hindlegs similar to male hindleg) ……... 34

Figure 3.5. Lectotype male (a) genitalia of *Lestagella penicillata* showing details of genital forceps (b) segment II and (c) segment III ……..... 36

Figure 3.6 – 3.8. Paralectotype nymph of *Lestagella penicillata*. Figure 3.6. Labrum showing (a) feathered setae; (b) remnant setal bases; (c) long, smooth setae (not feathered); Figure 3.7. Hypopharynx, dorsal view (right) and ventral view (left); Figure 3.8. Labium …….. 37

Figure 3.9 – 3.13. Paralectotype nymph of *Lestagella penicillata*. Figure 3.9. Ventral surface of mandibles; Figure 3.10. Left mandible; Figure 3.11. Right mandible; Figure 3.12. Right maxilla; Figure 3.13. Worn maxilla from a mature nymph ……... 39

Figure 3.14. Paralectotype nymph foreleg of *Lestagella penicillata* showing (a) spatulate setae; (b) variable medio-transverse gap on femur; (c) tarsal claw showing denticles and subapical setae …….. 41

Figure 3.15. Paralectotype nymph gills of *Lestagella penicillata*. (a) Filamentous first gill I on abdominal segment I; (b) operculate lamellate gill II found on abdominal segment II; (c) lamellate gill III; (d) lamellate gill IV……. 42

Figure 3.16. Regrowth of mouthparts of *Lestagella penicillata*. (a) Left mandible; (b) right mandible; (c) maxilla; (d) hypopharynx; (f) labium and (f) labrum ……... 44

Figure 4.1. Distribution of (a) localities where Teloganodidae samples across genera were collected and used for molecular analyses, (b) eight sampling areas of the Breede River catchment split into three basic groups; Breede-South, Breede-East and Breede-West, and (c) sampling localities in the Afrotropics, Ephemerythidae from Zambia and Limpopo Province (see Chapter Two, Figure 2.1), and *Manohyphella* from Madagascar ……. 50

Figure 4.2. COI Neighbour Net network showing relationships between 214 samples (centre network), relationships within genera are shown in coloured splits networks which are enlarged to enable visual detail of clustering. NN bootstrap values shown. Edge labels represent either river or region names where samples were collected (Appendix 4A), labels are colour coded according to catchment (refer to Figure 4.1). Long edges represent good support …… 65

Figure 4.3. The "Afr/SAm Group", ML best scoring tree with ML bootstrap support followed by BI posterior support. Nodes in the phylogram represent main division of "fringed" and "non-fringed" South African Teloganodidae. Species clades and subclades are deoicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.4 ……..…….. 68

Figure 4.4. Neighbour Net network with ML bootstrap support (from ML consensus trees) super-imposed onto the network, followed by NN bootstrap support (Grey lines drawn onto network to depict well-supported clades formed by the distance-based NN and ML bootstrap). The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge. Main clusters (within and between genera) denoted by coloured bubbles. Long edges represent good support ……. 69

Figure 4.5. Reticulation network for higher orders using the "Supernetwork" analysis of the five combined genes (COI/16S/12S/28S/H3). Edges are coloured in blue, indicating a reticulation event. "Supernetworks" do not need all trees to have the same taxa (for example, there is no 28S sequence for *Manohyphella*). Long edges represent good support 70

Figure 4.6. The "Afr/Mad Group", ML best scoring tree with ML bootstrap support followed by BI posterior support. Species clades and subclades are depicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.6 ……..…. 72

Figure 4.7. NN network with ML bootstrap consensus of "Afr/Mad Group". Both the bootstrap from ML tree and then bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge. Long edges represent good support …… 73

Figure 4.8. Consensus "Hybridisation" or reticulate network of the "Afr/Mad Group", representing four genes (COI/16S/12S/H3). Long edges represent good support …….. 74

Figure 4.9. The "Ephem Clade" ML best scoring tree with ML bootstrap support followed by BI posterior probability support. Species clades and subclades are depicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.10 ……. 77

Figure 4.10. Implicit NN network with ML bootstrap consensus of the "Ephem Clade". Both the bootstrap from ML tree and then bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch") Long edges represent good support …….. 78

Figure 4.11. "Ephem Clade": a) Reticulation network based on consensus network of ML gene trees and b) simplified Reticulation network based on 50 majority rule of ML gene trees ……. 79

Figure 4.12. "Lest Clade", ML best scoring tree with ML bootstrap support followed by BI posterior support. Well-supported clades are shown with colour-coded bars, which correspond to the coloured catchments on the included map of the sample area. The red bar on the map between L1 and L3 represents a genetic barrier observed between the two groups ……. 82

Figure 4.13. Implicit NN network with ML bootstrap consensus of the "Lest Clade". Both the bootstrap from ML tree and then bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch") Long edges represent good support …….. 83

Figure 4.14. "Lest Clade" (a) Reticulation network based on consensus network of ML gene trees and b) simplified Hybridisation network based on 50 majority rule of ML gene trees ……..... 84

Figure 4.15. "Nad/Lith Clade". ML best scoring tree with ML bootstrap support followed by BI posterior support. Tip labels for *Nadinetella* are colour-coded corresponding to the included map. Grey bars depict well-supported clades ……..... 87

Figure 4.16. Implicit NN network with ML bootstrap consensus of the "Nad/Lith Clade". Both the bootstrap from ML tree and the bootstrap result from NN distance analysis are presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch") Long edges represent good support ……..…… 88

Figure 4.17. "Nad/Lith Clade" (a) Reticulation network based on consensus network of ML gene trees and b) simplified reticulation network based on 50 majority rule of ML gene trees. Coloured labels correspond to catchments (see Figure 4.15) ……..…… 89

Figure 4.18. Species Tree estimation (a) using only mitochondrial genes COI/16S/12S, analysis done using 115 taxa (represented within the terminal taxon assignment groups above), Significance from Rosenberg analysis indicated as red circles (COI/16S/12S) and blue circles (COI only, when there were not enough samples) at the nodes. Rosenberg single representatives were removed. Rosenberg's probability of reciprocal monophyly, circled nodes significant to alpha < 0.05. Described species are shown in bold 94

Figure 4.19. Barrier Analysis for the *Ephemerellina* clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier …….. 98

Figure 4.20. Barrier Analysis for the *Lestagella* clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier …….. 99

Figure 4.21. Barrier Analysis Lithogloea clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier …….. 100

Figure 4.22. Barrier Analysis for the Species A clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier …….. 101

Figure 4.23. Barrier Analysis for the *N. brincki* and *N. crassi* complexes, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier 102

Figure 5.1. Pictorial reconstruction showing isobath up to 1000m below current sea level, and palaeo-drainage patterns from Late Cretaceous to LGM. Inland Early Tertiary drainage flows towards the Orange River. Data from sources: Partridge and Maud, 1987; Swartz *et al.*, 2008; Chakona *et al.*, 2013; Franceschini, 2003; Hendey, 1983; Allsop *et al.*, 2014 ……..... 119

Figure 5.2. "Afr/SAm Group" and "Afr/Mad Group" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Nodes with three circles represent the BBM, S-DEC and S-DIVA analyses in this order (to be read from left-to-right or top-to-bottom). Nodes with only one circle indicate that all three analyses returned identical results. Numbered nodes are discussed in the text for possible dispersal and vicariant events. Asterisks indicate 100 % support …….. 127

Figure 5.3. "Ephem Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast) …….. 131

Figure 5.4. "Lest Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast) ……... 134

Figure 5.5. "Nad/Lith Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast) ……... 138

Figure 5.6. Time-events algorithm graphs showing dispersal, vicariance and extinction events from all analyses (BBM, S-DEC and S-DIVA) between the different clade analyses. Timing and magnitude of sea level changes are depicted at the bottom of the graphs (modified from Siesser and Dingle, 1981). Green arrows represent timing of uplift events in the Miocene and Pliocene. Red star represents timing of earliest and highest number of dispersal and vicariant events. Trends are similar between analyses for each clade. Note that these graphs included all events estimated in the analyses, regardless of probability 140

Figure 5.7. Possible hypothesis for the Jurassic/Cretaceous distributions and divergences of the common ancestors of Teloganodidae, Melanemerellidae and Ephemerythidae; based on available evidence, Possible ancestral ranges during (a) the Triassic; (b) Jurassic, where growing desert band prevents north-south dispersal; (c) Late Jurassic, desert band and Lemuria separation causes barrier, isolating Asia and northern Afrotropical Africa; (d) Late Jurassic/Early Cretaceous, volcanism prevents north south dispersal, apparent dispersal from South Africa to Madagascar, possibly via the north western rim of Antarctica, (e) Cretaceous, Ephemerythidae/Melanemerellidae diverge as South America separates from Africa, volcanism prevents north-south dispersal, Madagascan and Asian lineages distinct and continue diverging, (f) after Mid Cretaceous, northern Afrotropical African Ephemerythidae lineage disperses towards the south as desert recedes and forests become widespread. See text for more detailed explanations ……..……. 147

Figure 5.8. Escarpment retreat in the Tertiary and watershed migration, (a) Early Tertiary escarpment was located near the coast (brown line), and moved inland with erosion or "scarp retreat" (purple line), the Amathole Mountains are remnant of the Mid- to Late Tertiary. 1000 m isobaths illustrated by dotted line. Note that drainage on the coastal side increases as the escarpment retreats, hexagons and red arrows depict possible areas where watershed migration may have occurred, (b) diagram of watershed migration redrawn from Skelton (1994) [Permission received 09/01/2016]. Species transferred to opposing drainage system and fragmented by scarp retreat without dispersion (range expansion taking place). Extinction in this process could mean the complete transfer of species from one system to another (e.g. from palaeo-Orange tributary to coastal rivers) …… 151

Figure 6.1. General Teloganodidae wings with venation labels for the (a) fore wing showing 16 landmark configurations chosen for this study, landmarks in red were removed for the *Lestagella* dataset analyses, (b) hind wing showing 13 landmark configurations, landmarks in red were removed for the *Lestagella* dataset analyses. ……... 167

Figure 6.2. Distribution map showing the localities for each of the *Lestagella* specimens included in the GMM analyses, each symbol represents river localities within the labelled catchments ……..…… 167

Figure 6.3 – 6.4. Maximum Likelihood phylogeny used to map morphometric data for (6.3) genus and (6.4) species. Bootstrap support values >70 are shown …….……... 172

Figure 6.5. Generic level fore wing shape variation of (a) standard PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate ……... 185

Figure 6.6. Generic level hind wing shape variation of (a) standard PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate ……... 186

Figure 6.7. Species level fore wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate …….. 187

Figure 6.8. Species level hind wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate ……..... 188

Figure 6.9. *Lestagella* populations separated by river catchment locality, fore wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate. Data analyses conducted with nine landmark configurations …… 189

Figure 6.10. *Lestagella* populations separated by river catchment locality, hind wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate. Data analyses conducted with nine landmark configurations ……..... 190

Figure 6.11. PCA-mapped phylogenies showing phylogenetic structure for uncorrected shape variation and changes for, (a) fore wing at species level, showing shape changes for PC1 – 3, and (b) hind wing at species level, showing shape changes for PC1 -3 ……...…….. 195

Figure 6.12. PCA histogram plots of Pooled within-taxa covariance matrix of wing shape, showing the first two PCs for (a) Fore wings at species level (showing scale factors of shape change from -0.04 to +0.04), and (b) hind wings at species level (-0.09 to +0.09) …….. 196

Figure 6.13. (a) evolutionary allometry between species in fore wing, (b) Evolutionary allometry between species in the hind wing (c) allometry within-taxa in the fore wing, (d) hind wing pooled within-taxa, (c and d) characterised by pooled within-group regression of shape of log-transformed centroid size. The scatter shows the individual deviations from the taxon means of shape and log transformed centroid size …….. 200

Figure 6.14. PCA-mapped phylogenies showing phylogenetic structure for size-corrected shape variation for, (a) fore wing at species level, showing changes for PC1 – 3, and (b) hind wing at species level, showing shape changes for PC1 –3. Shapes representing each genus after size correction can be seen in Appendix 6C. ……. 201

Figure 6.15. Patterns of evolutionary diversification in fore wing shape: a PCA for independent contrasts for complete variation in (a) the species level and, (b) PCA for the residuals from the regression of IC of shape on the IC of log transformed centroid size species …… 205

Figure 6.16. Patterns of evolutionary diversification in hind wing shape: PCA for independent contrasts for complete variation a) at species level and (b) PCA for the residuals from the regression of IC of shape on the IC of log transformed centroid size species ……... 205

Figure 6.17. Patterns of evolutionary integration between fore wing and hind wing shape: column a) PLS for independent contrasts for complete variation in in the species, column b) PLS (size-corrected) for the residuals from the regression of IC of shape on IC of log-transformed centroid size among species …….. 209

Figure 6.18. Teloganodidae in flight, demonstrating the amplexiform wing coupling, where the costal projection of the hind wing assists in coupling (taken by Geoff McIlleron, NIKON D200, 17/12/2008, at 20h51, permission granted: 12/01/2016) …….……. 214

Preface

This thesis is comprised of seven chapters. The first two chapters provide a brief introduction on the taxon selected, some background on the methods chosen, the taxon distribution and some biological notes observed in the duration of the study. Chapters Three to Six contain brief, specific introductions and aims, analyses and specific discussions. Chapter Seven provides a brief general discussion and suggests future research options.

Extra data and information that would have otherwise distracted the flow of the thesis were included in the appendix. Thus details of material examined or used for molecular analyses, data analyses sample lists and extra analyses for interest and comparative purposes are all included in the appendix.

Some of the work presented in this thesis has already been published. As this work has been written as a full thesis rather than as a series of papers, the content of published sections is included in the main body of the thesis and not necessarily in the same format as in the publication:

Chapter Three:

Pereira-da-Conceicoa, L.L. & Barber-James, H.M. (2013) Redescription and lectotype designation of the endemic South African mayfly Lestagella penicillata (Barnard, 1932) (Ephemeroptera: Teloganodidae). Zootaxa, 3750 (5), 450–464.
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Other publications anticipated from the work are outlined below:

1) Pereira-da-Conceicoa LL, Barber-James HM. A revision of *Lestagella* (Ephemeroptera: Teloganodidae) with new species descriptions. *Zootaxa.*

2) Pereira-da-Conceicoa LL, Barber-James HM. A revision of *Lithogloea* (Ephemeroptera: Teloganodidae) with new species descriptions. *Aquatic Insects.*

3) Pereira-da-Conceicoa LL, Barber-James HM. A revision of *Nadinetella* (Ephemeroptera: Teloganodidae) with new species descriptions. *Aquatic Insects.*

4) Pereira-da-Conceicoa LL, Barber-James HM. A revision of *Ephemerellina* (Ephemeroptera: Teloganodidae) with new species descriptions. *Aquatic Insects.*

5) Pereira-da-Conceicoa LL, Barber-James HM. Inferring phylogenies and evolutionary shape history using geometric morphometrics of Teloganodidae wing shape. *Biological Journal of the Linnean Society*.

6) Pereira-da-Conceicoa LL, Barber-James HM, de Moor FC. Freshwater macroinvertebrate surveys for EIAs: The importance of refined identifications of species for ecological management practices. *Journal of Insect Conservation.*

7) Pereira-da-Conceicoa LL, Barber-James HM. Historical biogeography and the drivers of high biodiversity in the Cape Floristic Region: A case study using Teloganodidae (Ephemeroptera). *BMC Evolutionary Biology*.

8) Pereira-da-Conceicoa LL, Barber-James HM. Combined morphological and molecular synthesis of Global Teloganodidae (Ephemeroptera) with a biogeographical perspective. *PLoS ONE*.

9) Pereira-da-Conceicoa LL, Chakona A, Barber-James HM, Gouws G, Swartz E. Evolutionary drivers of diversification and distribution of a southern temperate ephemeropteran family (Teloganodidae) and fish assemblage in the Cape Floristic Region. *BMC Evolutionary Biology*.

Open Access, interactive identification keys (using Lucid Builder 3.3) will be available for both nymphs and adults of southern African Teloganodidae. Will be accessible at the Scratchpads site (http://teloganodidae.myspecies.info).

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Declaration

The following thesis has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author unless otherwise stated.

Chapter One

Introduction with a Review of Taxonomic History

"Biodiversity starts in the distant past and points toward the future"

~ Frans Lanting

The Teloganodidae are a mayfly family with genera endemic to the southwestern Cape of South Africa, and relatives occurring in Madagascar and Asia. They have been considerably understudied in South Africa, and this investigation sets out to explore rivers in under-collected areas to find out just how well the teloganodid fauna has been represented in the literature up to this time. It also explores relationships between different clades within the family, as well as effects of deep time climatic and landform changes which have influenced the diversity and distribution seen today.

While the field of taxonomy is diminishing drastically in the world today, the need to discover and describe the remaining estimated 86 % of unknown species world-wide (Mora *et al.*, 2011) is increasing in urgency as developing and industrialised countries continue to degrade and destroy the environment at considerable rates. Research into biodiversity and biogeography allows an understanding of the earth's biota and why species occur in certain locations and not others. This knowledge can be used to develop strategies to preserve and monitor this biota and their habitats. Water is one of the most valuable resources on the planet. Functioning ecosystems within rivers are crucial to ensure clean, healthy water which is essential to sustain the natural and anthropogenic environment. It is imperative to understand how these systems operate and interact with the diversity of life found within them. Mismanagement of water systems places river-biodiversity under an ever-increasing threat of extinction. Ultimately, without the knowledge of species compositions and distributions, environmental managers cannot recognise biodiversity hotspots and conservation priority locations, and this lack of knowledge affects planning and development of reliable policies needed for safeguarding life and the environment.

This study aims to examine the phylogeny, historical biogeography and evolution of the Teloganodidae, a mayfly family with genera endemic to the south-western Cape of South Africa, and relatives occurring in Madagascar and Asia. This is approached using molecular

phylogeny, molecular phylogeography and geometric morphometrics. Background information giving an overview of the Teloganodidae is provided in this section to set the scene for the chapters following, including the scope and general aims of the study and reason for taxon selection.

1.1 Review of taxonomic history and distribution of family, Teloganodidae

Initially, the Teloganodidae were included in the family Ephemerellidae, which was officially established by Klapalek (1909), although Eaton (1883 – 1888) first recognised the group, including the genus *Ephemerella* Walsh, 1862 (which, at the time, included many of the Teloganodidae species that are known today). Historically speaking, the first teloganodid described was *Cloe tristis* (Hagen, 1858) from Sri Lanka which was placed in the genus *Teloganodes* by Eaton (1882). In 1924, Lestage (1924) erected the new genus *Ephemerellina* Lestage, 1924 (currently classified within Teloganodidae) and the new species *E. barnardi* Lestage, 1924 from the Hottentot-Hollands Mountains in Caledon, South Africa (Table 1.1). This was the first recorded genus of Ephemerellidae in southern Africa. Table 1.1 summarises the known members of the southern African Teloganodidae family at the commencement of this study.

Table 1.1. A summary of the current global members of the Teloganodidae (the South African taxa are the focus of this study). The current valid names of the species (indicated by ●) are followed by subordinate names when these exist (indicated by ○). The subordinate names are followed by an abbreviation in parentheses, indicating their status. All names, valid or subordinate, are followed by the actual author of that name and the date that name was published. The authors responsible for the latest names changes are indicated at the end of each list of synonyms. Author names are included in this table for first mention.

Family: TELOGANODIDAE McCafferty and Wang 1997 (= EPHEMERELLIDAE Ulmer 1920)

Genus: *Ephemerellina* Lestage, 1924

● *E. barnardi* Lestage, 1924 **[South Africa]**

○ *Ephemerellina barnardi* Lestage 1924 (orig.)

Genus: *Lestagella* Demoulin, 1970

● *L. penicillata* (Barnard, 1940) **[South Africa]**

○ *Lithogloea penicillata* Barnard, 1940 (orig.)

○ *Lestagella penicillata* Demoulin 1970 (comb.)

Genus: *Lithogloea* Barnard, 1932

● *L. harrisoni* Barnard 1932 **[South Africa]**

○ *Lithogloea harrisoni* Barnard, 1932 (orig.)

○ *Ephemerellina harrisoni* Demoulin, 1970 (comb.)

○ *Lithogloea harrisoni* McCafferty and de Moor, 1995 (comb.)

Genus: *Nadinetella* McCafferty and Wang 1998 (= *Nadinella* McCafferty and Wang 1997, preoccupied)

● *Nadinetella brincki* (Demoulin, 1970) **[South Africa]**

○ *Ephemerellina brincki* Demoulin, 1970 (orig.)

○ *Nadinella brincki* McCafferty and Wang, 1997 (hom. renam.)

○ *Nadinetella brincki* McCafferty and Wang, 1998 (renam.)

● *Nadinetella crassi* (Allen and Edmunds, 1963) **[South Africa]**

○ *Ephemerellina crassi* Allen and Edmunds, 1963 (orig.)

○ *Nadinella crassi* McCafferty and Wang 1997 (hom. renam.)

○ *Nadinetella crassi* McCafferty and Wang 1998 (renam.)

Genus: *Manohyphella* Allen, 1973

● *Manohyphella animosa* McCafferty and Benstead, 2002 [Madagascar]

○ *Manohyphella animosa* McCafferty and Benstead, 2002 (orig.)

● *Manohyphella keiseri* Allen, 1973 [Madagascar]

- *Manohyphella keiseri* Allen, 1973 (orig.)

● *Manohyphella sphyxia* McCafferty and Benstead, 2002 [Madagascar]

- *Manohyphella sphyxia* McCafferty and Benstead, 2002 (orig.)

Genus: *Derlethina* Sartori, 2008 (= Teloganodes sensu McCafferty and Wang, 1997; Kluge, 2004)

● *Derlethina eloisae* Sartori, 2008 [Borneo]

- *Derlethina eloisae* Sartori, 2008 (orig.)

● *Derlethina tamiraparaniae* Selvakumar, Sivaramakrishnan & Jacobus, 2014 [India]

- *Derlethina tamiraparaniae* Selvakumar, Sivaramakrishnan & Jacobus, 2014 (orig.)

Genus: *Dudgeodes* Sartori, 2008 (= *Teloganodes* sensu Ulmer, nec Eaton, 1882)

● *Dudgeodes celebensis* Sartori, 2008 [Sulawesi]

- *Dudgeodes celebensis* Sartori, 2008 (orig.)

● *Dudgeodes hutanis* Sartori, 2008 [Borneo]

- *Dudgeodes hutanis* Sartori, 2008 (orig.)

● *Dudgeodes lugens* (Navás, 1933) [China]

- *Teloganodes lugens* Navás, 1933 (orig.)
- *Dudgeodes lugens* Sartori, 2008 (comb.)

● *Dudgeodes pescadori* Sartori, 2008 [Philippines]

- *Teloganodes tristis* sensu Ulmer, 1924 (orig.)
- *Dudgeodes pescadori* Sartori, 2008 (orig.)

● *Dudgeodes stephani* Sartori, 2008 [Borneo]

- *Dudgeodes stephani* Sartori, 2008 (orig.)

● *Dudgeodes ulmeri* Sartori, 2008 [Java Sumatra]

- *Teloganodes tristis* sensu Ulmer, 1939 (orig.)
- *Dudgeodes ulmeri* Sartori, 2008 (comb.)

● *Dudgeodes bharathidasani* Anbalagan, 2015 [India]

- *Dudgeodes bharathidasani* Anbalagan, 2015 (orig.)

● *Dudgeodes palnius* Selvakumar, Sivaramakrishnan & Jacobus, 2014 [India]

- *Dudgeodes palnius* Selvakumar, Sivaramakrishnan & Jacobus, 2014 (orig)

Genus: *Indoganodes* Selvakumar, Sivaramakrishnan & Jacobus, 2014

● *Indoganodes jobini* Selvakumar, Sivaramakrishnan & Jacobus, 2014 [India]

- *Indoganodes jobini* Selvakumar, Sivaramakrishnan & Jacobus, 2014 (orig.)

Genus: *Teloganodes* Eaton, 1882 (= *Macafertiella* Wang, 1996 syn.)

- *Teloganodes dentatus* Navás, 1931 [India]
    - *Teloganodes dentatus* Navás, 1931 (orig.)
- *Teloganodes hubbardi* Sartori, 2008 [Sri Lanka]
    - *Teloganodes hubbardi* Sartori, 2008 (orig.)
- *Teloganodes insignis (*Wang and McCafferty, 1996) [Sri Lanka]
    - *Macafertiella insignis* Wang and McCafferty, 1996 (orig.)
    - *Teloganodes insignis* Sartori, 2008 (comb.)
- *Teloganodes jacobusi* Sartori, 2008 [Sri Lanka]
    - *Teloganodes jacobusi* Sartori, 2008 (orig.)
- *Teloganodes kodai* Sartori, 2008 [India]
    - *Teloganodes kodai* Sartori, 2008 (orig.)
- *Teloganodes tristis* (Hagen, 1858) [Sri Lanka]
    - *Cloe tristis* Hagen, 1858 (orig.)
    - *Leptophlebia tristis* Eaton, 1873 (comb.)
    - *Teloganodes tristis* Eaton, 1882, 1884 (comb.)
    - *Teloganodes major* Eaton, 1884 (hom. renam.)
    - *Teloganodes tristis* Ulmer, 1924, 1939 (renam.)
- *Teloganodes tuberculatus* Sartori, 2008 [Sri Lanka]
    - *Teloganodes tuberculatus* Sartori, 2008 (orig.)
- *Teloganodes sartorii* Selvakumar, Sivaramakrishnan & Jacobus, 2014 [India]
    - *Teloganodes sartorii* Selvakumar, Sivaramakrishnan & Jacobus, 2014 (orig.)

E. barnardi, described by Lestage (1924), was based only on the male (tarsal claws on forelimbs missing) and female imagos (all *in sicco*) and no nymphs were associated. Lestage also went into detail describing the colours of the imagos which can be problematic as dried specimens are much darker and do not reflect the true colour. There are no holotypes or topotypes for *E. barnardi* from Lestage (these types were never returned to the South African Museum), however Barnard (1932) examined another male imago specimen, unfortunately also without forelimbs. Barnard (1932) was also unable to link the nymphs and adults with absolute certainty, but found a "probable" match. In 1940, Barnard made additional records for *Ephemerellina barnardi*, however the additional descriptions he included for the imago stage (penis and dorsal abdominal spinules) were actually that of *Nadinetella brincki*

(Demoulin, 1970). Barnard (1932) also described another genus, *Lithogloea* and two new species *L. harrisoni* Barnard (1932) and *L. penicillata* Barnard (1940).

Allen and Edmunds (1963) synonymised *Ephemerellina* and *Lithogloea* referring to it as the *Ephemerellina-Lithogloea* complex. They also described a fourth species, *Ephemerellina crassi* [= *Nadinetella* McCafferty and Wang 1998] and also transferred a fifth species from the genus *Ephemerella* (*E. sinensis*), found from Kiangsi Province in China, into *Ephemerellina* based on the form of the genital forceps and the presence of three caudal filaments.

Edmunds *et al.*, (1963) subdivided the family Ephemerellidae into two subfamilies with six genera, namely the subfamily Ephemerellinae with genera *Ephemerella* (Palaearctic and Nearctic), *Ephemerellina* (presently only including a southern African teloganodid), *Teloganella* Ulmer, 1939 (Oriental), *Teloganodes* Eaton, 1882 (Oriental) and *Teloganopsis* Ulmer, 1939 (Palaearctic and Oriental) and subfamily Melanemerellinae (from Brazil, South America) with genus *Melanemerella* Ulmer, 1920.

Allen (1965) then noted that *Ephemerellina* and *Teloganodes* closely resemble primitive Tricorythidae (i.e. *Ephemerythus* Gillies, 1960 from Africa, which was placed in Tricorythidae at that time, but has since been moved into its own family, Ephemerythidae (McCafferty and Wang, 2000). The nymphs of these three genera have abdominal gills on segments II to V or II to VI, the male genitalia are also similar in that segment I and II of the genital forceps are subequal in length. In *Ephemerella*, abdominal gills occur on segments III to VII or IV to VII and the second segment of male genitalia forceps are more than three times the length of the first segment. Based on these major differences, Allen (1965) assigned *Ephemerellina, Teloganodes* and *Teloganella* (provisionally) to the separate subfamily, Teloganodinae.

At this time, *Teloganodes* was known from Indonesia and the Philippine Islands and *Ephemerellina* as it was defined then had a disjunct distribution in South Africa and China. The genus *Austremerella* Riek, 1963 was described from Queensland, Australia, and Allen (1965) suggested that *Austremerella* may be a junior synonym for *Ephemerellina* based on the almost-mature nymph and female imago. The imago forewing cubital area has three long basally-connected intercalaries in *Austremerella* while *Ephemerellina* has two long intercalaries where only one is basally connected. In the nymphal stage, *Austremerella* has

paired occipital tubercles and rudimentary maxillary palpi, whereas both are absent in *Ephemerellina*. These two characters have been known to vary considerably within individuals of the same subgenus within *Ephemerella* (Allen, 1965). Allen concluded that these characters are not sufficient to warrant separate genera, although the male imagos of *Austremerella picta* Riek were unknown and so he tentatively placed *Austremerella* as a subgenus within *Ephemerellina*.

Demoulin (1970) then described another species, *Ephemerellina brincki* which bears an exceptional resemblance to *E. crassi*, the only difference in the nymphal stage being that *E. brincki* does not possess an abdominal gill on segment I, while in *E. crassi*, abdominal segment I bears a rudimentary gill. Demoulin based his description of the imaginal stage of *E. crassi* [= *Nadinetella* McCafferty and Wang 1998] on Barnard's second, additional imago description of *E. barnardi* (an error made by Barnard, 1940; pg. 634; fig. 8). Demoulin also mentioned a *simplex* form with single, blunt dorsal tubercles on the abdomen as opposed to double/bifid tubercles. However, the description of *E. crassi* from Allen and Edmunds (1963) appears to be overlapping with Demoulin's *E. brincki* description in some aspects. Furthermore, Barnard's erroneous description (1940) could easily be considered as either *E. crassi* or *E. brincki*, as both descriptions lack important character comparisons such as presence and number of abdominal tubercles in the imago stage and presence or absence of nymphal lamellate gill markings, making distinction between the two species particularly difficult. However, based on the information at hand, and inspection of specimens from numerous localities, two major subdivisions were made and used throughout this study; a *Nadinetella brincki* complex and *Nadinetella crassi* complex.

Demoulin (1970) also erected a new genus *Lestagella* in which he placed *Lithogloea penicillata*, but he did not place the genus into any subfamily. By not including *Lestagella* in Ephemerellinae (Allen 1980; 1984), it was automatically assumed that it belonged to Teloganodinae.

Tshernova (1972) described a new genus, *Vietnamella* Tshernova, 1972, within the Ephemerellinae subfamily and a new species within the Teloganodinae subfamily, *Ephemerellina ornata* Tshernova, 1972, from two subimagos from the Yunnan Province in China based on their similarity to *E. sinensis* Edmunds and Murvosh (1995) transferred *Vietnamella* from Ephemerellinae to Teloganodinae. Wang and McCafferty (1995) then transferred both of the Oriental species from *Ephemerellina* and placed them in *Vietnamella*

due to the cephalic horn vestiges on the subimago and imago stages. This left *Ephemerellina* as a strictly southern African genus.

Allen (1973) described the genus *Manohyphella* Allen, 1973 from Madagascar and placed it in the subfamily Teloganodinae along with genus *Teloganella*. Hubbard (1990) listed the following genera in the Teloganodinae subfamily: *Ephemerellina*, *Lestagella*, *Manohyphella*, *Teloganella* and *Teloganodes*. Since this classification, Wang *et al.,* (1995) removed *Teloganella* from Ephemerellidae entirely and placed it within the family Tricorythidae. McCafferty and de Moor (1995) re-established the genus *Lithogloea* in Teloganodinae. McCafferty and Wang (1995) also removed *Manohyphella* and placed it within Tricorythidae. Wang and McCafferty (1996) described a new genus from the Oriental realm, *Macafertiella* Wang and McCafferty, 1996 from the subfamily Teloganodinae.

McCafferty and Wang (1997) recognised the two subfamilies Teloganodinae and Austremerellinae in their newly erected family Teloganodidae with the following genera: *Ephemerellina*, *Lestagella*, *Teloganodes*, *Macafertiella*, *Vietnamella*, *Austremerella* and new genus *Nadinella* McCafferty and Wang (1997) [= *Nadinetella* McCafferty and Wang 1998] (including the two species *Nadinella crassi* and *N. brincki*). McCafferty and Wang (2000) then placed *Manohyphella* back into Teloganodidae and transferred *Vietnamella* and *Austremerella* into the new family Austremerellidae.

Currently, Teloganodidae is placed as a sister group to the Holarctic-Oriental Ephemerellidae, in the superfamily Ephemerelloidea, along with ten other families (Jacobus and McCafferty 2006). Teloganodidae are pannote mayflies (McCafferty and Edmunds 1979; McCafferty and Wang, 2000) and can be distinguished from all other sister groups by shared derived characteristics of the abdominal gills (McCafferty and Wang 1997; 2000). Jacobus and McCafferty (2006) included that the stout and spatulate setae found on the margins of the coxal projections of the nymphs are apomorphic and characteristic of the family. The relationships between teloganodid genera are still uncertain and have differed between studies depending on the use and treatment of various characters utilised in the morphological analyses. McCafferty and Benstead (2002) used the characters and cladogram from McCafferty and Wang (1997) to produce a cladogram including the Madagascan genus Manohyphella Allen, 1973. In a later study, Jacobus and McCafferty (2006) published a larger study on Pannota (including the Teloganodidae genera), using more characters including egg morphology. Any autapomorphies and characters considered to be ambiguous,

prone to convergence or highly variable were excluded from the analysis (Jacobus and McCafferty 2006).

Teloganodidae have a disjunct distribution through the southern Afrotropical and Oriental regions (Sartori *et al.,* 2008). There are presently 28 species in nine genera of Teloganodidae described globally (Sartori *et al.,* 2008). Currently there are four genera of Oriental teloganodids: *Derlethina* Sartori *et al.,* (2008) with two described species (Sartori *et al.,* 2008; Selvakumar *et al.,* 2014); *Dudgeodes* Sartori *et al.,* (2008) with seven described species (Sartori *et al.,* 2008; Selvakumar *et al.,* 2014), *Teloganodes* Eaton with eight described species (Sartori *et al.,* 2008; Selvakumar *et al.,* 2014) and *Indoganodes* Selvakumar *et al.,* 2014 with one described species (Selvakumar *et al.,* 2014). In the Afrotropical region there are currently three species of described *Manohyphella* Allen from Madagascar; and within South Africa (Cape Province) there are currently five species in four genera (McCafferty and Wang 1997, 2000): *Lestagella penicillata* (Barnard, 1932); *Ephemerellina barnardi* Lestage, 1924; *Lithogloea harrisoni* Barnard, 1932 and *Nadinetella* McCafferty and Wang 1998 [= *Nadinella* McCafferty and Wang, 1997], with two species *N. brincki* (= *Ephemerellina brincki* Demoulin, 1970) and *N. crassi* (= *Ephemerellina crassi* Allen and Edmunds, 1963).

1.2 Species Conceptualisation and Delimitation, and Molecular Phylogenetics of Teloganodidae

"The species concept" is a controversial topic with many arguments that have resulted in numerous definitions. Mayden (1997) listed over 20 contemporary species concepts, many of which are incompatible in their definitions and can lead to different conclusions concerning species boundaries (De Queiroz, 2007). A unified species concept has been proposed by De Queiroz (2005a, 2005b, 2007) that incorporates an underlying conceptual theme found in all alternative concepts. This involves a common element, that species are segments of separately evolving metapopulation lineages (de Queiroz, 2007). Lineages are defined here as a single, unbroken line of direct ancestry and descent (not to be confused with monophyletic clades that contain many lineages), for example, genes, organisms, colonies and species are biological entities that form a lineage. In this unified concept, the variable elements traditionally considered as necessary properties of the varying species concepts (e.g. isolation, mate recognition, genealogical, evolutionary, phenetic) are reinterpreted as contingent properties instead, and not part of the theoretical concept (De Queiroz, 2007).

These properties are subsequently used as lines of evidence (operational criteria) for assessing the separation of lineages and do not disagree with the concept itself (De Queiroz, 2007). This makes the theoretical concept of species (as separately evolving metapopulation lineages) distinct from species delimitation (the methodological problem of inferring boundaries and numbers of species using operational criteria).

Under this unified perspective, all alternative species concept properties are seen as relevant to species delimitation. The presence of a relevant property provides evidence for lineage separation and the absence of this same property does not count against lineage separation. The greater the divergence between two lineages (species), the easier it will be to find more evidence (properties) separating the lineages, and thus the hypothesis that they represent different species is better substantiated. This concept shifts emphasis away from the traditional criteria for establishing species, which has led to many disagreements (De Queiroz, 2007), and describes a clear separation of the theoretical concept of species from the operational criteria that are used for its empirical application. So rather than a new species concept, the unified species concept just reiterates that all modern species concepts are variations of a common theme, and species delimitation involves the inference of species boundaries and numbers.

There are currently over 3000 species, 400 genera and at least 42 families of Ephemeroptera described (Barber-James *et al.*, 2008). Many more taxa have been estimated and not yet described, especially in the Afrotropics which presently comprises 122 genera and more than 400 species (Barber-James and Gattolliat, 2012). The use of molecular phylogenetics will continue to increase our knowledge of species and their relationships at a far more rapid rate than over the last century (Monaghan and Sartori, 2009; Barber-James and Gattolliat, 2012). The first studies to use molecular data to infer higher taxon relationships in Ephemeroptera (Ogden and Whiting, 2005; Sun *et al.*, 2006; Monaghan and Sartori, 2008; Ogden *et al.*, 2009) were able to show many instances of congruence and incongruence with previous morphology-based hypotheses. Combined morphological and molecular data analyses provide a more robust estimate of phylogenetic relationships, improving the resolution of phylogenies by reducing inclusion of morphological homoplasies (Ogden *et al.*, 2009). These molecular studies highlight areas and patterns in phylogenies in need of further investigation, thus identifying areas of future focus and research interest. Molecular systematics has revolutionised research on cryptic species diversity where morphological characters alone

cannot distinguish between groups. There are examples of strong evidence for cryptic species complexes within Ephemeroptera, particularly in Baetidae; *Baetis rhodani* Pictet (Williams *et al.*, 2006), *Baetis vernus* Curtis (Ståhls and Savolainen, 2008), *Baetis harrisoni* Barnard (Pereira-da-Conceicoa *et al.*, 2012) and other aquatic insects (Jackson and Resh, 1998), which has potential ecological and biodiversity implications for current and future studies concerning species relationships.

This is the first molecular study investigating the phylogeny of the Teloganodidae, focusing on the South African clade. Ogden *et al.*'s (2009) study on higher ephemeropteran classification which included Teloganodidae and close relatives, i.e. Melanemerellidae and Ephemerythidae, was unable to resolve these relationships, due to insufficient sample sizes. Accessibility of certain material is a common problem, as obtaining fresh material for molecular work is challenging.

1.3 Historical Biogeography and Ancestral Reconstruction

Mayflies have been considered to be ideal candidates for studying historical biogeography (Edmunds, 1972; 1975; Bae and McCafferty, 1991; McCafferty and Wang, 1997; Sartori *et al.* 2000) for four main reasons: 1) antiquity of the order, 2) restriction to freshwater as nymphs, 3) short-lived and fragile nature of the winged stages and 4) conservative vagility. These qualities are particularly useful for examining ancient patterns affected by vicariance (Croizat *et al.*, 1974), such as continental drift.

The Teloganodidae are regarded to be an ancient Gondwanan relict, having undergone tectonic vicariance and participated in a trans-Indian Ocean track (Craw, 1988; Edmunds, 1972; 1975; McCafferty and Wang; 1997; McCafferty, 1999; Sartori *et al.*, 2008; Selvakumar *et al.*, 2014). McCafferty and Wang (1997) traced the teloganodid lineage back to southern Pangea (ca. 200 Ma), based on inclusion of the Australian genus *Austremerella*, with the South African lineage considered as the old (palaeogenic) element in the Afrotropics (Harrison, 1965; McCafferty and Wang, 1997). McCafferty and Wang (1997) suggest that *Lestagella* (with Oriental *Teloganodes*) is the most derived clade and originated in temperate South Africa.

McCafferty and Benstead (2002) found that members of the African Teloganodidae (i.e. *Nadinetella*) shared close affinities to the Madagascan genus, and hypothesised that the

Manohyphella lineage originated earlier than the break-up of Madagascar from Africa (also supported by Selvakumar *et al.*, 2014). Stronger faunal similarities between Madagascar and Africa than between Madagascar and India have been observed in other mayfly groups, seen in many genera within Baetidae (McCafferty, 1999; Monaghan *et al.* 2005). There are uncertainties as to the timing of the isolation of India and Madagascar, which may make these observations significant. There has been suggestion that India split from Africa+Madagascar around 160 Ma (see Pielou, 1979), while others maintain that India and Madagascar remained together for 100 million years after separating from Africa (Brown and Lomolino, 1998). The relatively low number of shared mayfly genera between India and Madagascar, and the relatively high number of shared mayfly genera between Africa and Madagascar (McCafferty, 1999; Monaghan *et al.* 2005) supports the latter, which is now generally accepted among biologists (Sartori *et al.* 2000). As indicated in the references above, the Teloganodidae appear to support this hypothesis.

Rafting of the Indian subcontinent would then have carried the Gondwanan lineages to Asia which then spread through the Oriental regions as per the "Out-of-Africa" hypothesis (Datta-Roy and Karanth, 2009). Dispersal through Asia Minor during pluvial periods (ca. 17 Ma; Raven and Axelrod, 1974) has been hypothesised for other mayfly groups such as *Afromera*: (McCafferty and Gillies 1979) and *Povilla*: (Hubbard, 1984), however is unlikely for Teloganodidae due to their apparent absence from Asia Minor and east Africa (McCafferty and Wang, 1997).

Unfortunately, available fossil evidence for Ephemeroptera is scarce in the southern Hemisphere and the South African Triassic ephemeropteran-like fossils do not resemble any extant taxa (Riek, 1973, 1976; Hubbard and Riek, 1977).

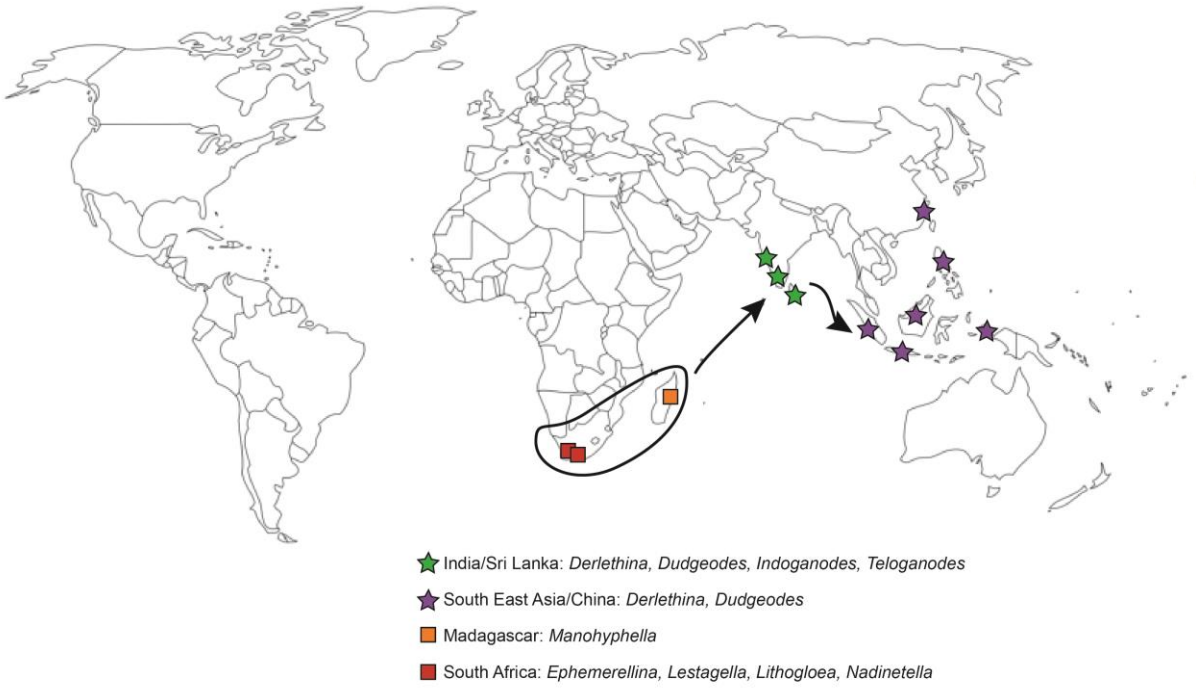


Figure 1.1. Current suggested dispersal/vicariance route for global Teloganodidae, with currently known global generic distribution (synthesized from literature Edmunds, 1972; 1975; McCafferty and Wang; 1997; McCafferty, 1999; Monaghan *et al.* 2005; Sartori *et al.*, 2008).

1.4 Geometric Morphometrics

Morphometrics refers to the measurement of form. It is a branch of biology that characterises organismal form and quantifies morphological variation in a manner that produces a more objective result (that is reproducible and testable), rather than a qualitative description. A quantitative method is also easier to pick up on more subtle differences that may be biologically relevant, and is hence more reliable than a simple qualitative inspection (Bookstein, 1985; Klingenberg, 2010).

The field of geometric morphometrics has expanded immensely over the last 40 years. Used traditionally by taxonomists to attain characters for phylogenetic analyses, it now incorporates ecological, functional, evolutionary and developmental facets. The question of whether morphometric data can be used to infer phylogenies is a controversial topic (Felsenstein, 1988; Zelditch *et al.*, 1995; Monteiro, 2000; Cardini and Elton, 2008). To avoid this controversy, a different approach to morphometrics and phylogeny is being developed,

where shape data is mapped onto independent and well-supported phylogenetic trees (e.g. produced using molecular methods). This approach can reconstruct the history of shape change and possibly provide insight into the process of shape evolution (e.g. Klingenberg and Ekau, 1996; Figueirido *et al.*, 2010; Klingenberg *et al.*, 2012).

Klingenberg and Gidaszewski (2010) used the wings of dipteran *Drosophila melanogaster* to develop a test for phylogenetic signal and homoplasy. Some studies have shown geometric morphometrics to successfully estimate phylogeny (Culicidae: Rohlf, 2002) while others fail in this respect (Klingenberg and Gidaszewski, 2010), despite high phylogenetic signal and low levels of homoplasy. While morphometric data may have significant phylogenetic signal, it may not be sufficient to reconstruct an entire phylogeny (Klingenberg and Gidaszewski, 2010). Geometric morphometrics in a phylogenetic context has also inspired research in comparative evolutionary studies in integration, modularity and allometry; to investigate evolutionary patterns and processes in various taxa (e.g. Klingenberg and Marugan-Lobon, 2013).

This study exploring geometric morphometrics in a phylogenetic context and patterns in evolutionary integration and allometry in the wings of South African Teloganodidae is the first of its kind in Ephemeroptera.

1.5 Scope

The focus of this thesis is on southern African Teloganodidae, with only a brief account of the family on a global scale. Lack of global sample material for genetic work has been a major limiting factor in this regard, although specimens were included in analyses where possible. Molecular data from five genes were used to investigate detailed phylogenetic relationships within and between genera, to reconstruct ancestral ranges within southern Africa in the greater context of a Gondwanan origin and to examine any connections between phylogeny and catchment locality using geometric morphometrics.

1.6 General Aims and Overview

This thesis aims to investigate the phylogenetic relationships and historical biogeography of Teloganodidae. While the research focuses on the southern African Teloganodidae, which is confined geographically to the southwestern Cape, it is contextualised on a global scale, looking both at the disjunct distribution of other teloganodid and closely related families.

Updated distributions and general biological notes are provided for the southern African lineage. This is followed by a detailed redescription of *Lestagella penicillata*, from a lectotype collected at the topotype locality.

A multi-faceted approach is used to investigate the general biodiversity of the southern African clade, and to what degree this diversity is associated with the main catchments formed in the folded mountains of the southwestern Cape. The mechanisms, drivers and timing affecting diversification are examined using detailed phylogeography and ancestral reconstructions.

More detailed aims and hypotheses are addressed in specific chapters.

Chapter Two

Sampling Methods, Distributions and Biological Notes

2.1 Introduction

The Cape Floristic Region (CFR) is a phytogeographic region (Figure 2.1a), first proposed by Grisebach (1872) and consequently refined by the works of many authors (Allsopp *et al.*, 2014), and is currently delimited by the distribution of the fynbos vegetation that is characteristic of the Cape in South Africa. Fynbos is heathland vegetation, occurring only in the Mediterranean-type climate zone of the southwestern Cape of South Africa (Allsopp *et al.*, 2014). The Greater Cape Floristic Region (GCFR) refers to the CFR and the surrounding Succulent Karoo Region, which are recognised floral and faunal biodiversity hotspots (Myers *et al.*, 2000; Born *et al.*, 2007) (Figure 2.1).

The African Teloganodidae are endemic to pristine, relictual forested, mountain streams found in the southern and western Cape of South Africa, mostly recorded within the CFR, with only one genus known to extend to the Amathole Mountains (Eastern Cape) (Figure 2.1b, Figure 2.2 “triangle” markers). They commonly occur on stones and vegetation such as the endemic *Wardia hygrometrica* (Wardiaceae) moss and *Isolepis digitata* (Cyperaceae) sedge, usually in swift currents and waterfalls (McCafferty and Wang, 1997). Harrison and Agnew (1962) noted that certain South African Teloganodidae are restricted to acidic waters found only in the southern and Western Cape regions due to the heath-like vegetation type (fynbos) in combination with the geology (Table Mountain Sandstone). These acid waters are a result of the geology, predominantly Table Mountain Sandstone, which produces acidic soils with low buffering capacity, also low in nutrient content (Allanson, 1990). The fynbos vegetation adds to the acidity by producing humic acids and other secondary compounds that are leached into rivers.

This chapter outlines the general methodology for field sampling, sample preparation and specimen housing, with notes on the distributions, habitat requirements and biological observations made over the course of the study.

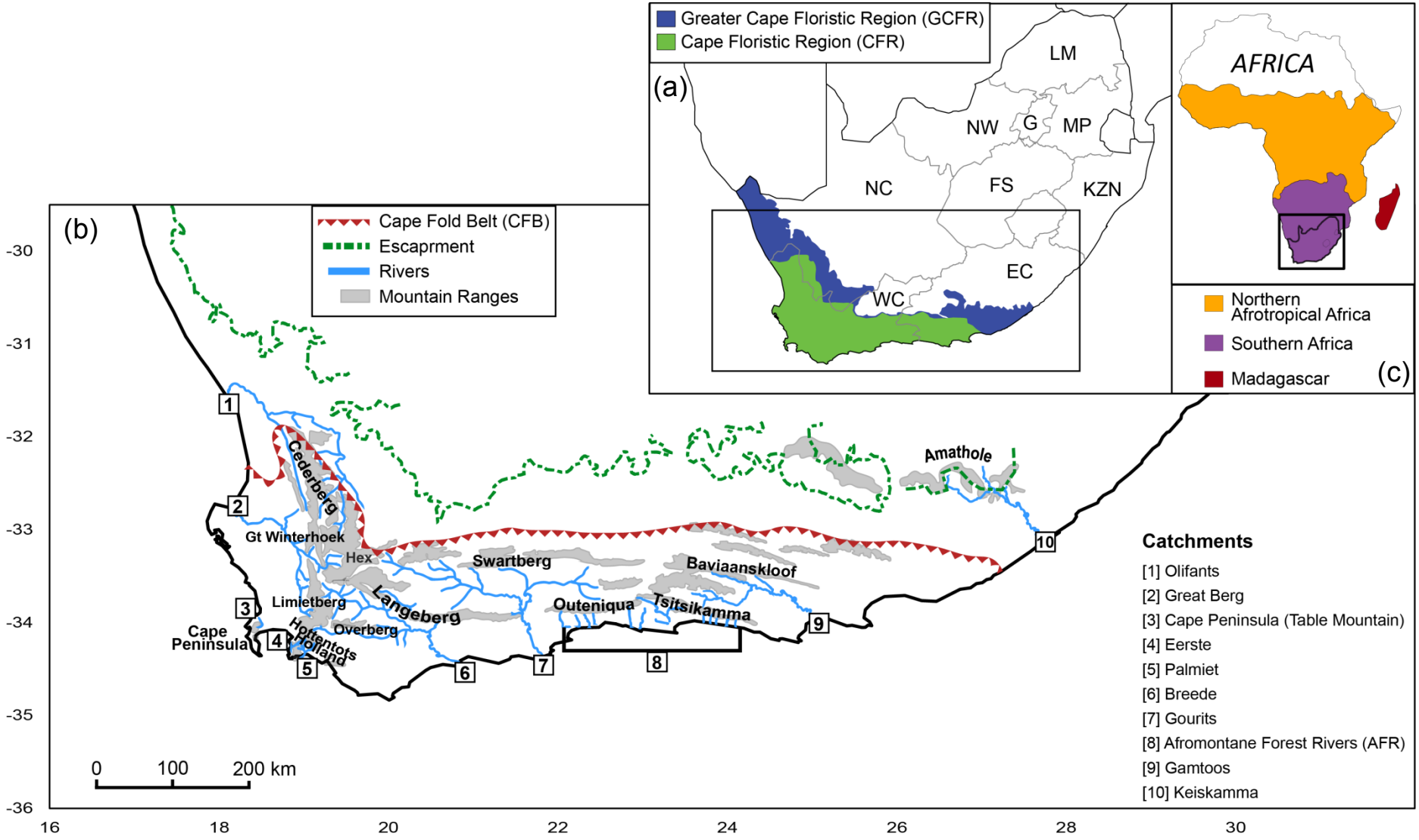


Figure 2.1. See legend overleaf

Figure 2.1. Reference map to the regions, boundaries and mountain ranges of the general study area, (a) Africa divided into three main sub regions referred to throughout this thesis, (b) the Greater Cape Floristic Region (GCFR) and Cape Floristic Regions defined in South Africa, and (c) map of the Cape Floristic Region where southern African Teloganodidae are recorded, showing the Cape Fold Belt and escarpment boundaries. Mountain ranges are labelled where main river catchments are located. Note that the Afromontane Forest Rivers include all coastal rivers in the Outeniqua and Tsitsikamma areas. Gt. Winterhoek = Groot Winterhoek Mountains, South African Provinces: WC – Western Cape, EC – Eastern Cape, KZN – KwaZulu Natal, FS – Free State, NW – North West, G – Gauteng, MP – Mpumalanga, LM – Limpopo.

2.2 General Methods

2.2.1 Sampling

Sampling sites were planned to incorporate a wide range of rivers representative of each main catchment (all sampling sites are denoted with red crosses "X" in Figure 2.2) throughout the GCFR. Catchments were sampled to include as many mountain ranges as possible (Figure 2.1). The Cederberg Mountains comprise the Olifants catchment which has headwater tributaries that flow in opposing directions, and has been divided into Olifants-East (easterly flowing rivers) and Olifants-West (westerly flowing rivers) for this study. The Breede catchment (Figure 2.1) has been divided for this study according to the different mountain range orientation (details in Chapter Four), where Breede-East includes the Langeberg Mountains, Breede-West includes the Limietberg Mountains and Breede-South includes the Overberg Mountains (Figure 2.1). The Outeniqua and Tsitsikamma Mountain Rivers have been grouped into one catchment category (Figure 2.1) and hereafter are referred to as the Afromontane Forest Rivers (AFR), after Swartz *et al.* (2008). The Tsitsikamma Rivers are the extreme east range boundary for the acid waters typical of the Western Cape Rivers.

Field collecting trips took place during Spring and Summer months, when emergence was expected, in order to collect adults and mature nymphs. Some of the mature nymphs were successfully reared through to adulthood for positive association of nymphs and adults. The penultimate instar stage in nymphs is used for identification and species descriptions throughout this work so as to remove any confounding morphological variation that may occur in younger instars; ultimate instars are also not suitable as important diagnostic features may undergo wear-and-tear, resulting in misleading morphology.

Standard collecting equipment was used to collect and preserve the immature stages, while light traps and emergence traps were used to trap the adults. Samples collected for DNA extraction were placed in 99.9% analytical ethanol to preserve the DNA while any material used for morphological work was placed into 70 – 80 % ethanol. All localities sampled over the duration of the study are shown in Figure 2.2. The GPS coordinates and river parameters including the pH, Total Dissolved Solids (TDS), Electrical Conductivity (EC) and temperature were recorded at each river (Appendix 2A). These insects were used for genetic and morphological work.

Current literature indicates that Teloganodidae are confined to the southern extent of South Africa (McCafferty and Wang, 1997; 2000), but little research has been done in the rivers of the Afromontane regions north of the southern Cape and Amathole Mountains. The mountainous streams of Karkloof (Kwazulu Natal Province, Figure 2.1), Lesotho and Bedford / Alicedale (Winterberg, Eastern Cape) have not previously been well-sampled or documented for Teloganodidae and other close relatives such as Ephemerythidae. In this study, rivers in these areas were sampled to determine the extent of the Teloganodidae distribution and find the boundaries. In Figure 2.2, sampling localities where Teloganodidae were found are marked with blue diamonds and a red-cross ("X"), sampling locations where no Teloganodidae were found are denoted as single red-cross and no blue diamond.

Samples of other Teloganodidae genera from Madagascar (*Manohyphella*) (Figure 2.1c) and Indonesia (*Dudgeodes*) were received from Dr Michel Sartori (Museum of Zoology, Lausanne, Switzerland), and used in these analyses. Samples from closely related families, South American Melanemerellidae, received from Assoc. Prof. Frederico Salles (Universidade Federal do Espirito Santo, Brazil) and Prof. Rafael Boldrini (Universidade Federal de Roraima, Brazil), and Afrotropical African Ephemerythidae, received from Dr Albert Chakona (South African Institute for Aquatic Biodiversity) and the AMGS collection, were utilized in this study.

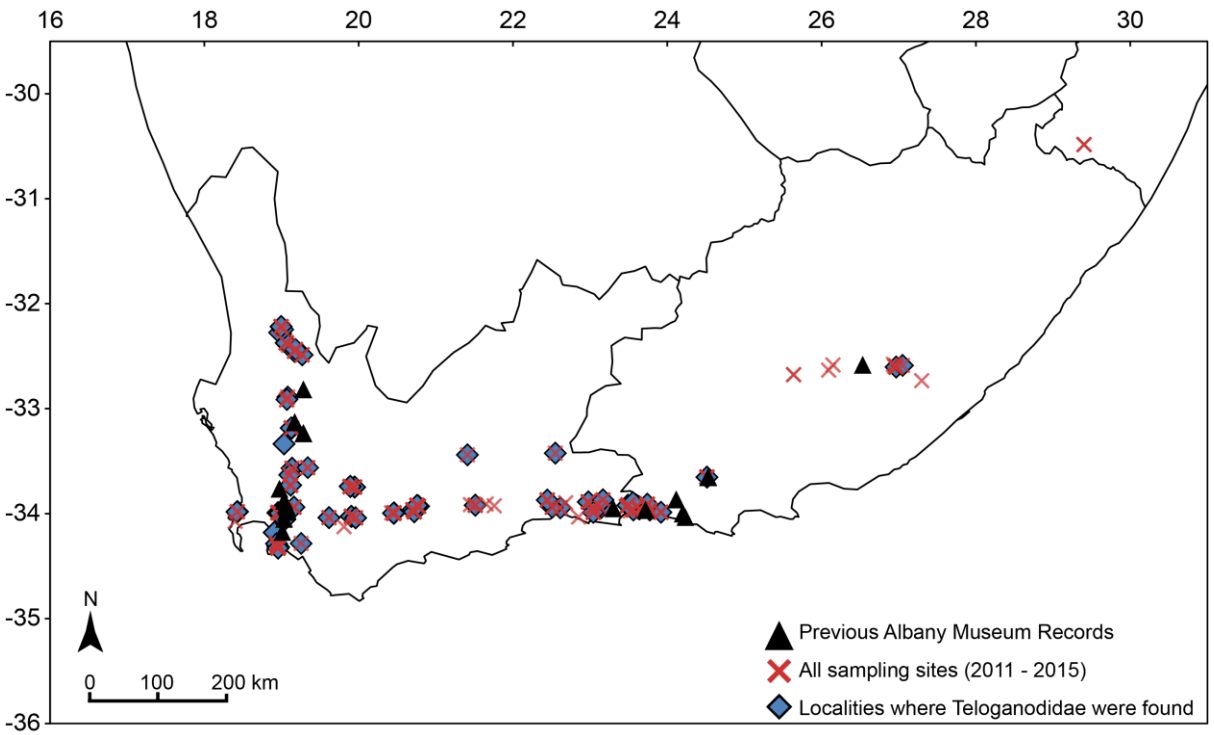


Figure 2.2. Map of Albany Museum Teloganodidae locality records (triangle shapes) obtained from the Albany Museum (AMGS) database at onset of study; all localities sampled throughout the study (red "X" shapes); and from these, the localities where Teloganodidae were found (blue diamond shapes). Sampling sites with only a red cross ("X") show sites sampled where Teloganodidae were absent.

2.2.2 Preparation of samples

Samples used for molecular analyses were at the nymphal stage. From the DNA extraction protocol (see section 2.3), the tissues were dissolved, leaving a fully intact nymphal exoskeleton which are used as the vouchers for the genetic dataset. The mouthparts, legs, gills and remainder of the body were slide mounted in Euparal. These vouchers as well as other specimens collected on field trips were used to assess variation and determine characters for future descriptions.

Photographs of specimens used for DNA extraction and for species descriptions were done using a Leica M125 dissecting Microscope and Leica DM1000 compound Microscope with a Leica DFC 295 mounted camera. All line drawings were prepared in Adobe Illustrator CS6.

Additional material used includes specimens from the Natural History Museum, London [BMNH], Iziko Museum collection, Cape Town [SAMC] and Albany Museum [AMGS] collection of Teloganodidae specimens and locality records All material collected in the duration of this study is housed at AMGS under the catalogue reference of "HMJ".

2.3 Distributions and Habitat Requirements

2.3.1 *Ephemerellina* Lestage

Ephemerellina barnardi Lestage 1924 has been observed to show peak emergences in mid-November and then again in mid-January (Barnard, 1932). New records from this study show that the genus occurs from the Cederberg Mountains, throughout the Cape Fold Belt and to the AFR (Figure 2.1), and is associated with aquatic sedge *Isolepis digitata* (Figure 2.3) in swift currents and waterfalls.

On examining slide mounts of *Ephemerellina* nymphs, their gut content contained numerous chironomid (Diptera) larval head capsules, and various antennae (possibly coleopteran). This indicates that the genus is predatory, which has not been recorded previously.

2.3.2 *Lestagella* Demoulin

The recorded distribution of *Lestagella* from this study; shows a range extending from the Cederberg area (Western Cape), throughout the Cape Fold Belt and to the Amathole Mountains (Eastern Cape) (Figure 2.1). Currently, *Lestagella* is the only African genus of Teloganodidae recorded from pH neutral rivers in the Amathole Mountains and in the Wit River, a tributary of the Gamtoos River in the Baviaanskloof, Eastern Cape (Figure 2.1).

2.3.3 *Lithogloea* Barnard

Barnard (1932) found that *Lithogloea harrisoni* has peak emergence at the beginning of August and which lasted until mid-March. This genus has not been recorded outside of the Western Cape, despite considerable sampling efforts throughout this study. Distribution is from the Cederberg Mountains, following the mountain ranges southwards to the Hottentots Holland and as far east as the Langeberg (Figure 2,1).

Larvae were found under stones in current, in fairly shallow streams, similar to *Lestagella* habitat type (Figure 2.3). McCafferty and Wang (1997) remarked that the genus had been

reported by other authors to occur on *Isolepis* sedge in swift currents. This genus has not been recorded in this habitat throughout this study, and this record is thought to have been mistaken for Species B (Table 2.1). Inspection of samples identified for environmental agencies in the Western Cape, show that samples of Species B were consistently misidentified as *Lithogloea*.

2.3.4 *Nadinetella* McCafferty and Wang

Nadinetella has a wider distribution than previously recorded (McCafferty and Wang, 1997), occurring from the Cederberg, throughout the Cape Fold Belt to the Tsitsikamma (AFR) (Figure 2.1). The genus has two described species (*N. brincki* and *N. crassi*) which are both strongly associated with *Wardia hygrometrica* moss, in swift currents and waterfalls (Figure 2.3).

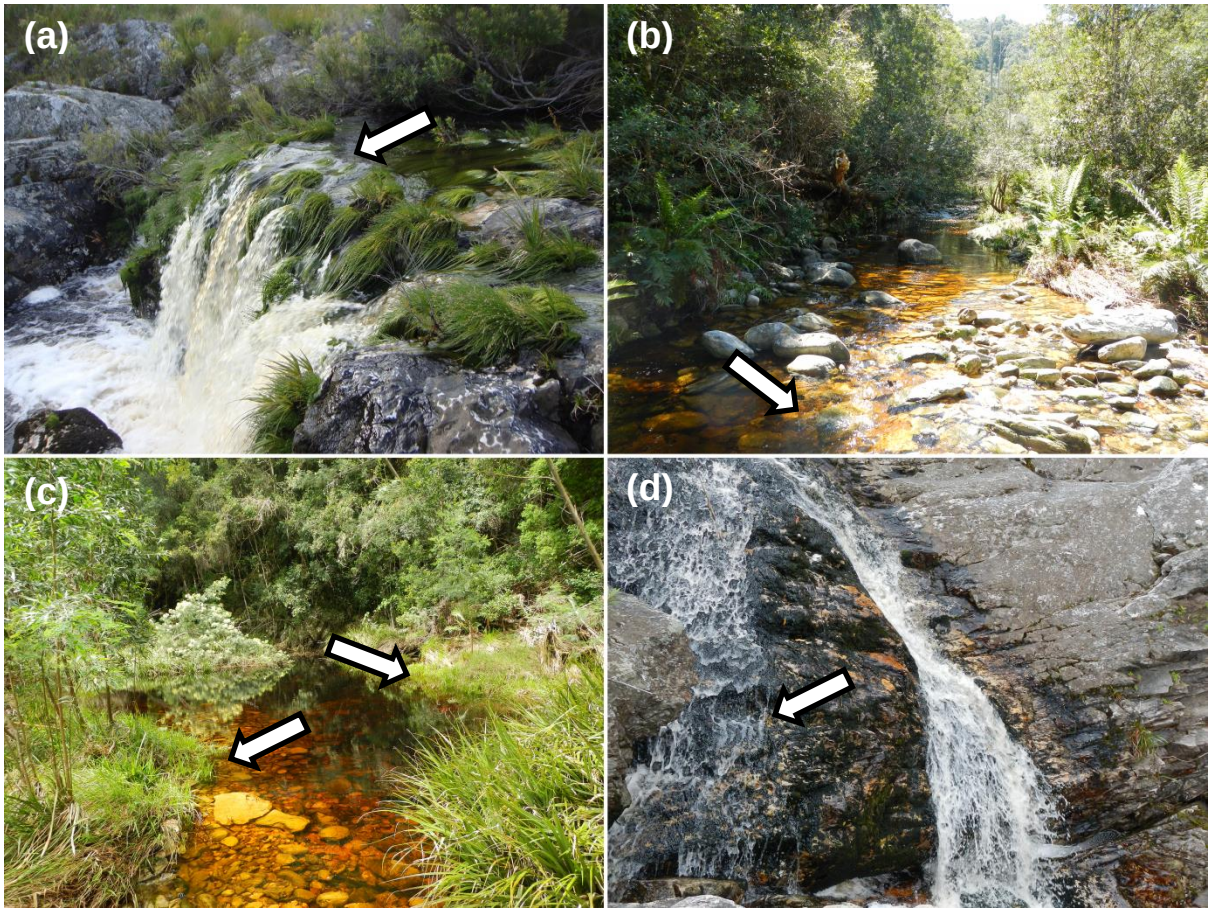


Figure 2.3. General habitats arrowed where southern African Teloganodidae have been collected, (a) *Isolepis digitata* in swift current about the waterfall, and in the waterfall, *Ephemerellina*, Species B

and Species C are found mostly in this vegetation type, (b) *Lestagella* and *Lithogloea* are found in shallow (ca. 30 cm depth) stones in current, (c) Species A is found in marginal vegetation in moderate to no current, and (d) *Nadinetella* is strongly associated with *Wardia hygrometrica* moss that grows in swift currents and waterfalls.

2.3.5 Previously unknown taxa

From this study, three taxon groups (at the nymphal stage) were unidentifiable according to available keys (Barber-James and Lugo-Ortiz, 2003) and were strikingly different from the currently described genera. These three groups were allocated alphabetic letters (Table 2.1) with the prefix "Species", as generic assignment could not be confirmed. These assignments, Species A, B and C, are used throughout the remainder of this thesis. Brief comments on their diagnostic characters and distributions are given in Table 2.1.

Table 2.1. Details of the three unknown Teloganodidae taxon groups, currently known distribution as a result of this study and a brief description of the most important diagnostic morphological characters and habitat preferences

Name	Known distribution	Brief description
Species A	Tsitsikamma and Outeniqua (Afromontane Forest Rivers [AFR]); Gourits River catchment, Olifants East catchment	Mottled, considerably hirsute, long filamentous gill I. Occurs in marginal vegetation out of current and in moderate current
Species B	Palmiet River catchment	Pale nymphs, no abdominal tubercles or postero-lateral processes, brown and yellow/white striped cerci Occurs in *Isolepis* sedge and other vegetation in moderate to swift currents
Species C	Breede River catchment, only in the Eastern (Barrydale) and Southern (McGregor) regions	Barrydale group: Body completely black, with white legs and striped (black and white) cerci McGregor variation: Body black, legs and cerci striped (black and white) Occurs in *Isolepis* sp. vegetation in swift currents and waterfalls

General notes

Barnard (1932) mentioned difficulty in finding the adults of *Lithogloea harrisoni* and *Ephemerellina barnardi* (note that these were the only two species described at this time) during the day and therefore suggested that both species may be nocturnal. Light trapping during this study, both at dawn, dusk and early evening, has not provided sufficient results to confirm the optimum emergence times. Subimagos of Species A (Table 2.1) in particular have been found throughout the day, resting on vegetation or rock overhangs. *Lestagella penicillata* has been known to emerge as imagos during the day (Vere Ross-Gillespe pers comm. December, 2013). When rearing mature nymphs in the laboratory, emergence to sub-

imago occurred during the day. Nymphs would sometimes crawl out of the water tub onto vegetation or a rock and break out of the nymphal shuck (Species A, *Ephemerellina*), and sometimes they would emerge straight from the water, leaving the nymphal shuck behind in the water (*Lestagella*, *Nadinetella*).

2.4 Conclusions

Historical records described the distributions of genera *Ephemerellina* and *Nadinetella* as restricted to the Western Cape, not including the southern Cape (Outeniqua and Tsitsikamma areas, Figure 2.1). This is clearly not the case, the distributions of South African Teloganodidae are more widespread than previously recorded, however the family is still shown to be confined mostly within the CFR and some parts of the GCFR, with the outlier *Lestagella* population in the Amathole Mountains of the Eastern Cape. A recent study of the Tsitsikamma area (2008 – 2011) has shown that there is a high degree of endemism and diversity of aquatic invertebrates within the Tsitsikamma, with many new and undescribed species (Dr Ferdy de Moor and Mr Terence Bellingan, pers. comm.). These biological notes on restricted distributions and habitats raise questions of how the South African Teloganodidae became endemic to the GCFR in terms of their origin and whether *Lestagella* was able to colonise the pH neutral rivers of the Amathole Mountains, or is a remnant of an older, more widespread ancestor.

Chapter Three

Lectotype Selection and Redescription of *Lestagella penicillata s.s.* (Barnard) 1932

Much of the contents of this chapter have been published. It is included here in the main body of this thesis (parts in Chapter One, Chapter Two and majority in Chapter Three) to show the mastering of the techniques essential for sound taxonomic work needed for the future descriptions of the previously unknown genera and species found during this research. It is important to note that this chapter was taken from the following publication:

Pereira-da-Conceicoa, L.L. and Barber-James, H.M. (2013) Redescription and lectotype designation of the endemic South African mayfly *Lestagella penicillata* (Barnard, 1932) (Ephemeroptera: Teloganodidae). Zootaxa, 3750 (5), 450–464.

Thus the aim of this chapter is to set a taxonomic-description "benchmark" for further detailed descriptions of new Teloganodidae species. The product of this description is the recognition of 173 morphological characters which are useful for species descriptions and future morphological phylogenetic research in the Teloganodidae (Appendix 3A).

3.1 Introduction and taxonomic background

Currently, *Lestagella* Demoulin, 1970 is the only African genus of Teloganodidae recorded from pH neutral rivers in the Amathole Mountains and in the Wit River, a tributary of the Gamtoos River, Eastern Cape. The recorded distribution of *Lestagella* (Figure 3.1) shows a range extending from the Cederberg area (Western Cape) through to the Amathole Mountains (Eastern Cape), with the exception of the Kogelberg region, where no *L. penicillata* were found in any of the rivers despite extensive sampling. Early instars of *Lestagella penicillata* from Table Mountain slopes (Western Cape) were originally misdiagnosed and described as *Lithogloea harrisoni* Barnard (1932). This was later corrected by Barnard (1940), and a second species of the genus *Lithogloea*, *Lithogloea penicillata* Barnard (1940) was described. However, no type specimen was allocated. Demoulin (1970) erected a new genus, *Lestagella*,

and removed *L. penicillata* from Lithogloea, placing it within the newly erected genus as the only described species. No imagos of *L. penicillata* had been adequately described, but Barnard (1940) indicated that the female subimago and male imago of *L. penicillata* are very similar to those described for *Lithogloea harrisoni*. The generic diagnosis has been modified from Demoulin (1970), McCafferty and Wang (1997) and Jacobus and McCafferty (2006), as some of the diagnostic characters are seen to be variable between individuals within a population of the same species. Wear-and-tear of mouthparts has also led to errors in identification where diagnostic characters of the nymphs are previously described as atrophied. For the purposes of this study and to avoid ambiguities, the term "atrophy" refers to a part or organ that is reduced or undeveloped, not worn down. In this study, a male adult of *Lestagella penicillata* is designated and described from the original syntype series from the Iziko Museum, Cape Town, South Africa. Detailed descriptions of the nymphal stage are presented using material from the original syntype series from the Iziko Museum and the Natural History Museum in London, England and from fresh material housed at the Albany Museum, Grahamstown, South Africa, with mention of the effects of wear-and-tear of mouthparts on species identification.

3.2 Methods and Materials

Specimens of *Lestagella penicillata* were examined from the original syntype series collected by Barnard in 1932 from both the Iziko Museum collection, Cape Town [SAMC] and the Natural History Museum, London [BMNH], as well as freshly collected material held at the Albany Museum, Grahamstown [AMGS]. One male imago from the Keppel Harcourt Barnard (K.H.B) material from the SAMC material, collected from the slopes of Table Mountain (Figure 3.1, represented as a cross on map), was slide mounted for descriptive purposes and is here designated as the lectotype for *Lestagella penicillata*. This study also examines and describes the nymphal stage from the paralectotype material. Fresh specimens of nymphs, subimagos and imagos from the lectotype locality were collected to aid in descriptions and for comparative purposes. Fresh imagos collected for this study were positively associated with nymphs by comparing a section of the Cytochrome oxidase subunit 1 [COI] gene for both stages, using primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The fresh material was then compared to the historical material and positively identified; also Barnard (1940) noted that *Lithogloea harrisoni* (the only other teloganodid that could be

confused with *Lestagella penicillata*) was not collected from the Table Mountain slopes, which further supports the association between nymph and imago. Euparal was used for all slide mounts with the exception of wings, which were slide mounted in ethanol to accentuate the wing venation, and once the ethanol was dry, the corners of the coverslip were glued in place with clear nail varnish. Wing venation follows the notation of Kluge (2004). Slide mounting can result in dissected specimen parts becoming fixed at skewed angles resulting in distortion and must be taken into careful consideration when describing species. Whole specimens of nymphs and imagos from the lectotype locality are presented as photographs (Figure 3.2), and line illustrations are used for more detailed structures (Figs. 3–15). The lectotype and paralectotypes described here belong to the SAMC collection, with additional paralectotypes from the same series held at BMNH.

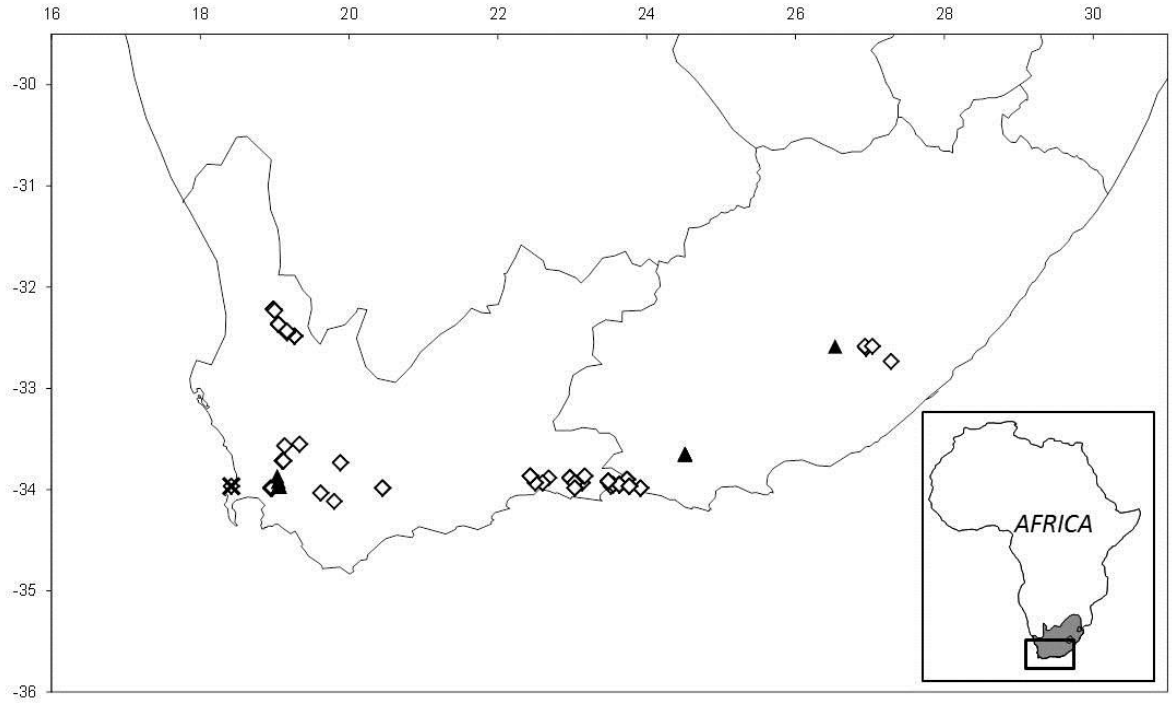


Figure 3.1. Map of recorded and sampled localities of the genus *Lestagella*. Diamonds represent sampled localities, triangles are previously recorded localities obtained from the Albany Museum (AMGS) database, and the cross ("X") denotes the lectotype locality from Table Mountain slopes.

DEscription Language for Taxonomy (DELTA) Editor (Dallwitz, 1980; Dallwitz *et al.*, 1999) was used to create a character list (Appendix 3A), which includes details of the following description.

3.3 Results

Lestagella Demoulin, 1970

Lestagella penicillata (Barnard, 1940) *Lestagella* Demoulin 1970: 130, Figure 3.15 (a–r); McCafferty and Wang 1997: 403–405, Figures 7, 16, 25, 34, 43, 52, 61, 68, 72, 86. Type species: *Lithogloea penicillata* by original designation.

Diagnosis. Nymph. *Lestagella* nymphs can be distinguished from other Teloganodidae by the following combination of characters: a well-developed marginal fringe of setae on the head (Figure 3.2c–e), spatulate setae on margin of prothorax, simple filamentous gills present on abdominal segment I, lamellate gills on segments II–IV with fully operculate gill on segment II, tibia lined with a single row of setae on the antero-dorsal surface, tarsal claw with well-developed subapical denticle, other denticles small. Abdominal segments with moderately developed posterolateral processes that are slightly separated from the base of the following segment, three caudal filaments with lateral, hair-like setae and a subequal median caudal filament. Imago. *Lestagella* adults can be distinguished from other Teloganodidae by the combination of the following characters: three caudal filaments, forewings with a relatively short, detached iMP and the presence of gill socket vestiges on abdominal segments II–IV.

Lithogloea harrisoni Barnard, 1932: 253, pro parte young nymphs, Fig. 43 a–c *Lithogloea penicillata* Barnard, 1940: 637 Ephemerellina *penicillata* Allen and Edmunds, 1963: 12 *Lestagella penicillata* Demoulin, 1970: 130 Material examined. SOUTH AFRICA: Lectotype, ♂ imago, Table Mt. slopes, XI-1932, K.H.B., approximately 33°58′S, 18°25′E. Condition: head, thorax and abdomen mostly disintegrated (kept in ethanol with syntype series). Slide preparations (EPH-A000411): genitalia, wings, mid- and hindlegs (forelegs missing), SAMC.

Paralectotypes. 5 ♀ nymphs, slide preparations of 3 ♀ nymphs (EPH-A000411): Gills (Slide 27), mouthparts (Slide 23, Slide 25, Slide 27) and legs (Slide 27), Table Mt. slopes, XI-1932, K.H.B., SAMC. 1 ♀ subimago, 1 ♂ subimago, 1 ♀ imago, 19 nymphs, (EPH-A000411), Table Mt. slopes, XI-1932, K.H.B., SAMC; 3 ♂ adults (BMNH(E) 1201855–BMNH(E) 1201857), 3 ♀ adults (BMNH(E) 1201858–BMNH(E) 1201860), 1 ♂ subimago (BMNH(E) 1201861), 29 nymphs (BMNH(E) 1201862–BMNH(E) 1201866; BMNH(E) 1201872–BMNH(E) 1201891; BMNH(E) 1239038–BMNH(E) 1239041), Table Mt. slopes, XI-1932, K.H. Barnard, BMNH. Other material examined. 3 ♂ adult, 1 ♀ adult, (BMNH(E) 1201851–

BMNH(E) 1201854) Orange Kloof Table Mt, 28-XI-1934, K.H.B. 2 ♀ nymphs, whole specimen photographed (HMJ 181A), slide preparations: HMJ 60A, whole specimen. 1 ♂ nymph, slide preparations: HMJ 60A, whole specimen. 2 ♂ imagos, slide preparations: genitalia, wings and legs (HMJ 181A), genitalia and wings (HMJ 181A), Table Mt. slopes, Window Stream 18-II- 2012, 14-XII-2012, AMGS. 2 ♂ nymphs, slide preparations: gills (HMJ 118A) and mouthparts (HMJ 118A), Table Mt. slopes, Skeleton Ravine, 29-X-2012, AMGS.

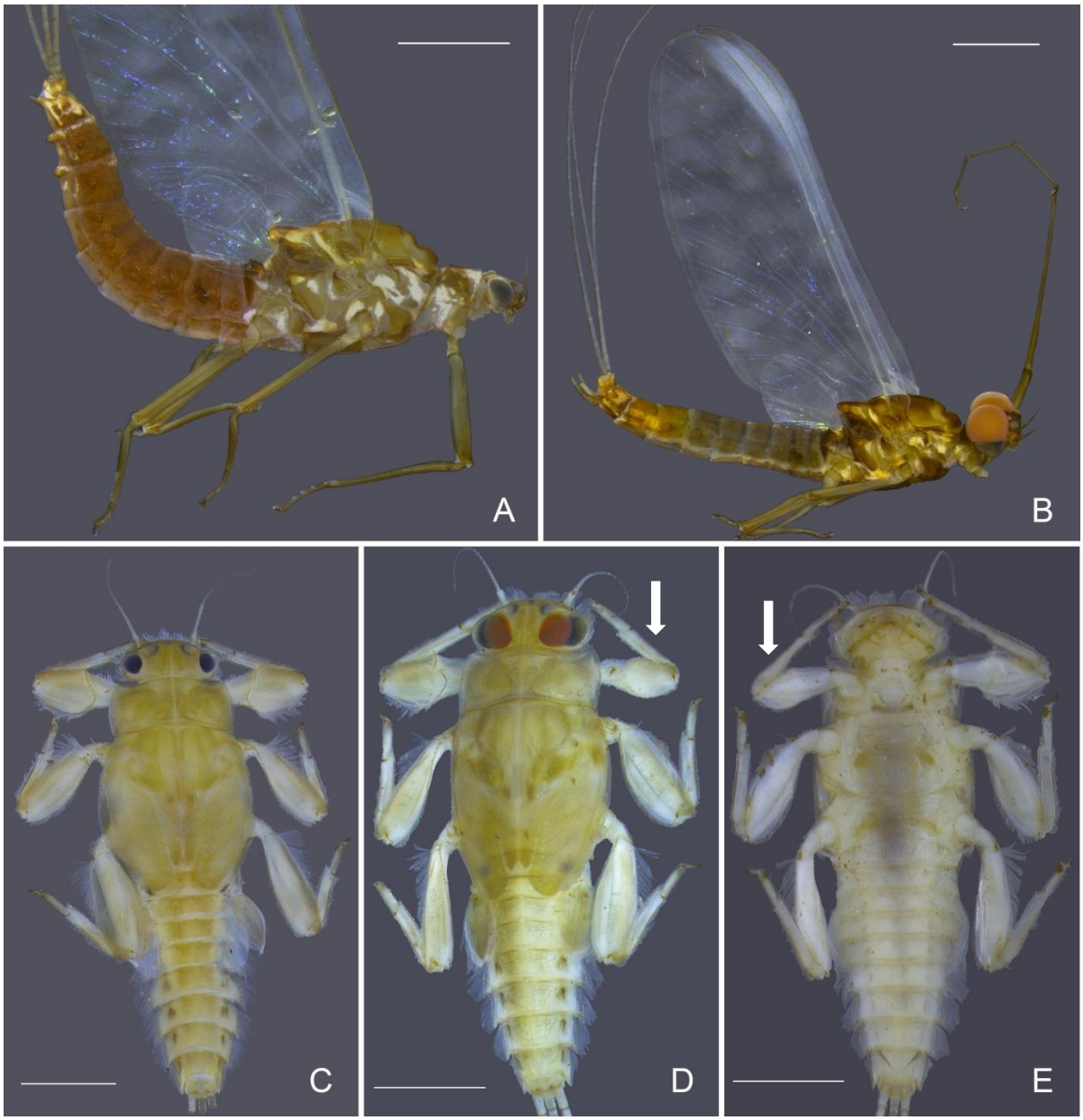


Figure 3.2. Paralectotypes of *Lestagella penicillata*. (a) Lateral view of adult female; (b) lateral view of adult male. Dorsal views of (c) female nymph and (d) male nymph, arrow showing foreleg regeneration where one foreleg is noticeably smaller than the other and without various types of setae. (e) Ventral view of male nymph. Scale bars = 1 mm.

Male Imago (Lectotype and paralectotypes). Head (Figure 3.2b). Antennae length half the width of the head capsule; dorsal portion of compound eyes large, reddish-brown and spherical; compound eyes only slightly separated dorsally (Figure 3.2b). Thorax. Forewings (Figure 3.3a) narrow with distinct pterostigma present with 8–10 crossveins, variations in

intercalaries denoted by dashed lines. RS basally faint. Marginal intercalaries present, sometimes paired, but not present between RA and RSa. Hindwings (Figure 3.3b) ovoid with costal elevation and slight medial depression in costal margin. Ventral margins of both fore- and hindwings jagged, possibly as a result of shedding of subimaginal falcate microtrichia.

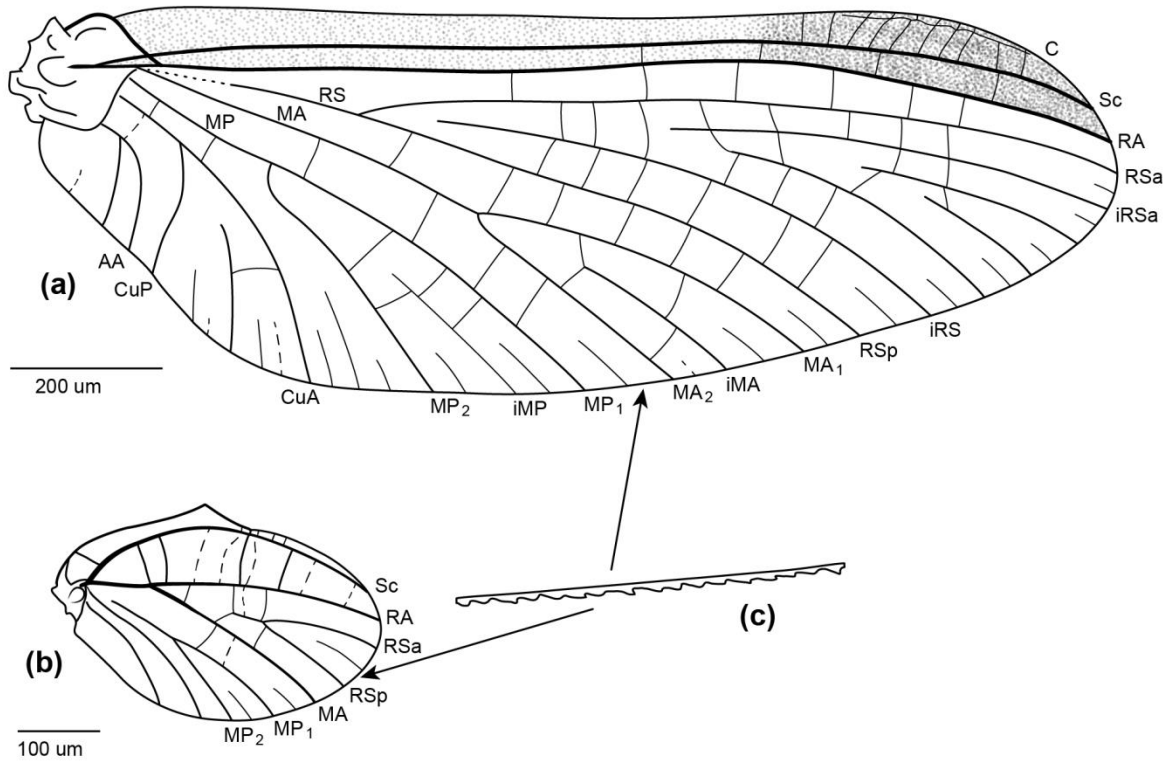


Figure 3.3.. Lectotype male imago of *Lestagella penicillata*. (a) Forewing with dotted line showing basally faint RS vein, dashed lines show variations in intercalaries seen in other specimens, intercalaries always absent between RA and RSa; (b) hindwing ovoid in shape with costal elevation and slight medial depression as depicted, variations in venation are shown as dashed lines; (c) Ventral margins of both fore- and hindwings showing jagged edge possibly as a result of shedding of subimaginal falcate microtrichia.

Forelegs (Figure 3.4a–d) with tibiae just over twice the length of femora and slightly longer than tarsi, segments I and II of tarsi subequal, segment III half-length of segment II and segment IV is ca. one third length of segment III. Femora with subtriangular, scale-like projections and few scattered setae (Figure 3.4b). Tibiae and tarsi with smaller scale-like

protrusions and scattered setae (Figure 3.4c). Claws modified into two pad-like lobes with flat scale-like dorsal surface and smooth, slightly grooved ventral surface (Figure 3.4d). Mid- and hindlegs (Figure 3.4e) with tibiae ca. 0.8 times femora length, few setae scattered on tibia becoming slightly more frequent nearer the tarsi (Figure 3.4f). Tarsi one third the length of tibiae, tarsal segments I–III subequal in length with many short simple setae, segment IV twice the length of segment III. Distal end of tibia and tarsal segments I–III with distinct spike on ventral surface (Figure 3.4g). Claws with one pad-like lobe and one hook, dorsal surface with flat scales as in foreleg.

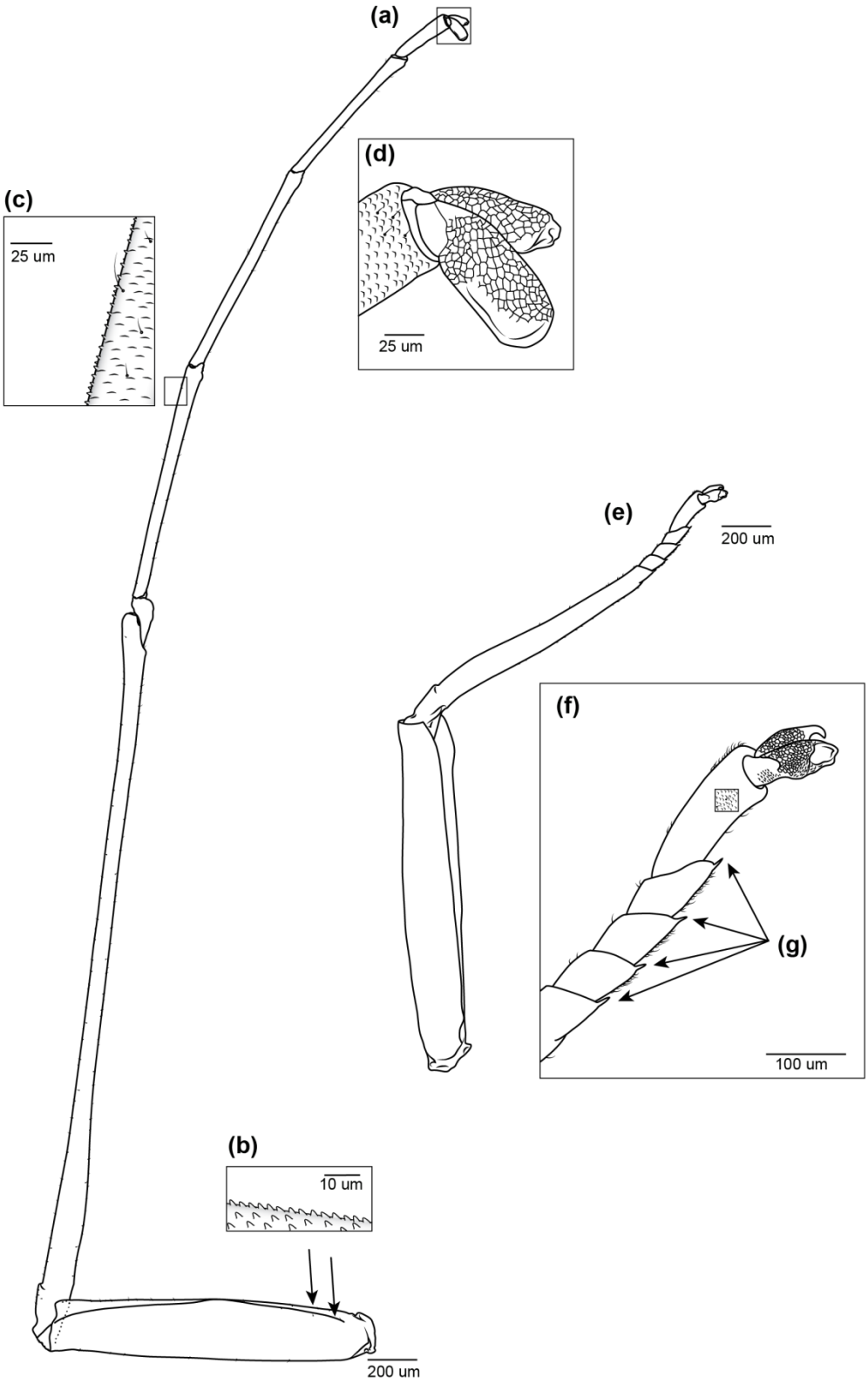


Figure 3.4. Paralectotype male imago of *Lestagella penicillata*. (a) Foreleg showing details of (b) femora; (c) tarsus and (d) tarsal claw. (e) Hindleg showing (f) details of the tarsi and claw and (g) spine-like processes of tarsi (female imago fore-, mid-and hindlegs similar to male hindleg).

Abdomen with gill socket vestiges present on segments II - IV. Male abdomen ca. two thirds thickness of female abdomen. Male genitalia (lectotype (Figure 3.5a–c)), genital forceps three-segmented, segment I more broad than other segments and ca. two-thirds the length of segment II (Figure 3.5a). Segment III reduced, bending inwardly, nearly forming a right angle, inner lateral margin of genital forceps with ovoid scale-like processes on all three segments (Figure 3.5a). The ventral, dorsal and outer margin surfaces of segment I and II covered with few simple setae scattered and small subtriangular scales (Figure 3.5b) while the third segment has many larger, slightly more elongate subtriangular scale-like processes (Figure 3.5c). Penis lobes elongated but shorter than genital forceps, fused with a small medial notch or indentation, slightly broadened distally, longitudinal groove vestigial. Styliger plate distinctly convex. Three caudal filaments ca. subequal in length and one third longer than body length, few setae scattered all over caudal filament surface.

Female Imago. Head (Figure 3.2a). Antennae as in male; without dorsal portion of compound eye, compound eyes relatively small and laterally situated. Thorax. Forewings as in male; fore-, mid- and hindlegs similar in structure to mid- and hindlegs of male. Abdomen with gill socket vestiges present. Caudal filaments as in male.

Nymph. Colouration of immature nymphs pale, straw-coloured and slightly darker as nymphs mature with darker abdominal markings (Figure 3.2c–d). Body dorso-ventrally flattened. Head with well-developed marginal fringe of setae extending to lateral margin of head with setae longer anteriorly and shorter laterally (Figure 3.2 c–d). Male nymphs show developing dorsal compound eyes (Figure 3.2d). Labrum (Figure 3.6) width 3 times length. Lateral margins rounded, anterior with slight emargination. Basal margin with elongated, squared notch medially. One third of apico-dorsal margin with densely clustered, feathered setae; similar feathered setae scattered along lateral margin (Figure 3.6a). Dorso-medial transverse row of setae 3–4 times larger than apico-dorsal setae and not feathery and possible remnant setal bases situated towards dorso-basal margin (Figure 3.6b). Dorso-lateral margin with single cluster of setae 1.5 times length of dorso-medial transverse setae (Figure 3.6c). Hypopharynx (Figure 3.7), lingua and superlinguae of similar size, both rounded apically, narrowing towards the base. Lingua with variable distomedial notch, ranging from deep indentation to none (Figure 3.7d). Dorsal surface with tuft of fine setae antero-laterally; very short brush setae covering dorsal to ventral anterior surface and a small cluster of larger setae postero-laterally (Figure 3.7e). Superlinguae with long, feathery setae on outer antero-lateral

margin (Figure 3.7f) becoming shorter and non-feathery on outer lateral margin closest to base. Small, short setae sparsely covering inner antero-lateral surface extending from antero-dorsal to inner latero-ventral surface, covering two thirds toward base. Labium (Figure 3.8) with partly divided glossae and paraglossae. Paraglossae slightly falciform, larger than glossae which are rounded apically. Apical surfaces of glossae and paraglossae with short-feathered setae (Figure 3.8g). Lateral outer surface of paraglossae with long, thin-feathered setae 2 times the length of apical setae (Figure 3.8h). Dorsal surface of paraglossae and glossae with short, scattered setae and 2 rows of longer setae situated obliquely from paraglossal base towards base of glossae (Figure 3.8i). Labial palps three-segmented, segment I as long as segment II and III combined. Articulation between segment II and III distinct. Segment II narrows slightly distally, segment III small with slight medial constriction and tapered distally. Lateral surfaces of segment II and III with scattered setae. Prementum and postmentum as seen in Figure 3.8; postmentum covered with sparsely scattered simple setae and disc-shaped sensory pits or setal bases.

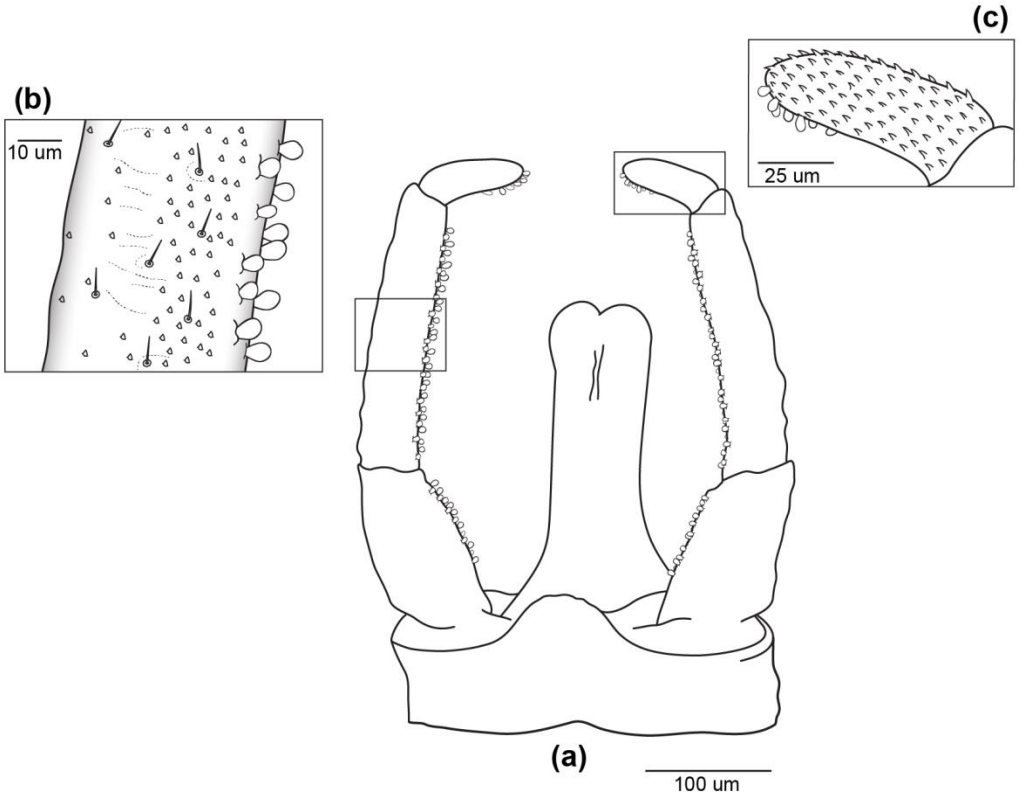


Figure 3.5. Lectotype male (a) genitalia of *Lestagella penicillata* showing details of genital forceps (b) segment II and (c) segment III.

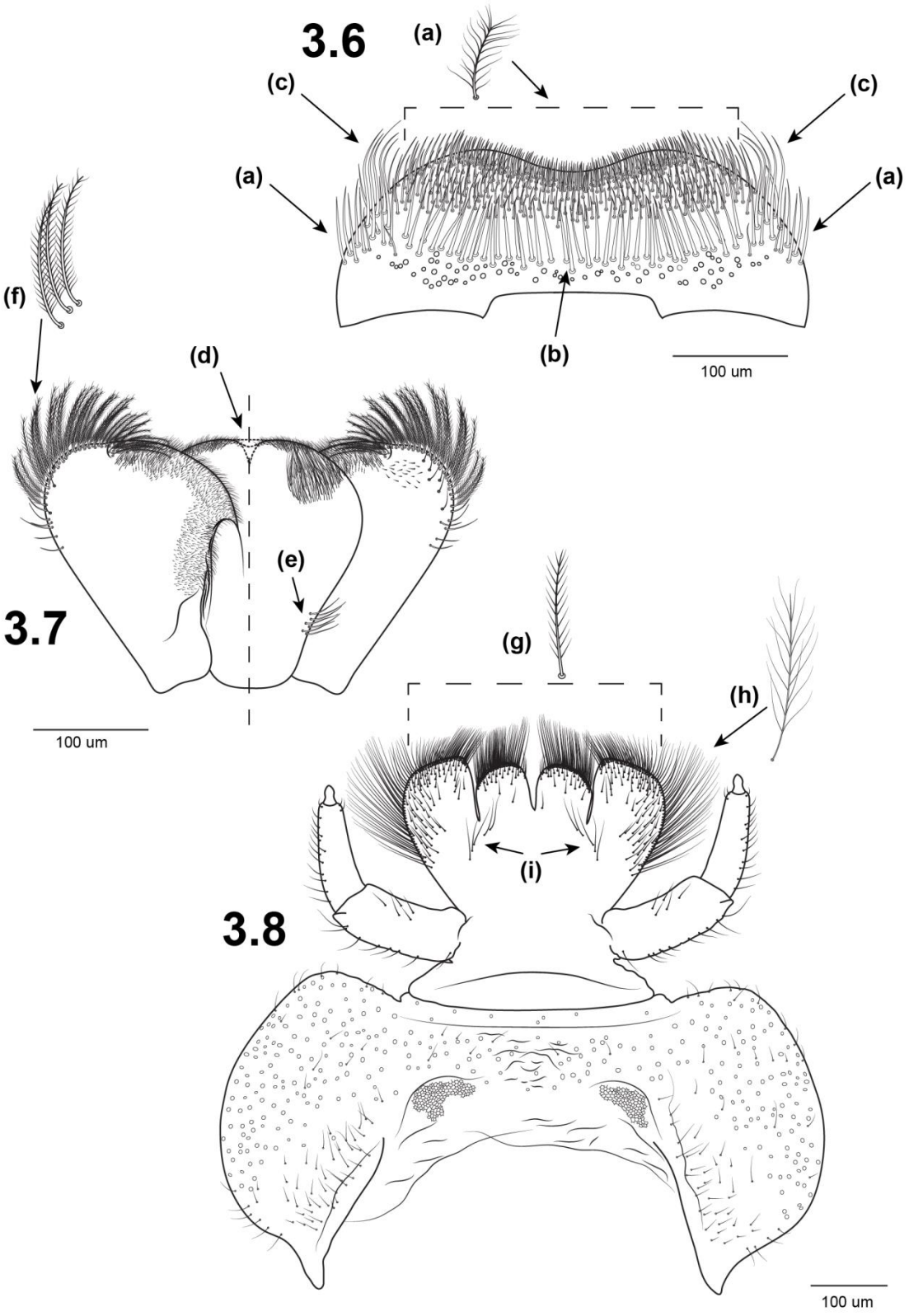


Figure 3.6 – 3.8. Paralectotype nymph of *Lestagella penicillata*. Figure 3.6. Labrum showing (a) feathered setae; (b) remnant setal bases; (c) long, smooth setae (not feathered); Figure 3.7. Hypopharynx, dorsal view (right) and ventral view (left); Figure 3.8. Labium.

Mandibles (Figure 3.9–11) elongate, length almost 4 times width. Few long socketed-setae (< 10) from middle of outer lateral margin towards the base. Incisors single, prominent, with small tuft of very fine setae on outer edge of incisor near distal end (Figure 3.10–11a). Ventral surface of mandibles (Figure 3.9) with many disc-shaped sensory pits or setal bases, medio-transverse groove with small triangular scales or sclerotized projections (Figure 3.9b). Left mandible (Figure 3.10) with prostheca close to incisor, well-developed into long sclerotized setae with the longest seta feathered or brush-like (Figure 3.10c). Molar region prominent with 2–3 long thin setae below mola. Right mandible (Figure 3.11) with prostheca well-developed, with branched sclerotized setae, with a single feathery or brush-like seta protruding out towards the mola (Figure 3.11d); molar region with 3 sclerotized seta-like projections distally (Figure 3.11e) and an elongated, thumb-like proximal ridge (Figure 3.11f); row of setae (< 10) below mola. Maxillae (Figure 3.12–3.13) uniform with maxillary palp absent. Canines fused into a single elongate canine, with a depression along inner margin containing a single seta (Figure 3.12g). Cluster of long, thin setae at base of canine on outer face. Apex of galea-lacinia with two dentisetae (Figure 3.12h, 3.13h) and 6–7 long socketed setae. Proximal dentiseta slightly serrated on the inner margin. One large seta present on inner dorso-lateral surface below galea-lacinia apex and 5 thin simple setae on inner lateral surface below large single seta as seen in Figure 3.12. A few large and thin, simple setae present on lower, outer lateral margin.

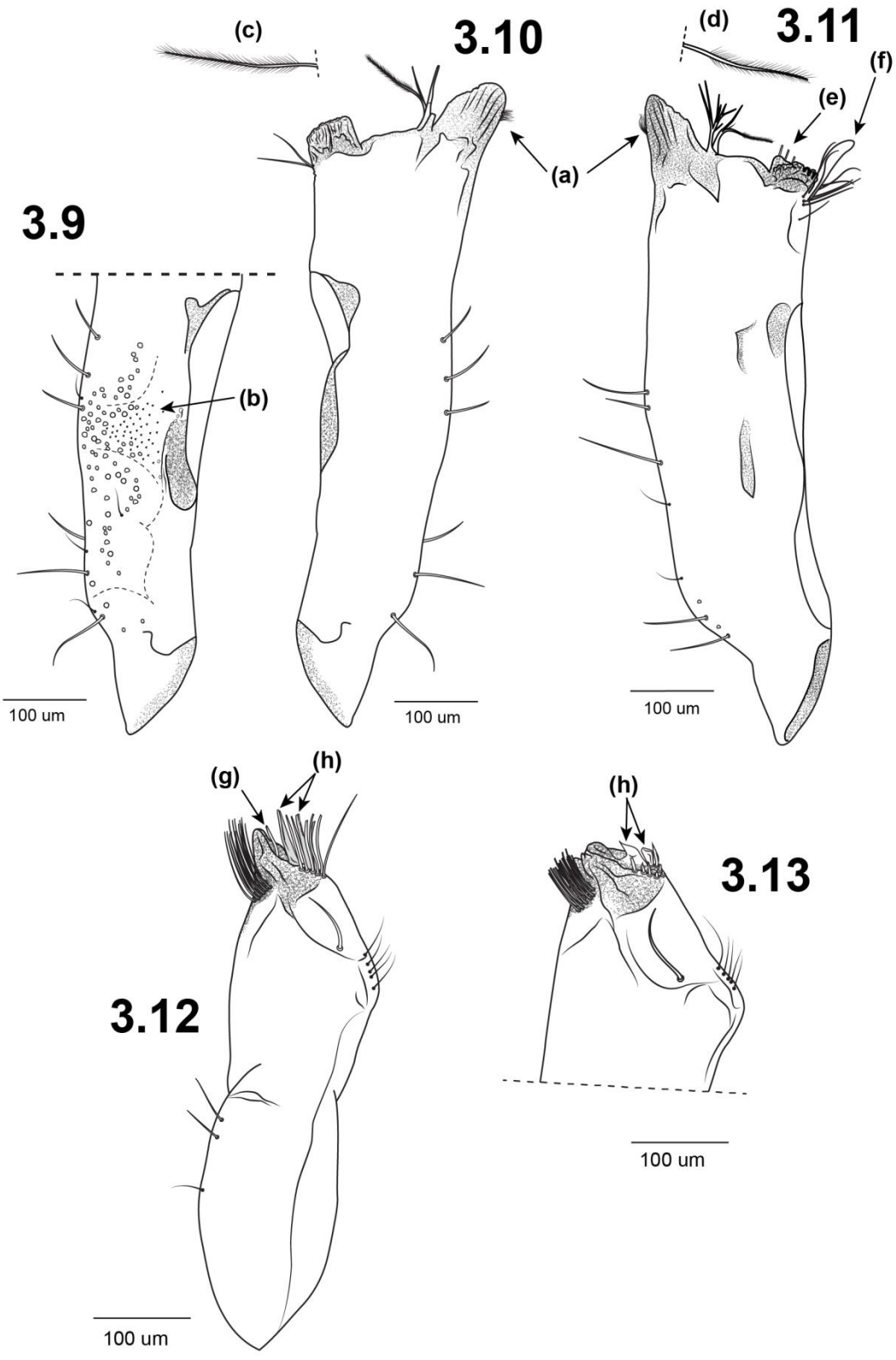


Figure 3.9 – 3.13. Paralectotype nymph of *Lestagella penicillata*. Figure 3.9. Ventral surface of mandibles; Figure 3.10. Left mandible; Figure 3.11. Right mandible; Figure 3.12. Right maxilla; Figure 3.13. Worn maxilla from a mature nymph.

Thorax. Outer edges of prothorax lined with spatulate setae similar to spatulate setae shown in Figure 3.14a on forefemur. Foreleg (Figure 3.14), femur subequal to tibia in length, medially broadened, narrowing toward distal and proximal ends. Medio-transverse spatulate setae (Figure 3.14a) present along transverse ridge on dorsal surface of forefemur; size of medial gap between transverse ridges of spatulate setae variable (Figure 3.14b). This ridge extends distally along the postero-lateral margin where the setae become longer (over 3 times length of spatulate setae). Short spatulate and lanceolate setae are present along antero-lateral margin with few long setae scattered near proximal base. Dorsal surface of tibia with single row of long, perpendicular setae, lateral margins interspersed with small, simple setae. Tarsus ca. half the length of tibia, entire surface interspersed with small, simple setae. Tarsal claw (Figure 3.14c) elongate with a single row of 4–6 variably sized smaller denticles followed by one large apical tooth. A single row of 4 small, subapical setae present apicolaterally on each side of claw. Ventral base of tarsal claw with small sparsely scattered setae. Mid- and hindlegs as seen in Figure 3.2c–e, femora slightly more elongate and without transverse ridge of stout setae, antero-lateral margins with long setae and postero-lateral margins with spatulate or stout setae. Tibia, tarsi and claws similar to foreleg.

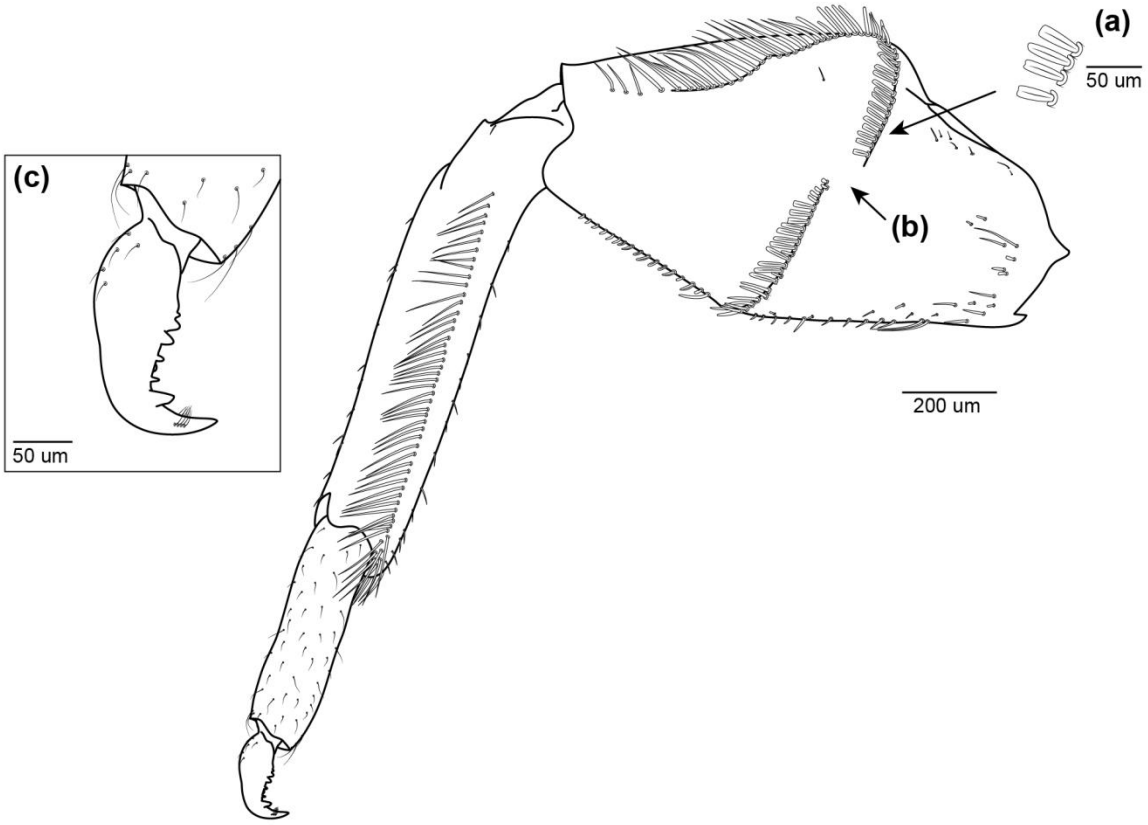


Figure 3.14. Paralectotype nymph foreleg of *Lestagella penicillata* showing (a) spatulate setae; (b) variable medio-transverse gap on femur; (c) tarsal claw showing denticles and subapical setae.

Abdomen with many fine setae covering the ventral surface, becoming more hirsute closer to the lateral margins. Abdominal segments with series of dark spots dorso-laterally, largest occurring on segment II (Figure 3.2c–d). Postero-lateral processes subtriangular, moderately developed and slightly separated from base of following segment, processes with single row of long setae. Filamentous gill I (Figure 3.15a) present on abdominal segment I; three-segmented with long, thin, fine setae scattered around segment III. Lamellate gills present on segments II to IV. Gill II (Figure 3.15b) fully operculate, upper lamella ovoid with thin setae present starting ca. two thirds from base, setae longer anteriorly and shorter laterally; lower lamella bifid and highly lobed. Gill III (Figure 3.15c) upper lamella more circular in shape with thin setae present starting ca. three quarters from base, setae longer anteriorly and shorter laterally, lower lamella not bifid, singular and highly lobed. Gill IV (Figure 3.15d) almost semicircular, lower lamella absent, thin setae present along lateral margin to apex,

setae longer anteriorly and shorter laterally. All three caudal filaments banded at base, sparsely setose with setae becoming slightly longer distally, medial caudal filament well-developed, cerci ca. two thirds length of body.

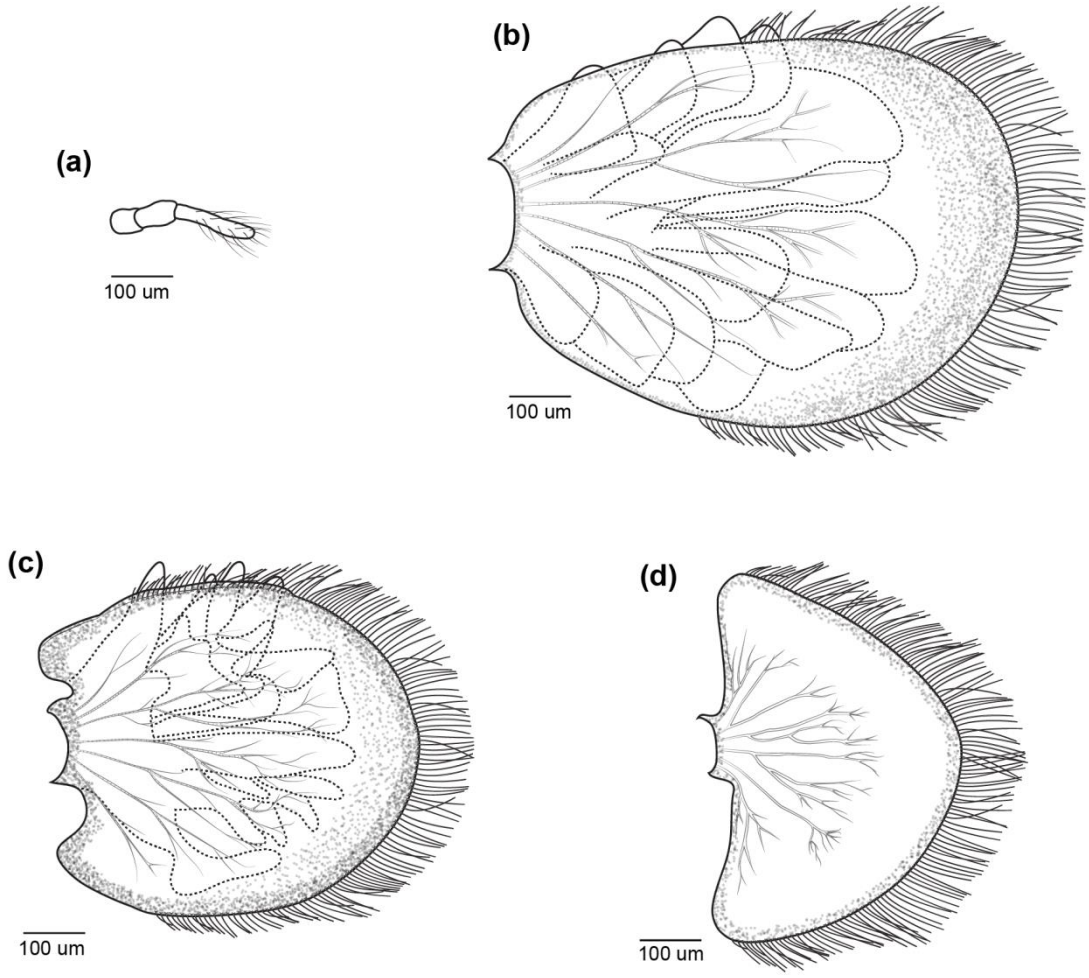


Figure 3.15. Paralectotype nymph gills of *Lestagella penicillata*. (a) Filamentous first gill I on abdominal segment I; (b) operculate lamellate gill II found on abdominal segment II; (c) lamellate gill III; (d) lamellate gill IV.

3.4 Discussion

L. penicillata shows a high degree of variability in the lingual medial notch of the hypopharynx (Figure 3.7), other factors such as wear-and-tear of mouthparts (Figure 3.16), regeneration of limbs (Figure 3.2d, arrowed) and different moulting stages can affect the interpretation of morphological characters and identification of the species. However, the imago stage is less variable than the nymphal stage and differences have been seen in the adult stages of potential new and undescribed species of *Lestagella*. Edmunds (1959) attributed the differences in distinctiveness between life stages to different rates of evolution between the nymphs and short-lived adults and emphasised the importance of knowing the adult and nymphal stages when recognising genera and species groups (Edmunds, 1962). McCafferty and Wang (1997) stated that the presence of a medial notch on the lingua of the hypopharynx was diagnostic of the genus. However, in the material examined for the species *L. penicillata* alone the hypopharynx was found to be variable and not a suitable character for generic or species diagnosis. Demoulin (1970) and McCafferty and Wang (1997) both described the mandibles of *L. penicillata* as being atrophied. The use of the term "atrophy" in both studies is ambiguous and unclear. Demoulin (1970) noted that the nymphal mandibular and maxillary canines underwent "progressive atrophy" with age. McCafferty and Wang (1997) suggested that the mandibles are apically atrophied while the maxillae have modified apices, which could refer to either the dentition being reduced, or the thickness and width of the mandibles. In this particular case, it is assumed that McCafferty and Wang (1997) were referring to the reduction in dentition, as the width of the mandibles were already mentioned as being narrow, and both left and right mandibles clearly show worn-down dentition in the illustrations.

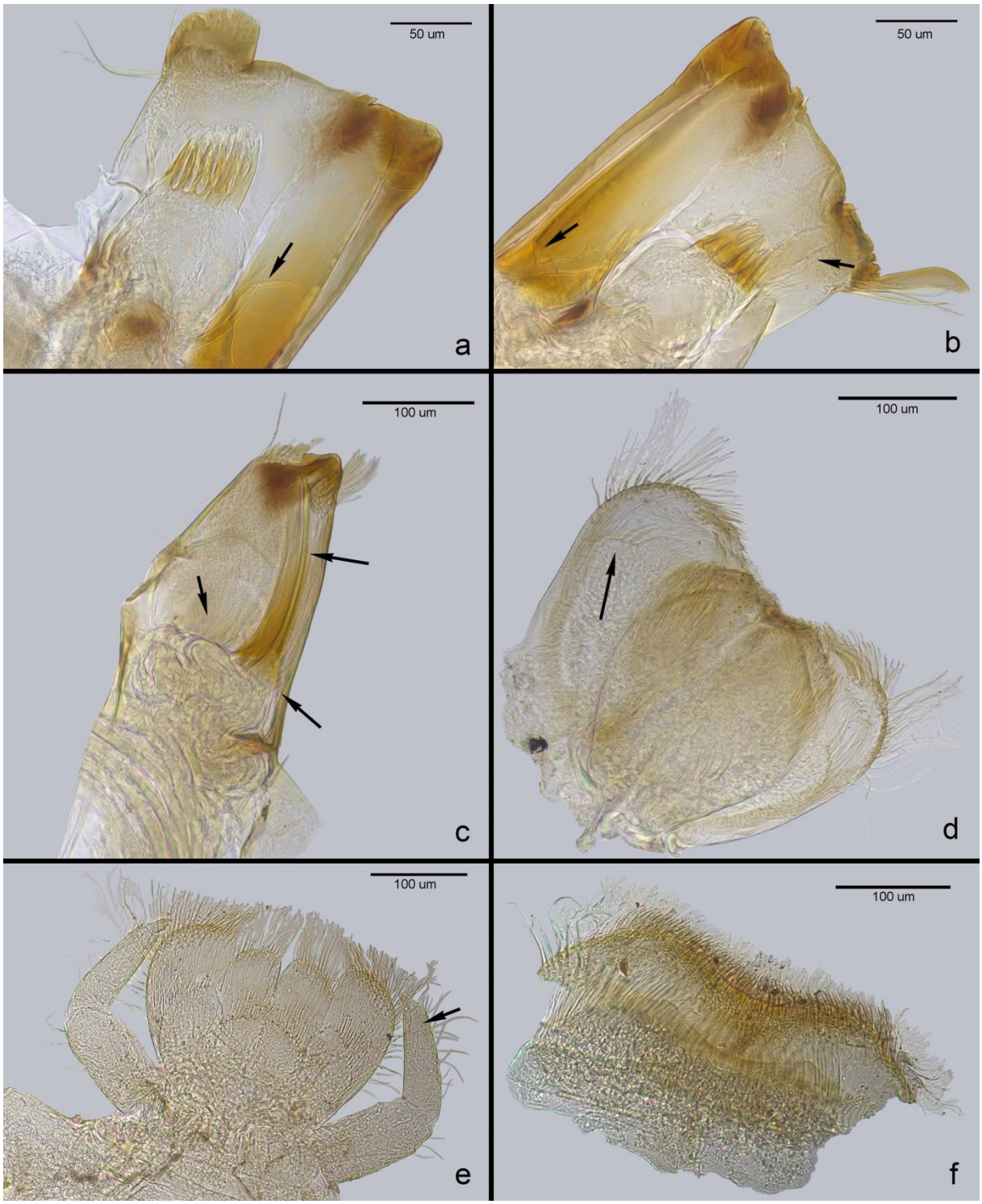


Figure 3.16. Regrowth of mouthparts of *Lestagella penicillata*. (a) Left mandible; (b) right mandible; (c) maxilla; (d) hypopharynx; (f) labium and (f) labrum.

Demoulin (1970) examined two nymphs at a younger and older stage, and it is by chance that the younger nymph had moulted more recently than the older nymph, therefore having less worn-down mandibular and maxillary canines, thus making it appear that there is a process of "progressive atrophy" in these mouthparts. It is therefore important to ensure that freshly moulted specimens are used for slide mounting when used for identification purposes, as stressed in other taxonomic studies (e.g. Kluge, 2004, Sartori *et al.*, 2008). By the aforementioned definition of atrophy in the introduction section, neither of these reported "atrophies" are true for *Lestagella*; this "atrophy" is due to wear-and-tear and is seen and well-documented in various other groups [Baetidae (Muller-Liebenau, 1973); Ephemeroidea (Elpers, 1997); and other stream animals such as Plecoptera, Coleoptera, Diptera, an isopod and snail (Arens, 1990)]. When the *Lestagella* nymph approaches the next moult, the mouthparts are well worn down (appearing atrophied), but the new mouthparts are evident (Figure 3.16). As McCafferty and Wang (1997) only described the ultimate nymphal stage, it is most likely that the nymphal mouthparts were worn down and they would not be replaced due to final nymphal moult to the subimaginal stage. Figure 3.16(a–f) shows the mouthparts of a *L. penicillata* nymph approaching the next moult. New, well-developed incisors and molars are evident in the left and right mandibles (Figure 3.16a and b respectively, arrowed), which appear to be initially distinctly separated; new setae and molar projections are arrowed. The maxilla (Figure 3.16c) regrowth shows a very well-developed, fused incisor and the dentisetae and other setae are clearly visible (see arrows). Newly developed mouthparts can also be seen for the hypopharynx, labium and labrum as seen in Figure 3.16(d–f) respectively.

3.5 Perspectives

This comprehensive account of *Lestagella penicillata*, including descriptions of all life stages, and the importance of choosing freshly moulted penultimate nymphs with unworn mouthparts for species diagnosis, sets a benchmark for all further studies of not only new species of *Lestagella*, but of all Afrotropical Teloganodidae. A comprehensive character list (Appendix 3A) for use in the DELTA software is made available for comparative purposes for future descriptions.

Chapter Four
Molecular Phylogenetics and Species Delimitation

SUMMARY

This chapter uses an array of methods to explore species delimitation under the unified species concept (Chapter One) in terms of phylogenetic monophyly, genealogy and isolation. These include bifurcating phylogenetic tree methods such as Maximum Likelihood and Bayesian Inference; network analyses and underlying reticulation patterns based on parsimony methods; species tree estimation under the coalescent model; reciprocal monophyly according to Rosenberg's test, the effects of isolation by distance (IBD) and isolation by barriers (IBB) in landscape genetics. Reticulation networks highlighted conflicting signal (hybridisation, introgression, homoplasy or incomplete lineage sorting) within phylogenies. This affected the resolution at higher taxon nodes, making phylogenetic inference difficult. Melanemerellidae and Ephemerythidae form a monophyletic clade; however their relationship to Teloganodidae is uncertain. The placement of the Asian teloganodid (*Dudgeodes*) is uncertain and Madagascan *Manohyphella* groups with the non-fringed South African Teloganodidae; however reticulation weakens the support in this relationship. For South African Teloganodidae, gene and species trees recovered 27 well-supported, reciprocally monophyletic species and possibly six genera across the family. Further studies are needed to confirm morphological distinctiveness between each group. IBD seems to have played a role only in *Lithogloea*, to a small but significant degree. IBB analyses highlighted genetic barriers separating some clades between certain catchments and not in others, while some barriers were common for several different species. This complexity of barriers may be attributed to the dispersal abilities of the different genera, and the considerable length of time that some lineages have been isolated for (allopatric speciation). Orientation and direction of mountain ranges possibly also affects dispersal ability and population isolation. The Breede catchment can be roughly divided into three zones where taxon groups are genetically distinct: Breede-East, Breede-West and Breede-South. These zones correlate with rainfall patterns; 'aseasonal arid', 'winter mesic' and 'aseasonal mesic'. This may affect adult emergence times, interrupting mating periods or limiting dispersal to other zones due to unfavourable conditions. The results presented reveal complex biodiversity patterns, which are unlikely to be unique to Teloganodidae alone, thus highlighting the need for more biodiversity studies of other taxa in the region.

4.1 Introduction

Evolutionary history is our explanation for the origin of biodiversity (Morrison, 2010), and phylogenetic trees are currently the preferred way of depicting this history and organising this knowledge, mostly because it is an intuitive process that reveals how closely organisms are related to each other and helps with group recognition for a hierarchical taxonomic system.

In any biological study, a stable taxonomy, preferably based on a robust phylogeny, is required. Real evolutionary groups (hereafter referred to as "clades") need to be determined and taxonomically named, this can then be used in any comparative biological study, quantitatively examining biodiversity patterns and evolutionary processes. The aim is to achieve a well-accepted phylogeny in which evolutionary relationships are not subject to change as new evidence accumulates (Morrison, 2010). A valuable use of phylogenetic classification is that it can help predict and explain features shared between closely related organisms, even if we have not yet observed them (Farris, 1979), therefore maximising information content. It is becoming widespread for phylogenetic inference to be based on datasets of multiple, unlinked gene sequences (Edwards, 2009).

All of this is however, is based on the popular assumption that a universal "Tree of Life" exists, whereby evolution is tree-like (bifurcating) throughout history (Lecointre and Le Guyader, 2006). Yet others believe organismal relationships to be more reticulate or "net-like" (Ragan, 2009) as there are many mechanisms (such as hybridisation, introgression, recombination, genome fusion and incomplete lineage sorting) for genetic exchange that disrupts genealogical trees (see Doolittle, 1999; Legendre, 2000; Huson and Bryant, 2005; Kunin *et al.*, 2005; Goker and Grimm, 2008; Koonin, 2009).

The genealogical species concept (Baum and Shaw, 1995) proposes that species are delineated by monophyly of genetic lineages of their members, that is, they share an exclusive genealogical relationship (Baum and Shaw, 1995; Shaw, 1998; Hudson and Coyne, 2002). Genealogical speciation can therefore arise from all typical evolutionary processes such as genetic drift and selection, influenced by population size and phenotypic adaptability, and is independent of the effects of geography, that is, they can be recognised in sympatry or allopatry (Shaw, 2001). These mechanisms can affect the rate of differential gene lineage extinction that develops the boundaries around genealogical species (Shaw, 2001). This property of species delimitation (see "unified species concept" in Chapter One) is explored

using the coalescence theory model (Kingman, 1982) with singular value decomposition (SVD) quartets (Chifman and Kubatko, 2014; 2015) and reciprocal monophyly (Rosenberg, 2003, 2007). Furthermore, genetic discontinuities and their association with landscape features are investigated using isolation by distance and isolation by barrier methods.

This chapter aims to investigate the phylogeny, distribution and biodiversity of South African Teloganodidae, using a variety of methods including standard bifurcating tree phylogenies built using character-based methods such as maximum likelihood (ML) and Bayesian Inference (BI), and parsimony to test congruency. Distance methods, although long criticised for the loss of phylogenetic information through transformation (Penny, 1982), were used in combination with network analyses which are generally found to represent the data as effectively as character-based methods (Huson and Bryant, 2005). These networks represent many reticulate evolutionary processes more comprehensively than trees, as evolution is not necessarily tree-like.

Current literature reports only four genera and five species (McCafferty and Wang, 1997). It is hypothesised that the true diversity of teloganodids could actually be higher than is currently recorded. The isolated nature of the river catchments within the extensive Cape fold mountain ranges of the Cape Floristic Region (CFR) and other Great Escarpment ranges (up to the Amathole range in the Eastern Cape), coupled with the poor dispersal ability of most mayflies due to short adult lifespan and poor flying ability (e.g. Brittain, 1982) could result in considerable speciation due to fragmentation of populations.

The aim of exploring the genealogical species concept is to illuminate diversity patterns in an evolutionary context, which takes us one step closer to determining which organisms should be considered as distinct species.

To a lesser extent, this chapter also aims to provide insight into the relationships between the Madagascan, Asian and South African teloganodid lineages. The Ephemerythidae, a family with strikingly similar morphology to the Teloganodidae, occurs only in the more tropical regions of South Africa, extending northwards throughout Afrotropical Africa. The relationship between Ephemerythidae and Teloganodidae was investigated to determine the relationship between these two families. Due to the anticipated antiquity of the Teloganodidae (considered to be a Gondwanan relict family by McCafferty and Wang, 1997), a closely related family from South America, Melanemerellidae, was included in the analyses

to investigate whether the relationships between these families reflect the ancient gondwanan connections between Africa and South America. Molineri and Dominguez (2003) provided a cladistics analysis based on nymphal and egg morphology, of the systematics of *Melanemerella brasiliana*, placing it within superfamily Ephemerelloidea, elevating it into its own monotypic family, Melanemerellidae, due to the uncertainty of its relationship with other families. As *Ephemerythus* is no longer placed within Tricorythidae (McCaffery and Wang, 2000), the cladistics tree produced (Molineri and Dominguez, 2003) shows further complications regarding familial relationships, molecular markers are used to add some clarification to the relationship of these to families with Teloganodidae.

4.2 Methods and Materials

4.2.1 Field work and laboratory protocols

A total of 255 specimens from 85 sites (75 sites from South Africa, see Figure 4.1 map) from 72 rivers (62 rivers from South Africa) were used for molecular analyses (Appendix 4A). The other ten sites were from rivers in Zambia (Ephemerythidae), Madagascar (*Manohyphella*), South America (Brazil, Melanemerellidae), India (*Dudgeodes palnius*), Indonesia (*Dudgeodes ulmeri*) and Japan (outgroup Ephemerellidae - *Serratella*). Note that *D. palnius* was used only for the COI network (SplitsTree analysis) to show any close links between Madagascan and Indian teloganodids, as this was the only marker available (on GenBank) for an Indian *Dudgeodes* species. *Dudgeodes ulmeri* (Indonesia) was used for all other analyses as the representative of the Asian genus due to more markers being available. The rivers were categorised according to the catchment to which the river belonged (Figure 4.1, Appendix 4A). Laboratory protocols were initially conducted in the molecular laboratory at the Zoology and Entomology Department, Rhodes University, Grahamstown (at that time managed by Dr Alicia Timm), then in the molecular laboratory at the South African Institute for Aquatic Biodiversity (SAIAB), managed by Ms Taryn Bodill.

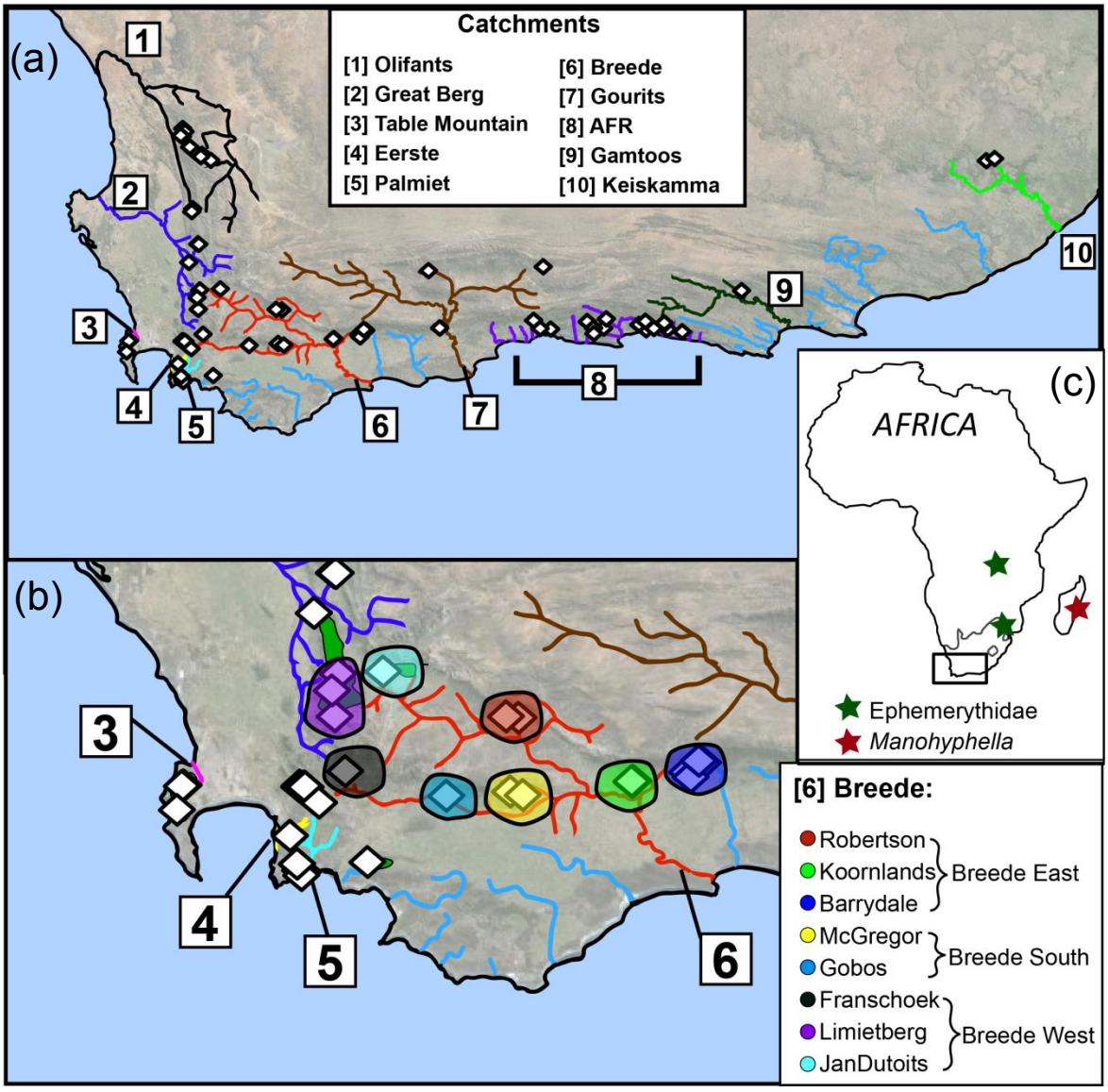


Figure 4.1. Distribution of (a) localities where Teloganodidae samples across genera were collected and used for molecular analyses, (b) eight sampling areas of the Breede River catchment split into three basic groups; Breede-South, Breede-East and Breede-West, and (c) sampling localities in the Afrotropics, Ephemerythidae from Zambia and Limpopo Province (see Chapter Two, Figure 2.1), and *Manohyphella* from Madagascar.

All specimens used for molecular analysis were photographed before DNA extraction. Photographs included the dorsal, ventral and sometimes lateral sides, with magnified images of interesting features such as medial abdominal tubercles and gills. DNA was extracted by internal body digestion using the Invisorb extraction kit (Invitek). The mouthparts, legs and

gills were dissected out from the left-over exoskeletons after extraction and slide mounted, the rest of the body was also slide mounted and kept as a voucher.

Partial segments of five gene regions were amplified and sequenced, three mitochondrial loci (cytochrome c oxidase subunit I [COI], the small subunit ribosomal 16S rDNA [16S] and 12S ribosomal DNA [12S]) and two nuclear (Histone 3 [H3] and 28S ribosomal DNA [28S]). The primers used for PCR and sequencing reactions are listed below, forward primer presented first, followed by the reverse primer and author: COI – LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994); 16S – 16Sar (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Palumbi *et al.*, 1991); 12S – 12Sai (5'-AAA CTA GGA TTA GAT ACC CTA TTA T-3') and 12Sbi (5'-AAG AGC GAC GGG CGA TGT GT-3') (Palumbi, 1996); H3 – HexAF (5'-ATG GCT CGT ACC AAG CAG ACG GC-3') and HexAR (5'-ATA TCC TTG GGC ATG ATG GTG AC-3') (Colgan *et al.*, 1998); and 28S – EP4a (5'-CGT CTT GAA ACA CGG ACC AA-3') and EP5b (5'-TCC TGC TGT CTT AAG CAA CC-3') (Ogden & Whiting 2005).

The COI locus is widely used for barcoding, and so is an informative and relatively fast evolving gene, thus providing a better resolution towards the tips of the phylogenetic treesSometimes samples were collected from different localities along the same rivers to assess any population differences within the river. Representative samples were then selected for the more-conserved genes; including sequences from Genbank (for Teloganodidae or closely related families) for the selected genes (See Appendix 4A). Genes were first analysed separately and then combined to form mutlilocus datasets. Different genes were used in different matrices depending on the number of taxa available for the respective gene, for example, 28S was replaced by the less conserved 16S gene for within-genera, intraspecific analyses, due to availability of more operational taxonomic units (OTUs) for the 16S marker.

All PCR reactions were performed in a 25 µl volume using the following thermal cycling profile: initial denaturation of 94ºC for 5 min, followed by 35 - 40 cycles of denaturation at 94ºC for 30s, primer annealing at 48ºC (COI, 16S and 12S), 49ºC (28S) or 52ºC (H3) for 45s, elongation at 72ºC for 1 min 30s; followed by a final extension period of 72ºC for 10 min. PCR product amplifications were confirmed by a 1 % agarose gel electrophoresis using 5 µl of product mixed with 5 µl of tracking dye and stained with either SYBR® Green or ethidium bromide, then visualised on a UV trans-illuminator.

PCR products were sent to either MACROGEN Korea, Central Analytical Facilities (Stellenbosch University, South Africa) or SAIAB (South African Institute for Aquatic Biodiversity, Grahamstown, South Africa) for PCR product clean up, purification and cycle sequencing conducted under BigDyeTM terminator cycling conditions and then precipitations run through an Automatic Sequencer. Trace files were checked and edited using GeneStudio ™ Professional Edition v.2.2.0.0 (GeneStudio, Inc). Sequences were initially aligned using Multiple sequence alignment software, MAFFT version 7 (Katoh and Standley, 2013) on the online server at http://mafft.cbrc.jp/alignment/server/. Gaps in 16S and 12S were treated as missing. See Appendix 4A for a list of samples with corresponding gene loci sequenced.

4.2.2 Phylogenetic analyses

4.2.2.1 Gene Tree analyses

The Incongruence Length Difference (ILD) test (Farris *et al.,* 1994) evaluates the congruency and combinability among genes. This was assessed using a partition homogeneity test (PHT) in PAUP* (Swofford, 2002) with 1000 replicates. The phylogenetic signal of the combined gene datasets was verified in PAUP* using the skewness test statistic (g1) which measures the random variation in characters (noise) in the datasets. The test statistic takes the number of taxa and informative characters into account, which are compared to the 95 % and 99 % critical values published by Hillis and Huelsenbeck (1992). Data with g1 values lower than these critical values have significantly more phylogenetic signal than random noise. Saturation (multiple substitutions of transitions and transversions) within each gene was examined by plotting the transitions and transversions against the K80 distance in DAMBE v5.3.10 (Xia, 2013).

Models of molecular evolution for single gene datasets were estimates using jmodeltest v.2.1.6 (Guindon and Gascuel, 2003; Darriba *et al.,* 2012) on XSEDE via the CIPRES (Cyberinfrustructure for Phylogenetic Research) Science Gateway v3.3 (Miller *et al.,* 2010) which is supported by the San Diego Supercomputer Center (SDSC) and the University of California (UC San Diego). PartitionFinder (Lanfear *et al.,* 2012) was used to select the best-fit partitioning schemes and molecular evolution models for the combined nucleotide

alignments. The first, second and third codon positions were defined (as partitions) for the protein coding genes: COI and H3. The Akaike Information Criteria (AIC; Akaike, 1974) was used to find the most appropriate model of molecular evolution for each subset for Maximum Likelihood (ML) and Bayesian Inference (BI) analysis.

The partitioned schemes versus non-partitioned schemes for Bayesian Inference analyses were then tested by comparing the Bayes Factors (Kass and Raftery, 1995; Nylander *et al.,* 2004; Brandley *et al.,* 2005). All Bayesian Inference analyses were run in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) through the CIPRES Science Gateway. Each analysis was run with two independent runs of five to ten million generations each with a sample frequency of 1000 generations. Random starting trees with four chains (one cold, three hot) were used with trees sampled every 1000 generations. All parameters, excluding branch lengths and topology, were unlinked across partitions. Partitions were set using the *charset* and *partition* command, and models were set using the *lset* and *prset* commands associated with the models in Table 4.2. Stationarity was assessed in Tracer v1.5.0 (Rambaut and Drummond, 2007) after a 10 % burn-in and confirmation of acceptable effective sample size (ESS) scores, potential scale reduction factors (PSRF) at a value ~1.00, average standard deviation of split frequencies < 0.005 and likelihood score plots were observed. The first 10% of trees sampled were discarded from each run as burn-in. The majority rule consensus Bayesian topology and posterior probability values were then computed from the remaining sampled trees. Posterior probability support values from MrBayes analyses were plotted onto the corresponding nodes of the Maximum Likelihood (ML) 'best-tree' phylogram.

All Maximum Likelihood analyses were conducted using RAxML-HPC v.8 (Stamatakis, 2014) on XSEDE through the CIPRES Science Gateway. RAxML methods are slightly different from other ML programs; these use randomized stepwise-addition-order parsimony starting trees on which it initiates a Maximum Likelihood based optimization, whereas many other ML analyses use (distance method) Neighbour Joining (NJ) starting trees. This is an advantage of RAxML as it is more likely to find good ML trees if executed several times because ML optimisations of topology have a distinct starting point in the vast tree space (details can be found in the RAxML 8 manual; Stamatakis, 2014). Identical sequences were identified in RAxML and removed before the bootstrap and ML searches were conducted. All model parameters are estimated and optimized in the RAxML program. A rapid bootstrap

analysis of 1000 replicates and search for best-scoring ML tree using 12 fast, 12 slow and 12 thorough independent ML searches was performed on each dataset.

4.2.2.2 Splits Networks – Data summary and Reticulation

A phylogenetic tree attempts to resolve conflicting information in data patterns to infer evolutionary relationships while a network tries to highlight this conflicting information. Network analyses enable a higher resolution at an exploratory level, where features (tree-likeness, ambiguities and conflicting signal) can be deduced from the data without restricting attention to a single tree (Bryant and Moulton, 2004), as would be expected from the conventional maximum likelihood and Bayesian inference analyses. Splits networks represent incompatible and ambiguous signals in a dataset that potentially arise from reticulation, or a result of homoplasy. As the Neighbour-net (NN) networks are not rooted, there is no "direction" or evolutionary history associated with the nodes, hence the network is referred to as a summary of the data; or an exploratory (implicit) network analysis (Kloepper and Huson, 2008). NN networks are prone to Long Branch Attraction LBA, however, unlike bifurcating trees, networks can represent the both LBA and the signal of the underlying phylogeny (Clements *et al.,* 2003).

All network analyses were conducted in SplitsTree v.4.14.2 (Huson and Bryant, 2005). Neighbour-net (NN) networks were selected as it is a rapid approach that often results in higher resolution than other methods such as split decomposition (Bryant and Moulton, 2004).

The phylogenetic quality (tree-likeness) of distance matrices was tested using the delta index (Göker *et al.,* 2007; Auch *et al.,* 2006; Holland *et al.,* 2002). This approach makes use of delta scores and Q-residuals, which assesses the tree-likeness of a set of data, and can identify non-treelike or noisy datasets arising from processes such as parallel evolution and recombination (Holland *et al.,* 2002). The average delta scores and Q-residuals for each network analysis were computed. These indicate the extent of tree-likeness in the datasets, the closer to zero the scores, the more a tree fits the data. Any deviation from tree-likeness in the data was examined in the networks.

Firstly, to explore and summarise lower taxonomic relationships (species and population levels), NN networks for the entire COI dataset were calculated. The distance-based NN network was calculated based on the GTR model (Table 4.1), with 1000 bootstrap replicates

and followed by visual representation of the confidence interval (CI) at the 95 % level, by opacity of "edge" (similar to branch) lengths. For each of the combined datasets (i.e. the datasets used in ML and BI analyses, see Table 4.2), NN network analyses were conducted under the appropriate model with 1000 bootstrap replicates and visual representation (opacity of edges) of the 95 % CI. For comparative purposes, the NN bootstrap values and the maximum likelihood bootstrap values were mapped onto the networks. A consensus network from the RAxML bootstrap trees was created in SplitsTree and these support values were mapped onto the NN network. The NN networks with the ML and NN bootstrap support mapped onto the network show extra information lost between branch tips that cannot be seen on the conventional phylogram.

To investigate the genealogies and reticulation within and between the taxa, reticulation networks were computed. "Hybridisation Network" is the term used in the SplitsTree program and "Hybridisation" is a mathematical term for these reticulation network analyses, rather than implying hybridisation in the biological sense. Therefore to avoid confusion, the "Hybridisation network" from hereafter will be referred to as "reticulation network", as the analyses presented do not differentiate between the different causes of reticulation in the networks. These networks estimate any reticulations between samples by taking the unrooted graph, adding a root and then finding the minimum number of reticulation nodes needed to replace each reticulate region (McBreen and Lockhart 2006). This results in an evolutionary or explicit network (Kloepper and Huson, 2008). While one can draw evolutionary mechanisms and conclusions from the analyses, the data should be interpreted with caution and at least when drawing conclusions, *a priori* biological information is important, as it is difficult to determine whether a reticulation network is due to hybridization, incomplete lineage sorting or homoplasy; all of which can affect the conclusions of the results.

To calculate the reticulation networks; independent, ML best-scoring trees of each separate gene locus were imported into SplitsTree. A rooted consensus or "Supernetwork" network and reticulation network was computed on the datasets. "Supernetworks" are advantageous in this respect as they require only partially overlapping datasets (Huson *et al.*, 2004), allowing for inclusion of more gene trees. "Supernetworks" were computed for all five available genes to examine any reticulations in the higher taxon datasets. To further simplify the reticulations present where necessary, a majority rule tree was made in PAUP of the ML bootstrap trees.

These were combined into the "Supernetwork" with a total of 2 to 5 runs, following the methods of McBreen and Lockhart (2006).

Reticulation networks were used to investigate whether or not there has been reticulation or gene flow between sampled taxa, which may explain discrepancies in other analyses (such as the phylogenetic tree analyses). Further analyses on population genetics are needed to investigate details of these reticulations (hybridisation, introgression, recombination, incomplete lineage sorting or homoplasy), which is not the focus of this study.

4.2.2.3 Species Tree Estimation

A species tree was estimated using multilocus sequence data under the coalescent model and singular value decomposition anlaysis using SVDquartets (Chifman and Kubatko, 2014; 2015) in PAUP* (Swofford, 2002). The coalescent model (Kingman, 1982) assumes that events that occur in one population are independent of those occurring in other populations within the phylogeny and that coalescent events within populations are independent of other populations (Liu *et al.*, 2009). In addition, the model assumes that all pairs of lineages are equally likely to coalesce within a population, no gene flow occurs following speciation and no other evolutionary processes (such as horizontal gene flow or gene duplication/loss) have led to incongruence between gene trees and species trees (Liu *et al.,* 2009).

There is concern that as more genes are added, the process of estimating species trees from concatenated data can be statistically inconsistent and may converge to the wrong tree when a gene tree that is topologically incongruent with the species tree has the highest probability (Chifman and Kubatko, 2014; 2015). This can be overcome by explicitly modelling the coalescent process, which is done here by using the SVDquartets method, which utilises full data as the input is aligned sequences and not just branch lengths or topologies. This is an advantage as a fully model-based framework is used and allows for assessment of uncertainty using a non-parametric procedure. In addition, the SVDquartets method is faster than most other methods with comparably accurate results, for these reasons, this approach was chosen over other methods. The SVDquartets method calculates which of three possible splits is the true split for a set of four taxa, using the information provided by the observed site pattern distribution. Scores are computed for each split in a given quartet of taxa and the program selects the split with the best (lowest) score (Chifman and Kubatko, 2014; 2015).

Both the species-level and lineage-level inferences were considered using multigene datasets, particularly for the mitochondrial dataset (COI/16S/12S) as this dataset contained the most individuals per assigned species group (a total of 115 taxa in the mitochondrial dataset). Other datasets used (for comparative purposes) included those used in the phylogenetic tree analyses. Individuals in the sequence alignments were assigned to species groups; therefore each terminal taxon in the species tree phylogram is represented by more than one sample. Individuals with uncertain placement were not assigned a group (left as individuals in the analysis) or were grouped with similar individuals from the same locality. A species-tree analysis was conducted using 100 000 randomly generated quartets with 100 bootstrap replicates.

Taxonomic distinctiveness was examined using Rosenberg's test (2007), applying the "Spider" package (Brown *et al.*, 2012) within the R statistical environment (R Core Team, 2013). This package makes use of distance measurements to calculate taxonomic distinctiveness. Rosenberg's test was applied to the combined mitochondrial dataset (COI/16S/12S) and the COI dataset, as these had the highest number of taxa. Rosenberg (2007) uses this test to examine whether monophyly in phylogenetic analyses has been produced by evolutionary processes or chance (and insufficient sampling). The null hypothesis is that monophyly has occurred by chance, rather than distinctiveness of the lineage (Rosenberg, 2003; Rosenberg, 2007) and can also manage smaller sample sizes representative of certain groups in the analyses. This overcomes the problem of the Likelihood Ratio Test (Huelsenbeck *et al.*, 1996), which investigates monophyly by using constrained and unconstrained DNA sequence sets, which cannot discriminate between chance and true monophyly.

4.2.2.4 Landscape genetics

Landscape genetics refers to the way geographical and environmental features structure genetic variation at both population and individual levels. The aim is to detect genetic discontinuities and their correlation with landscape features (Cushman *et al.*, 2006).

If genetic differences are related to landscape structure, the *isolation by barrier* hypothesis determines whether the "populations" are genetically distinct as a result of some form of barrier to gene flow. Alternatively, selective pressures may exist on a geographic distance gradient, where genetic dissimilarities increase with increasing distance, which is the basis

for the *isolation by distance* hypothesis. These tools can help to evaluate land use impacts (on gene flow) and identify areas of important connectivity (e.g. corridors) for focal species and conservation.

Considering only the South African Teloganodidae for landscape genetics analysis, the hypotheses of *isolation by distance* and *isolation by barrier* were examined to see which of these factors may be contributing to genetic dissimilarities within the species groups.

The contribution of isolation by distance (IBD) for each genus/species group was assessed using the Isolation By Distance Web Service (IBDWS; Jensen *et al.,* 2005) which makes use of the Mantel test (Mantel, 1967) to determine the significance of the correlation (r) between the log-transformed genetic distance [Slatkin's Similarity Index M = $((1/Phi_{ST})-1)/4)$] and log-transformed geographic distance, with 1000 randomisations (distances of zero were set to 0.0001 before logging), using the uncorrected proportions parameter for genetic distances between sequences for populations. The COI dataset was used as it is the most informative gene region with the most number of representative. Geographic distances between sample sites were calculated using the online Geographic Distance Matrix Generator v1.2.3 (Ersts, Accessed 2015/10/28).

Isolation by barriers was estimated using Barrier v.2.2 (Manni *et al.,* 2004), which identifies areas with significant genetic differentiation. This analysis was conducted on each currently described taxonomic group separately. That is, *Ephemerellina* ("E2" group), *Lestagella*, *Lithogloea*, *Nadinetella brincki* complex, *N. crassi* complex and the Species A group. Species B and Species C were excluded due to insufficient sample sizes. Firstly, 100 bootstrapped datasets of the molecular sequence dataset were created in SeqBoot (PHYLIP v3.69; Felsenstein 1989). DNAdist (PHYLIP v3.69; Felsenstein 1989) was used to convert the 100 bootstrap sequence alignments into 100 genetic distance datasets; this was then imported into Barrier for analysis. Geographic coordinates in decimal degrees were converted into X and Y coordinates which were then imported into the Barrier program. The 100 genetic distance replicates allowed the program to calculate confidence values for the barriers, which were also visually supported by the thickness of lines representing where the strongest barriers were found. The number of barriers chosen *a priori* was dependent on the number of catchments represented by the dataset.

4.3 Results

4.3.1 Data characteristics

A total of the 227 (of the 255) samples for the COI gene were successfully amplified, which included more than one sample from each river. The characteristics for each gene dataset are summarised in Table 4.1. The percentage of parsimony-informative sites within each gene fragment ranged from 59 % (12S) to 21 % (28S; Table 4.1). The g1 statistics (in Table 4.1 under "phylo signal") show that there is phylogenetic signal in all loci, with a minimum amount of random noise. According to the partition homogeneity tests, the combined datasets represented in Table 4.2 were not found to be significantly incongruent ($P > 0.05$ for all presented gene combinations in Table 4.2).

Combined datasets were separated into generic and specific levels (Table 4.2), the reason being two-fold; firstly the conserved gene loci (such as 28S) have only few representatives (generic level) that were sequenced (see Table 4.1, where 28S has only 34 taxa in the dataset) and secondly, it reduces the random noise within the analyses (such as saturation of genes due to distantly related taxa being included) and focuses on fewer taxon ranks at a time. These combined datasets were used for all further phylogenetic tree analyses unless otherwise stipulated. Reference names for each dataset can be found in Table 4.2 and will be used from this point forward. The list of taxa used in each of these combined datasets can be found in Appendix 4A.

Saturation plots did not show any serious signs of saturation (Appendix 4B). The Species C clade branch appears to be highly diverged for the COI gene, indicating a possible pseudogene for COI as the other gene loci do not exhibit a similar pattern; this may affect certain phylogenetic results and must be kept in mind when interpreting results.

The partitions from PartitionFinder (using AIC criterion, suitable for Maximum Likelihood, but not necessarily Bayesian analyses) are listed in Table 4.2. The log-likelihood (lnL) values from the data partition sets (data not shown) resulted in the best lnL values for these partitions. Partitions for the Bayesian analyses were determined using Bayes Factors (following Nylander *et al.,* 2004) with equation $2log_e(B_{10})$ where "1" is an unpartitioned dataset and "0" is partitioned. The factor value was found to be lower than 2 in all cases which indicates that there is little difference between the partitioned and unpartitioned datasets, which ultimately is "not worth more than a bare mention" (Kass and Raferty, 1995).

As a result the Bayesian analyses did not require partitions and separate models in the dataset alignments.

Table 4.1. Data characteristics showing model selection after identical sequences were removed from the various alignments.

Partition	No. Terminals	No. Sites	No. Variables	No. Pars Info	% Pars Info	Phylo signal	Model
CO1	214	677	395	332	49	+	GTR+I+G
COI_1		226	118	87	39	+	SYM+G
COI_2		226	55	25	11	+	GTR+I+G
COI_3		225	222	220	98	+	GTR+I+G
16S	142	535	294	243	45	+	GTR+I+G
12S	139	391	263	229	59	+	GTR+G
28S	34	649	258	136	21	+	GTR+G
Histone 3	122	351	147	110	32	+	GTR+I+G
H3_1		117	9	2	1.7	+	K80+I
H3_2		117	29	13	11	+	GTR+I+G
H3_3		117	109	95	81	+	SYM+G

Table 4.2. Partitions, congruent and incongruent datasets used for analyses. Code names for each dataset are listed below and are used in the manuscript from this point forward. Mitochondrial datasets and 5-gene datasets listed are used for splits and reticulation networks. Model selection for ML analyses calculated in PartitionFinder, and models according to Bayes Factor results (Bayes factors < 2 for all datasets, therefore only one model was necessary).

Code	No. Terminals	No. Sites	Gene combination	Maximum Likelihood Model	Bayesian Model
"Afr/SAm Group"	27	2068	COI/12S/28S/H3	GTR+I+G	GTR+I+G
			COI_1	SYM+G	
Incl.			COI_2	GTR+G	
Melanemerella			COI_3	GTR+I+G	
			12S	GTR+I+G	
			28S	GTR+G	
			H3_1	JC+I	
			H3_2	GTR+G	
			H3_3	SYM+G	
"Afr/Mad Group"	43	1954	COI/16S/12S/H3	GTR+I+G	GTR+I+G
			COI_1	GTR+I	
Incl.			COI_2	GTR	
Manohyphella			COI_3	GTR+I+G	
			16S	GTR+I+G	
			12S	GTR+G	
			H3_1	JC+I	
			H3_2	GTR+I	
			H3_3	GTR+G	
"Ephem Clade"	30	1954	COI/16S/12S/H3	GTR+I+G	GTR+G
			COI_1	GTR+I	
Incl. Species A			COI_2	GTR	
Species B			COI_3	GTR+G	
Species C			16S	GTR+G	
			12S	GTR+G	
			H3_1	JC+I	
			H3_2	GTR	
			H3_3	GTR+G	
"Lest Clade"	25	1954	COI/16S/12S/H3	GTR+I+G	GTR+I+G
			COI_1	SYM+G	
			COI_2	F81	
			COI_3	GTR+G	
			16S	GTR+G	
			12S	HKY+G	
			H3_1	JC+I	
			H3_2	GTR+I	
			H3_3	GTR+G	
"Nad/Lith Clade"	42	1954	COI/16S/12S/H3	GTR+I+G	GTR+I+G
			COI_1	SYM+G	
			(COI_2, H3_2)	GTR+I+G	
			COI_3	GTR+G	
			16S	GTR+I+G	
			12S	GTR+G	
			H3_1	JC+I	
			H3_3	GTR+G	
Mitochondrial	115	1603	COI/16S/12S	(incongruent)	
5 Genes	31	2603	COI/16S/12S/28S/H3	(incongruent)	

Af = Africa; SAm = South America; Mad = Madagascar; Ephem = *Ephemerellina*; Lest = *Lestagella*; Nad = *Nadinetella*; Lith = *Lithogloea*.

4.3.2 Phylogenetic and Data-summary Analyses

Neighbour-net (NN) networks represent the complexity and ambiguity of data, and give an indication of the general trends and structure in the data, without limiting it to a single bifurcating tree. A NN network on the full COI dataset is presented first as it shows some of the basic trends in the overall phylogeny (Figure 4.2).

Maximum likelihood (ML) analyses of the datasets yielded a most-likely fundamental tree for each of the combined datasets (as mentioned in Table 4.2). The Bayesian Inference (BI) posterior probabilities were superimposed onto the ML best-scoring trees. The bifurcating tree as a result of ML and BI analyses for each dataset is then followed by a NN network of the appropriate dataset. At species level in the ML and BI phylograms, it was difficult to determine clear species groups within certain clades, particularly in *Ephemerellina* and *Lithogloea*.

All Neighbour-net (NN) networks exhibit a prevailing tree-like structure, demonstrated by the Delta and Q-residual scores (Table 4.3) for each dataset which show fairly low scores (comparable to low scores reported by Göker and Grimm, 2008) that indicate that all networks are considerably tree-like and the Neighbour-net splits trees show similar results to the phylogenetic tree analyses.

Table 4.3. Delta and Q-residuals scores for each dataset used in SplitsTree Network analyses

Dataset	Delta Score	Q-residual Score
"Afr/SAm Group"	0.2137	0.005433
"Afr/Mad Group"	0.1229	0.003098
"Ephem Clade"	0.1269	0.001873
"Lest Clade"	0.1570	0.002344
"Nad/Lith Clade"	0.1348	0.003250
"COI All"	0.2145	0.007676

The "Afr/SAm Group" and "COI All" datasets have the highest scores. The portion contributing to the non-treelikeness is investigated using NeighbourNet networks for each dataset. The tree-likeness is also highlighted the NN network figures (Figures 4.1, 3.4, 4.7, 4.10, 4.13, 4.16) visually by CI shading on networks (faint lines mean low CI support) and ML consensus bootstrap values superimposed on the networks. All networks presented identify the same highly supported clusters (>80% NN bootstrap), which at least identifies the genera and main divergences within certain genera.

4.3.2.1 Neighbour Network for COI dataset

The COI dataset NN network is tree-like (Table 4.3, Figure 4.2), however as the generic clusters are examined more closely, some distinct clusters within are evident while others display many conflicts and ambiguities. As each cluster is examined more closely, the more the conflicting splits between samples increase. This is to be expected as gene flow between species and populations is more recent and can encompass various reticulation events that can cause the ambiguity (such hybridisation, recombination, introgression, homoplasy, incomplete lineage sorting or sampling error). It is important to remember that these networks do not necessarily represent any evolutionary relationships, but can rather be seen as data summaries, to pick up trends and formulate hypotheses. It is also possible that the NN network can represent the evolutionary history, but this is not for certain, and currently cannot be tested (Morrison, 2010).

In terms of higher taxon relationships, the NN network in Figure 4.2 links Melanemerellidae (South America) and Ephemerythidae (Africa). There also appears to be a connection with *Manohyphella* (Teloganodidae from Madagascar) and Melanemerellidae, while the Indian teloganodid (*Dudgeodes palnius*) does not show a close connection to the Madagascan teloganodid. Most of the conflicting signal is found at the base of the network (at the higher taxon level) and as NN networks are prone to long-branch attraction (LBA), it is uncertain at this point whether it is a result of LBA or if it is in fact phylogenetic signal in the data. More samples are needed to resolve these relationships.

In terms of the South African teloganodids, Species C had to be removed from the analysis as the sequences produced extremely long-branch/ edge lengths which distorted the data (possible pseudogene of the COI locus). This taxon was therefore removed from this analysis to prevent any LBA.

Clusters formed well according to the genera, with high support values in both new (Species A, Species B and Species C) and existing genera. *Lestagella*, *Nadinetella*, *Ephemerellina* and the new genera have deep generic divergence splits (Figure 4.2). Two splits can be seen in *Ephemerellina* (from this point forward referred to as ‘E1’ and ‘E2’), three splits in *Lestagella* (‘L1’, ‘L2’ and ‘L3’) and three splits in *Nadinetella* (‘*N. brincki* complex’, ‘*N. crassi* complex’ and ‘*N.* ‘Robertson’’).

Lestagella is generally well-separated according to locality in cluster L2 and contains a group from the southern and western parts of the Breede River catchment. Samples from cluster L1 do not overlap in rivers, but do exist in the same catchment (Breede catchment, see Figure 4.2), with particular reference to the Koornlands, Robertson and Barrydale areas of the Breede catchment (also refer to map in Figure 4.1).

Ephemerellina E1 (not shown enlarged) forms a cluster distinct from E2. The enlarged E2 cluster shows three well-separated groups, from Robertson, Olifants/AFR and a group including many catchments (Figure 4.2).

Species A forms at least two distinct clusters, separated into AFR catchment as one clade, and Gourits, Barrydale, Breede-West, Palmiet and Olifants-East as another. Species B is well-supported, however there are few samples from few sites representing this cluster.

Nadinetella has three deeply diverged clusters, found in overlapping rivers (i.e. sympatric). The *N. brincki* complex shows that a Berg/Olifants group is clearly separated from the rest. The *N. crassi* complex shows some evidence of well-supported distinct clustering between catchments, with one Kogelberg (Palmiet catchment) sample distinctly separated from the other Kogelberg sample (Figure 4.1, Appendix 4A). The *N.* 'Robertson' group is markedly separate from the other groups, and has only been recorded from this one area of the Breede River catchment (Figure 4.1).

Lithogloea has considerable conflicting signal, which could be as a result of homoplasy (the ambiguities show within-gene conflict in this NN analysis). Separation of the Eerste, Palmiet and Olifants river-catchment taxa from the ambiguous Breede catchment cluster has moderate to high support.

As data is tree-like (Table 4.3), traditional bifurcating phylogenies for the various groups are investigated with more confidence. Congruent datasets (Table 4.2) are used to resolve higher taxonomy status for *Manohyphella* (Madagascan teloganodid), Melanemerellidae (South American close relative), Ephemerythidae (African close relative) and Asian Teloganodidae lineage ("Afr/SAm Group" and "Afr/Mad Group"). These phylogenetic analyses are conducted to resolve relationships between genera and species as far as possible. Within the conflicting signal data within the genera, a more detailed data-summary (implicit) NN network is examined and explicit reticulation network analyses are used to resolve the relationships that are observed in Figure 4.2.

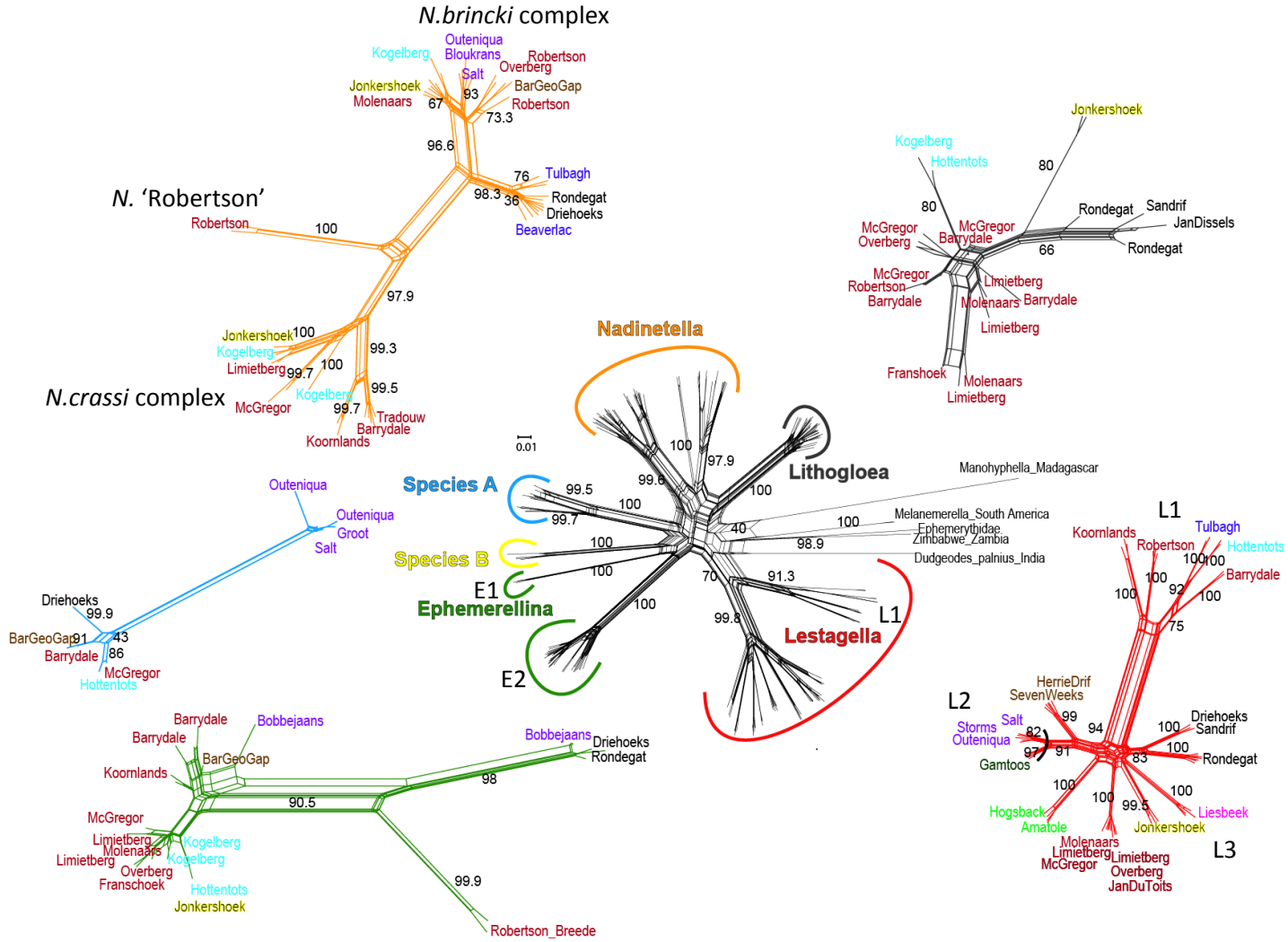


Figure 4.2. COI Neighbour Net network showing relationships between 214 samples (centre network), relationships within genera are shown in coloured splits networks which are enlarged to enable visual detail of clustering. NN bootstrap values shown. Edge labels represent either river or region names where samples were collected (Appendix 4A), labels are colour coded according to catchment (refer to Figure 4.1). Long edges represent good support.

4.3.2.2 African/South American/Asian Group ("Afr/SAm Group")

In the generic level ML and BI phylogeny of "Afr/SAm Group" (Figure 4.3) which includes Asian Teloganodidae (*Dudgeodes ulmeri*), East African *Ephemerythus* (Ephemerythidae) and South American *Melanemerella* (Melanemerellidae), there is an interesting link between the East African *Ephemerythus* and South American *Melanemerella*. They form a well-supported monophyletic clade that is positioned as a sister clade to the South African (Western Cape) Teloganodidae, leaving the family paraphyletic when considering the Asian species, *D. ulmeri.* All nodes show good support from the combined dataset of four genes, including mitochondrial and nuclear DNA data (Table 4.2). The phylogeny for South African Teloganodidae splits into two groups (arrowed on phylogeny in Figure 4.3), with the *Lestagella* clade splitting from the other genera. The nymphs of the *Lestagella* clade have a characteristic head-fringe which is unique to this lineage (Figure 4.3). Hereafter these are referred to as the "fringed" clade, while the remaining genera are referred to as the "non-fringed" clade. The Madagascan teloganodid, *Manohyphella*, does not possess this head-fringe.

The L1 and L2/L3 clades seen in the NN network within genus *Lestagella* are well supported in this analysis. *Lithogloea* forms a close sister clade to the *Nadinetella* genus, which has three distinct clades within the genus (as seen in the NN network, Figure 4.2), all with high support from both ML and BI. *Ephemerellina* has two distinct, deeply diverged and well-supported clades within the genus (E1 and E2), while Species A, Species B and Species C also form distinct clades with high support, and may represent distinct genera.

The NN network (Figure 4.4) shows good support for the Melanemerellidae/Ephemerythidae clade, which also groups with Asian lineage. Although these groups have long branches, they have high support from ML, BI and NN analyses, so long-branch attraction is unlikely to be an issue.

The reticulation network (Figure 4.5) was conducted on the five gene dataset (Table 4.2), using the ML trees produced in RAxML. This allowed for all representatives of the higher taxon level to be investigated in one analysis. Ancestral nodes of *Teloganella* (closely related family, the Teloganellidae) and *Dudgeodes ulmeri* (Asian teloganodid) share a reticulate connection, as do Ephemerythidae and Melanemerellidae ancestors (Figure 4.5). However, more samples are needed to further infer any relationships within the higher taxa.

Manohyphella (Madagascar) appears to be separated from the Asian group, as was seen in the NN network, there is ancestral reticulation between *Ephemerellina* and the base group comprising *Lithogloea*, *Nadinetella* and Species A (a possible effect of incomplete lineage sorting).

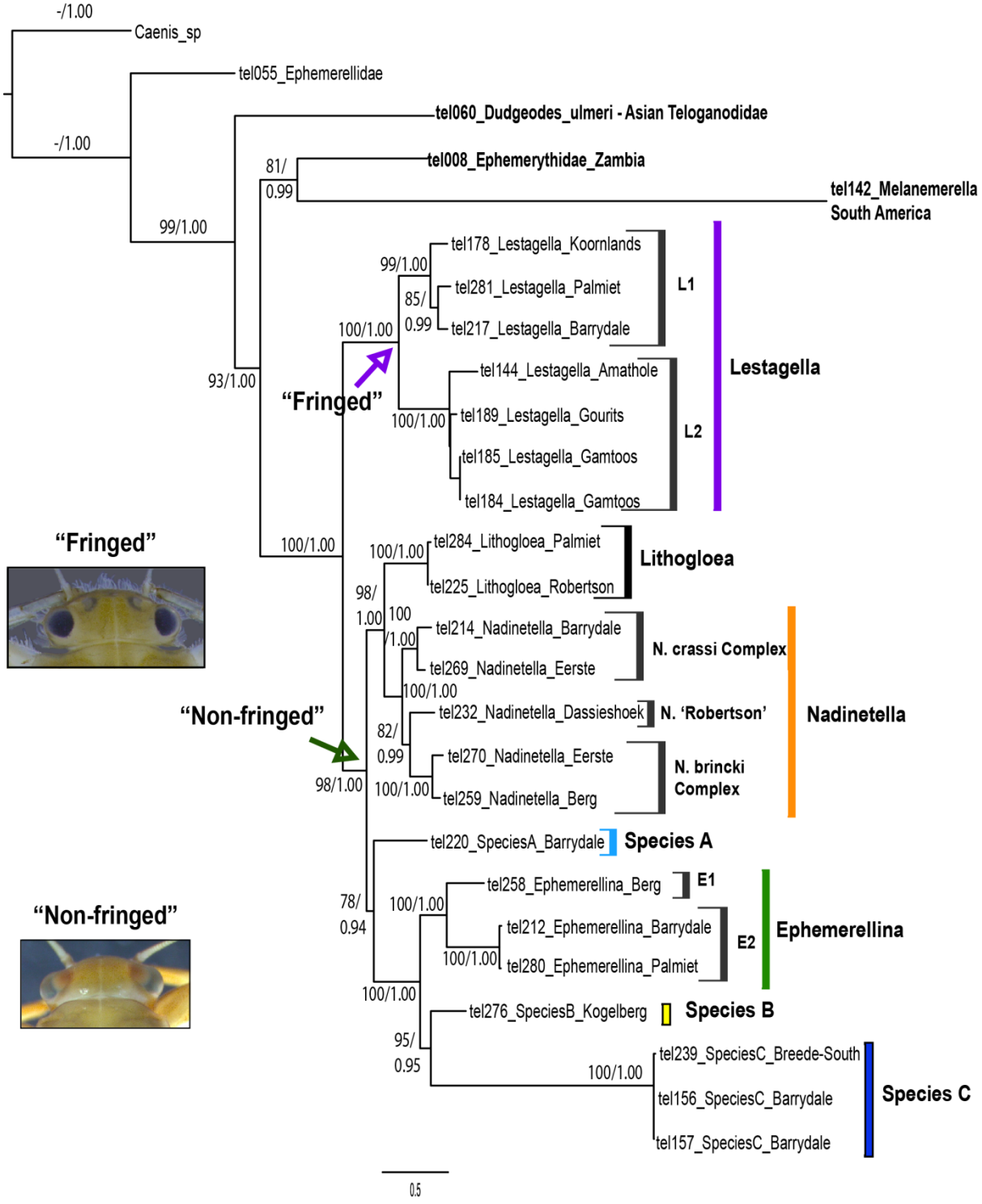


Figure 4.3. The "Afr/SAm Group", ML best scoring tree with ML bootstrap support followed by BI posterior support. Nodes in the phylogram represent main division of "fringed" and "non-fringed" South African Teloganodidae. Species clades and subclades are deoicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.4.

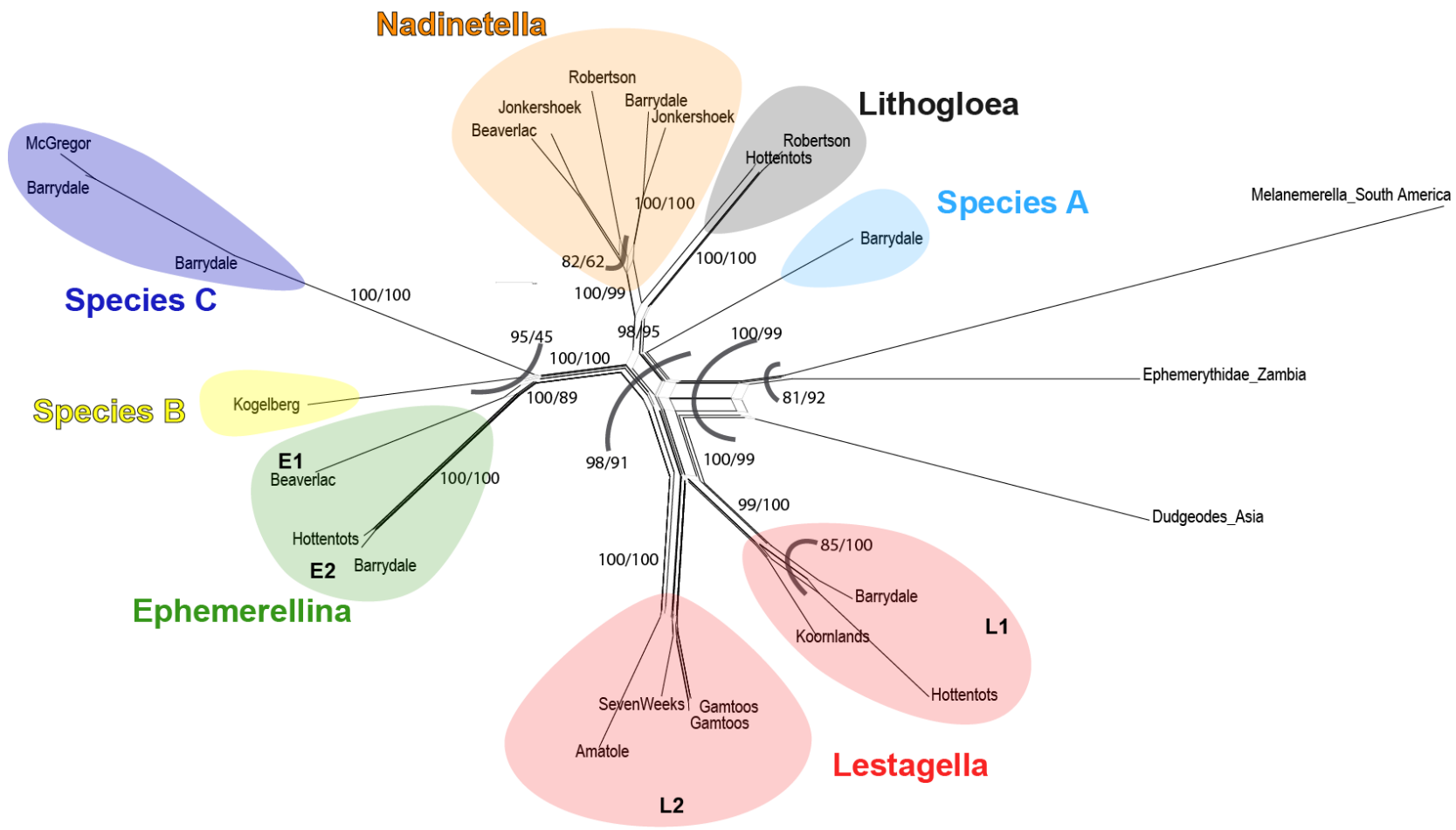


Figure 4.4. Neighbour Net network with ML bootstrap support (from ML consensus trees) super-imposed onto the network, followed by NN bootstrap support (Grey lines drawn onto network to depict well-supported clades formed by the distance-based NN and ML bootstrap). The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge. Main clusters (within and between genera) denoted by coloured bubbles. Long edges represent good support.

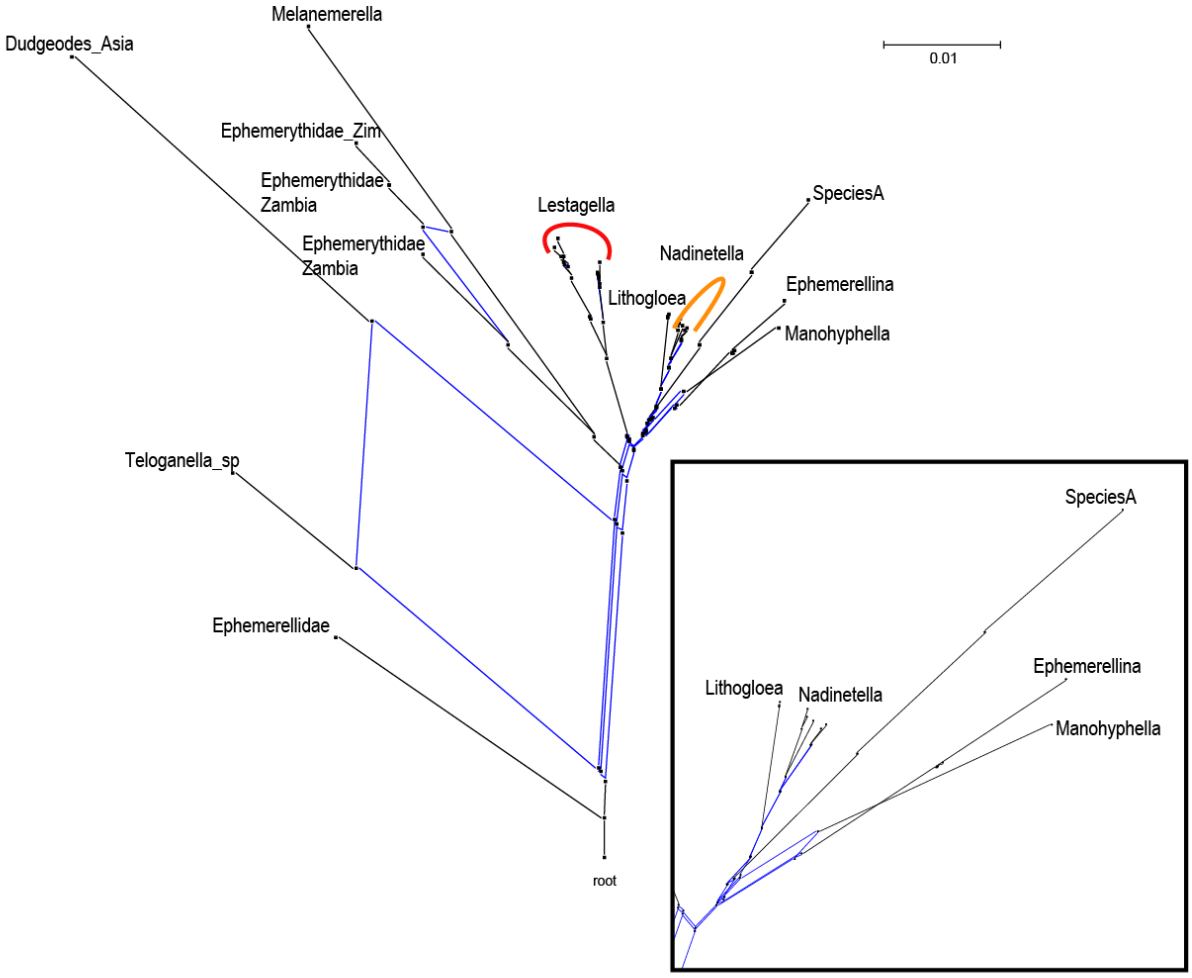


Figure 4.5. Reticulation network for higher orders using the "Supernetwork" analysis of the five combined genes (COI/16S/12S/28S/H3). Edges are coloured in blue, indicating a reticulation event. "Supernetworks" do not need all trees to have the same taxa (for example, there is no 28S sequence for *Manohyphella*). Long edges represent good support.

4.3.2.3 African/Madagascan Group

A 28S sequence for *Manohyphella* from Madagascar was not available on Genbank and not successful in laboratory amplification, so separate generic level analysis (including the Madagascan *Manohyphella*) was performed on a dataset excluding 28S and including 16S. Due to incompatible genes available for analysis, the Asian teloganodid (*Dudgeodes ulmeri*) was excluded from the "Afr/Mad Group".

The "Afr/Mad Group" (Figure 4.6 – 4.8) provides a more detailed view of the Western Cape Teloganodidae and its relationship with *Manohyphella*, using 4 gene loci (COI/16S/12S/H3; Table 4.2). The genera still form well supported clades, similar to previous figures. It is important to note here that *Manohyphella* appears to group with the Western Cape teloganodids, excluding the *Lestagella* clade, however with only moderate support from ML and low support in the BI analysis, so exact placement of the Madagascan genus is uncertain from this analysis. However there is good support that includes *Manohyphella* with the well-supported node for this group of South African teloganodids, and still forms a monophyletic clade with the South African teloganodids (Figure 4.6, 4.7). Although the branch with Species C shows much variation, it has very good support and is unlikely to be affected by long-branch attraction (LBA). *Manohyphella* on the other hand may be affected by LBA, and this is possibly be the reason for the uncertainty of placement within this group. The data summary (NN network, Figure 4.7) indicates a tree-like structure, CI and bootstrap result shows a fairly weak link between *Manohyphella* and Ephemerythidae (seen by faintness of the edges connecting these two groups), this link was also not supported by the ML consensus. CI and bootstrap support show higher support for grouping *Manohyphella* with "non-fringed" southern African teloganodids, and Ephemerythidae pulling more towards *Lestagella*, with moderate to good support from NN and ML bootstrap analyses. However, conflicting edges (box-like structures seen at the base of the network) are apparent, and may be a result of homoplasy. A reticulation network analyses (from a "Supernetwork" of the ML gene trees) was performed on the data to try and resolve this (Figure 4.8). There appears to be a reticulation event (similar to Figure 4.5) between the lineage that gave rise to the Species A, Species B, Species C and *Ephemerellina* clade, and the lineage that gave rise to an ancestor of Madagascan *Manohyphella*. This event (attributable to hybridisation, deep coalescence or introgression) is the likely cause of the problems placing the Madagascan species within the bifurcating tree and the data-summary network.

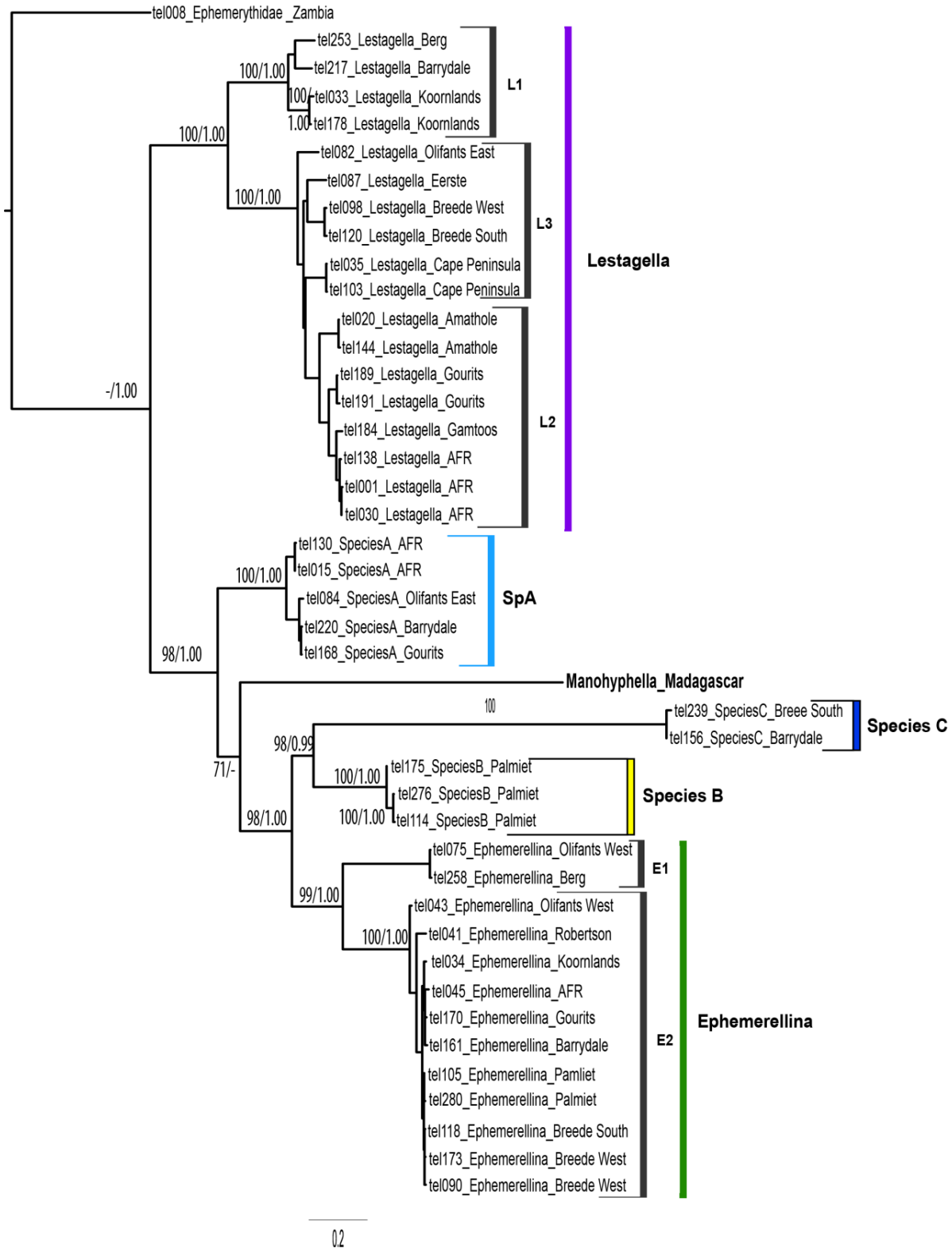


Figure 4.6. The "Afr/Mad Group", ML best scoring tree with ML bootstrap support followed by BI posterior support. Species clades and subclades are depicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.6.

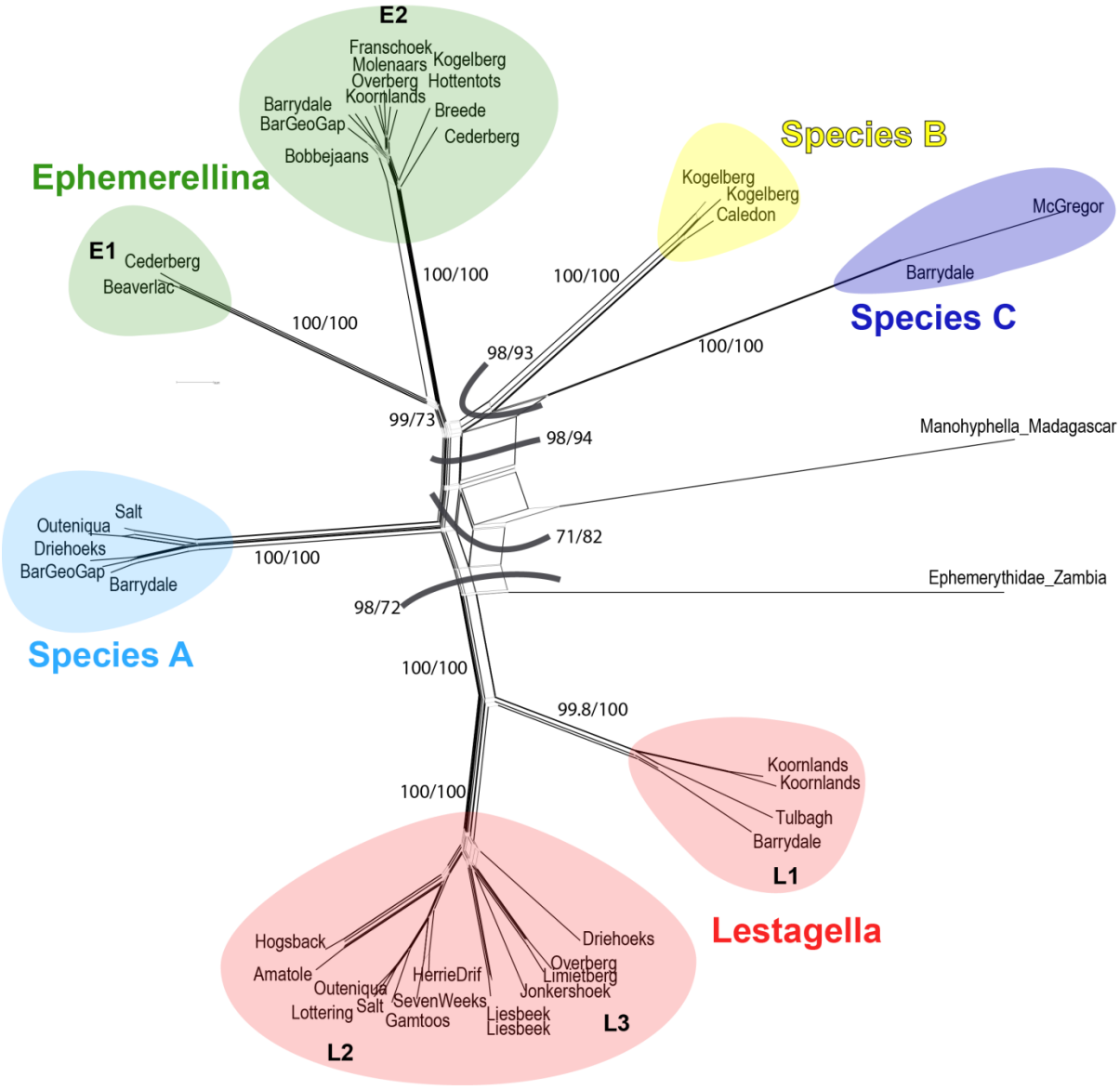


Figure 4.7. NN network with ML bootstrap consensus of "Afr/Mad Group". Both the bootstrap from ML tree and the bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge. Long edges represent good support.

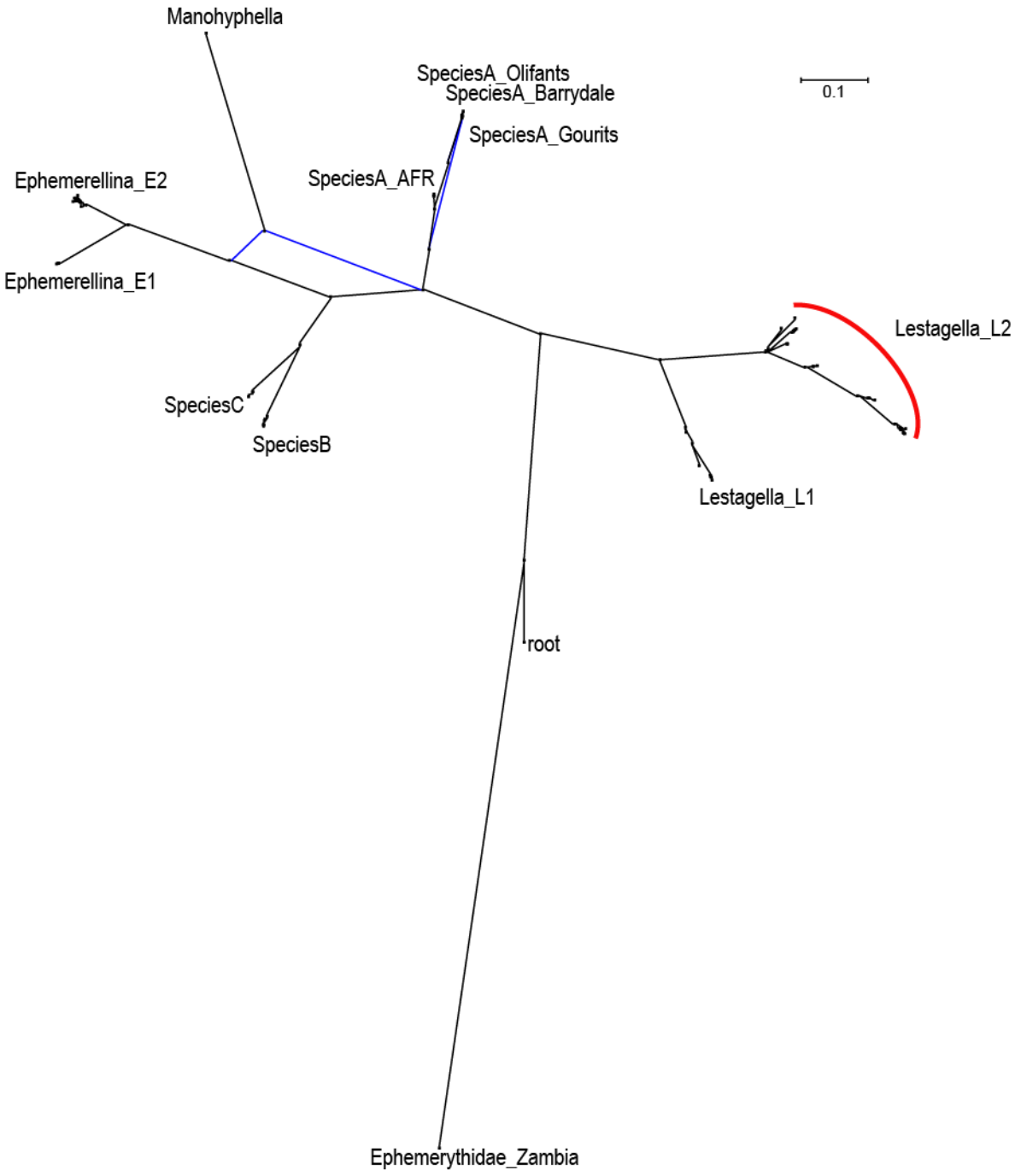


Figure 4.8. Consensus "Hybridisation" or reticulate network of the "Afr/Mad Group", representing four genes (COI/16S/12S/H3). Long edges represent good support.

4.3.2.4 *Ephemerellina*/Species A/SpeciesB/Species C Group ("Ephem Clade")

A combined, four gene dataset (Table 4.2) was analysed to resolve lineage relationships within the "Ephem Clade" (Table 4.2), which includes *Ephemerellina*, Species A, Species B and Species C. (Figures 4.9 – 4.11).

ML and BI analyses (Figure 4.9) show good support for main clades Species A, Species B, Species C and *Ephemerellina*. Species A shows a distinct split into two clades, AFR separated from the rest; Olifants-East has good support for divergence from the Barrydale/Gourits group, however the resolution is not good.

Species A forms two distinct and well supported clades, indicating that it may comprise more than one species, the AFR forming a separate group to the group occurring further West (comprising of the Olifants, Breede and Gourits River catchments). Inference beyond this point is difficult in this analysis.

Species B, currently only known from the Kogelberg and Caledon (Palmiet River catchment) and Cederberg (Olifants River) forms a well-supported clade. Species C seems to be highly divergent for the COI locus (possible pseudogene). However, the strong support at each node suggests that LBA is unlikely. The species is only known from two localities despite extensive sampling (Figure 4.1), McGregor (Breede-South) and Barrydale (also Breede River catchment).

The *Ephemerellina* group, 'E1' from the Jan Dissels River (Olifants River catchment) and Ratel River (Berg River catchment) is distinctly separate from group 'E2' (which is more widespread and diverse). *Ephemerellina* 'E2' is a well-supported monophyletic clade, with poor internal resolution.

In the NN network analysis (Figure 4.10), there is very strong support (ML and NN) for splitting all genera in a tree-like fashion (see Delta scores in Table 4.3). Species A splits into AFR and Barrydale/Gourits/Olifants catchments with good support, and there is evidence of Olifants River lineage splitting away from Gourits lineage with good support. Species B and Species C resemble the phylogenetic trees (Figure 4.9).

In the *Ephemerellina* 'E2' NN network (Figure 4.10), Breede-Robertson and Olifants River lineages separate from the remainder of the group (Breede-East, Breede-West, Palmiet,

Gourits and AFR catchments). There also appears to be some separation between Breede-East (and Easterly catchments Gourits and AFR) and Breede-West (and westerly Palmiet catchment).

In the reticulation network (Figure 4.11a), Species A, Species B and Species C show high levels of reticulation and the relationships are difficult to interpret in detail. The consensus network could not be simplified to display the relationships in all the optimal gene trees used to build a reticulation network. Species A shows a reticulation event between the ancestor of the Gourits catchment and AFR lineages to produce the Olifants group. There is another reticulation event between the ancestor of the whole clade and a Gourits ancestor to produce the Breede-Barrydale lineage. *Ephemerellina* E2 complex shows a connection between ancestors of the Olifants catchment and Robertson area, while the rest of network is comprised of conflicting signal.

A simplified network using only majority rule trees (50%) made in PAUP* from the existing ML bootstrap trees was estimated. These were combined into the "supernetwork" with a total of 2 to 5 runs, and a simplified reticulation network proposed the reticulation events seen in Figure 4.11b.

The simplified reticulation network shows only three hybrid events for the entire clade. It would appear that the common ancestor of the lineage giving rise to the Breede-West, Breede-South, Palmiet and Eerste catchments is the result of a possible reticulation between the common ancestor lineage of Breede-Barrydale/Gourits/AFR and the Breede-Koornlands lineage.

The reticulation history within Species A is complex, the two reticulation edges (out-degree of 2) gives rise to a connected network (out-degree > 2). This suggests that there are 'sub-histories' that cannot be reconstructed and represented in this simplified analysis. This network suggests that there is possible hybridisation, incomplete lineage sorting or homoplasy between Species C and common ancestors of Species A and Species B.

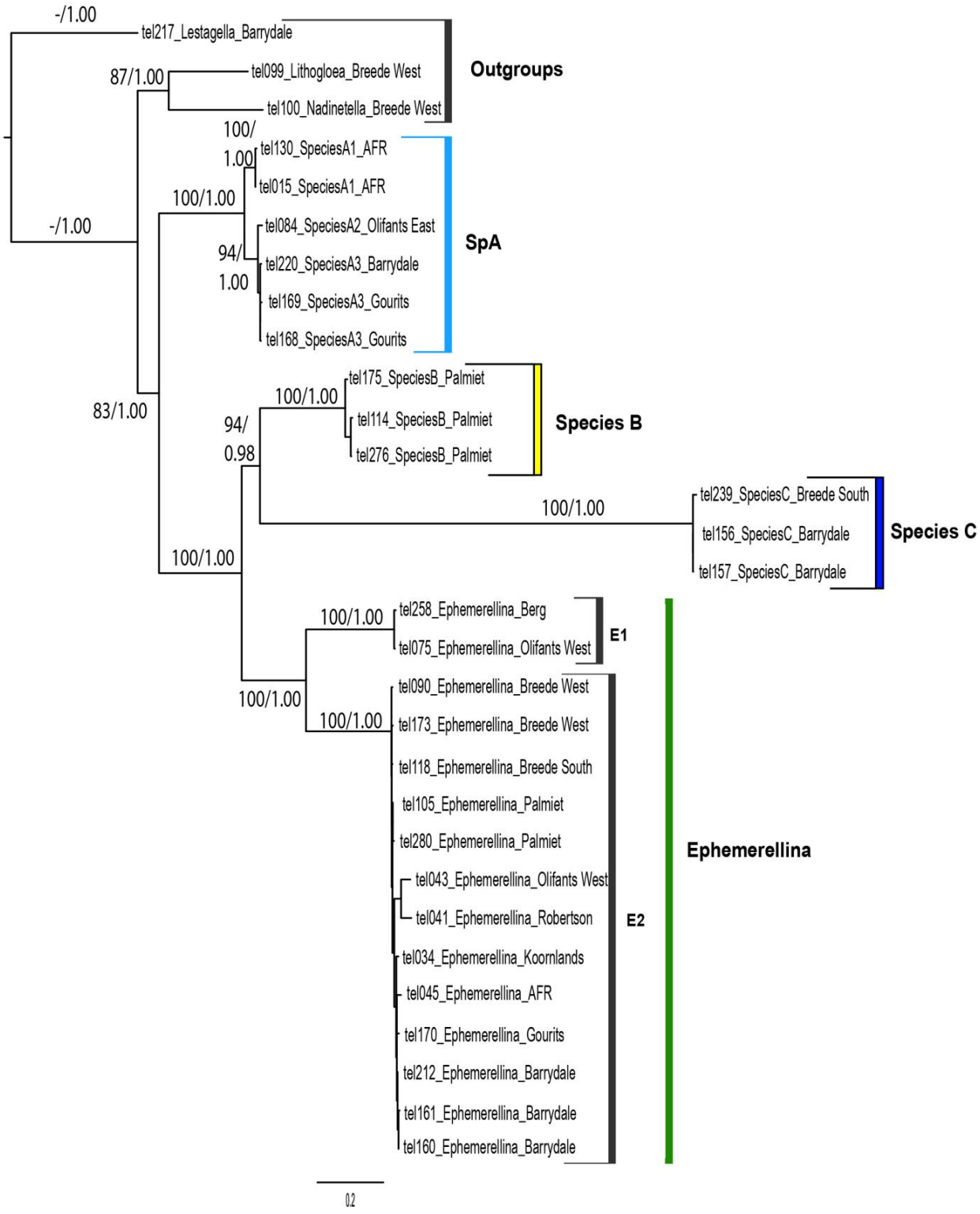


Figure 4.9. The "Ephem Clade" ML best scoring tree with ML bootstrap support followed by BI posterior probability support. Species clades and subclades are depicted by coloured bars which correspond to the NN network in Figure 4.2 and 4.10.

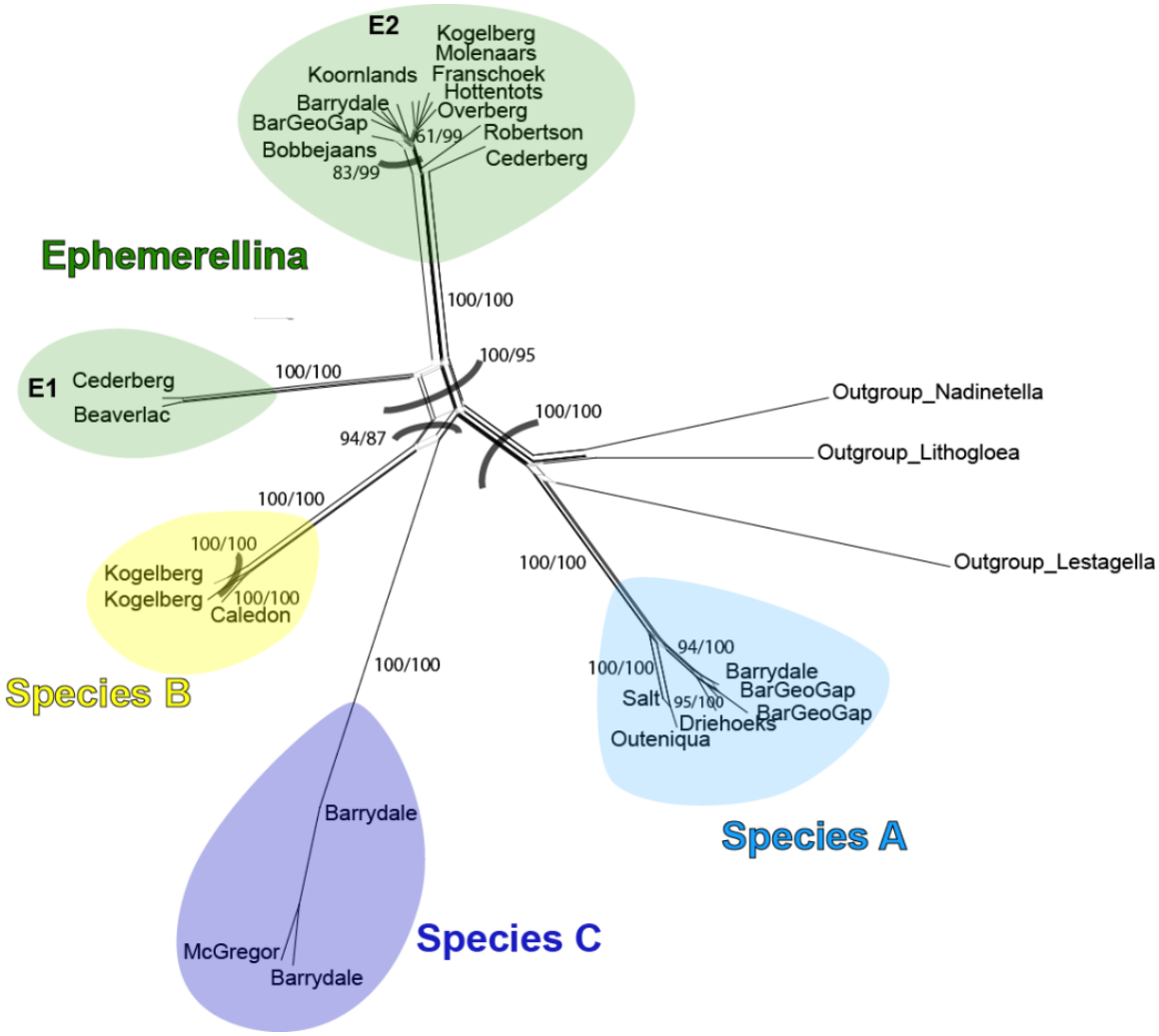


Figure 4.10. Implicit NN network with ML bootstrap consensus of the "Ephem Clade". Both the bootstrap from ML tree and then bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch") Long edges represent good support.

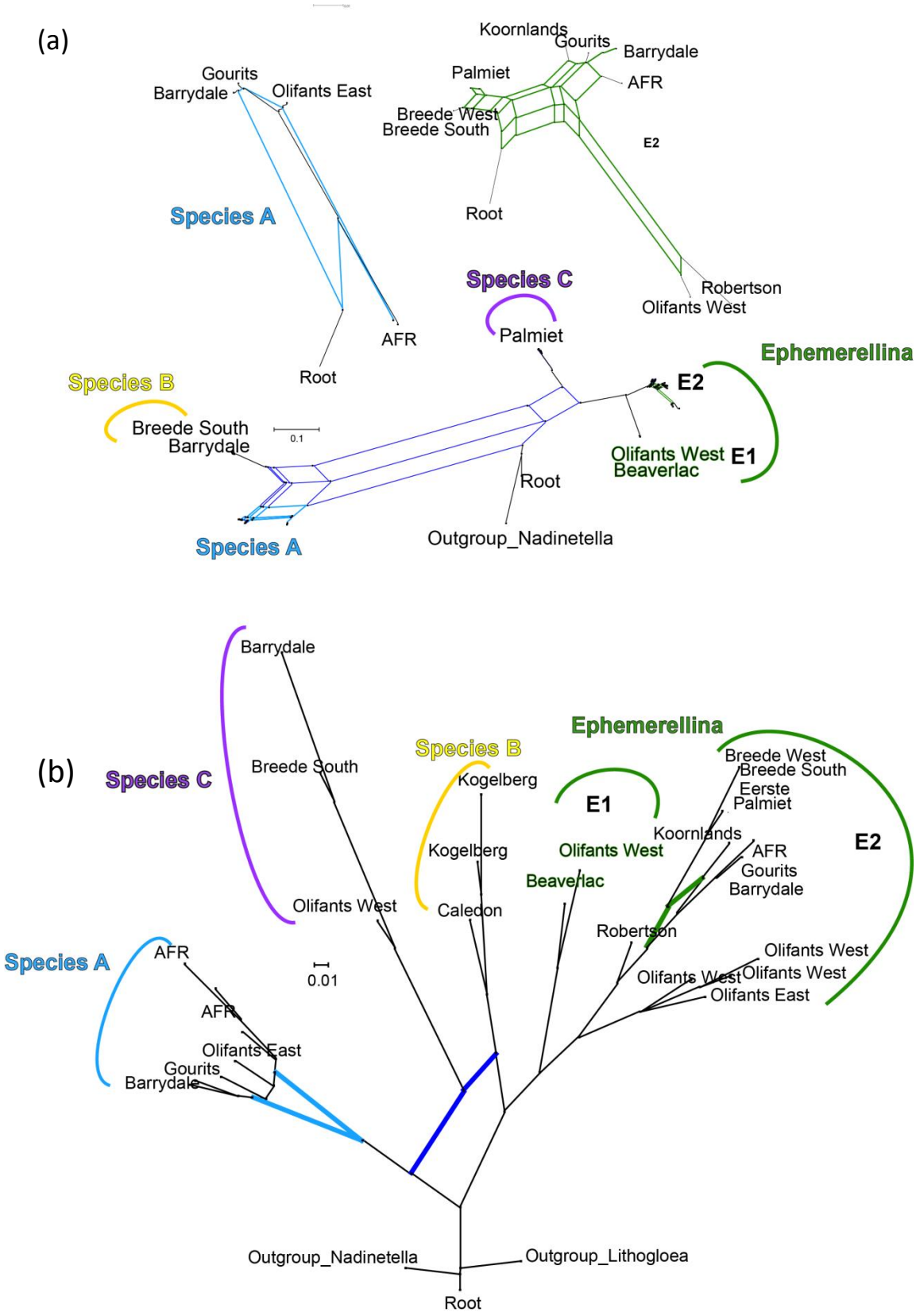


Figure 4.11. "Ephem Clade": a) Reticulation network based on consensus network of ML gene trees and b) simplified Reticulation network based on 50 majority rule of ML gene trees.

4.3.2.5 *Lestagella* Clade ("Lest Clade")

The "Lest Clade" phylogeny (Figure 4.12) presents a well-supported, deeply diverged split between two groups within the genus *Lestagella* (L1 and L2/L3). These groups appear to be highly associated with catchment locality. Within the Breede catchment there is a deep divergence between Breede-East (L1) and Breede-West/South (L3) which represent the two catchment tributary branches, one flowing eastwards and one westwards, which meet to form the main Breede River (Figure 4.12). The catchments associated with L1 are also not situated geographically close presently, where L1 is found in Breede-East, Berg and Palmiet (not seen in Figure 4.12) catchments, while L2/L3 occur in all other catchments in the southern Cape.

Within the L2/L3 clade, there is another distinct divergence between the East (L2: Gourits, AFR, Gamtoos and Keiskamma) and West (L3: Breede, Eerste, Table Mountain and Olifants) of the southern Cape. These are also highly associated with locality, though within the L3 clade there also appears to be genetic distinction within the Olifants catchment between East and West (Figure 4.12). This Olifants catchment split shows a remarkable amount of genetic variation between two well-supported clades where the rivers are only 10 km apart and with a possible barrier of a ~200 m relief, however it is important to note the rivers flow in opposing directions (the Rondegat River to the West and Driehoeks to the East).

The data summary, NN network is very tree-like and mostly mirrors the good support from the ML bootstrap consensus (Figure 4.13). The only differences are where Breede-Barrydale and Berg catchments have low support for ML and high support for NN network bootstrap. Also the base of the L3 cluster has a higher ML support than there is for the NN network. There is low support for the splitting of Liesbeek (Cape Peninsula), Breede-West/Breede-South and Eerste catchments. Otherwise all clusters are well supported, with the Gamtoos catchment showing a closer connection with AFR than either of these to the Gourits catchment.

The reticulation network using the consensus tree (Figure 4.14a) had some complex reticulations, L1 showed a possible case where complex sub-histories could not be estimated by the analysis. L2/L3 shows high levels of conflicting signal. However it appears that there was a reticulation event between populations in the Breede-West/South catchment and the Cape Peninsula rivers, giving rise to the ancestral lineage of the Eerste catchment population.

In the simplified reticulation network (Figure 4.14b) using a "Supernetwork" (from 50 % majority-rule trees), L1 shows the Breede Barrydale lineage as a hybrid between the Palmiet/Berg ancestor and the Breede Koornlands/Robertson ancestor. Within L2, a reticulation event occurred between Gourits and AFR common ancestors, which have given rise to the Gamtoos lineage. It would appear as though there were reticulation events linking Olifants-East and West.

As mentioned before, "supernetworks" have the distinct advantage where datasets only need to overlap partially, which allows for the inclusion of samples that are not represented in all the genes. In Figure 4.14b, there is a Tulbagh (Berg catchment) sample that is currently grouped with the Jonkershoek (Eerste River catchment) cluster (while all other Tulbagh samples group within the L1 clade, labels in bold in Figure 4.14b). This sample in particular (TEL252, see Appendix 4A) was only successfully sequenced for two genes (16S and 12S) and was so removed from the gene comparative analyses. Separate analyses (not shown here) indicate that both 16S and 12S result in TEL252 being grouped in L3. TEL252 was collected in a different area of the same river in Tulbagh. This indicates a sympatric occurrence of deeply diverged taxa L1 and L3 within *Lestagella*. More samples are necessary to investigate this result.

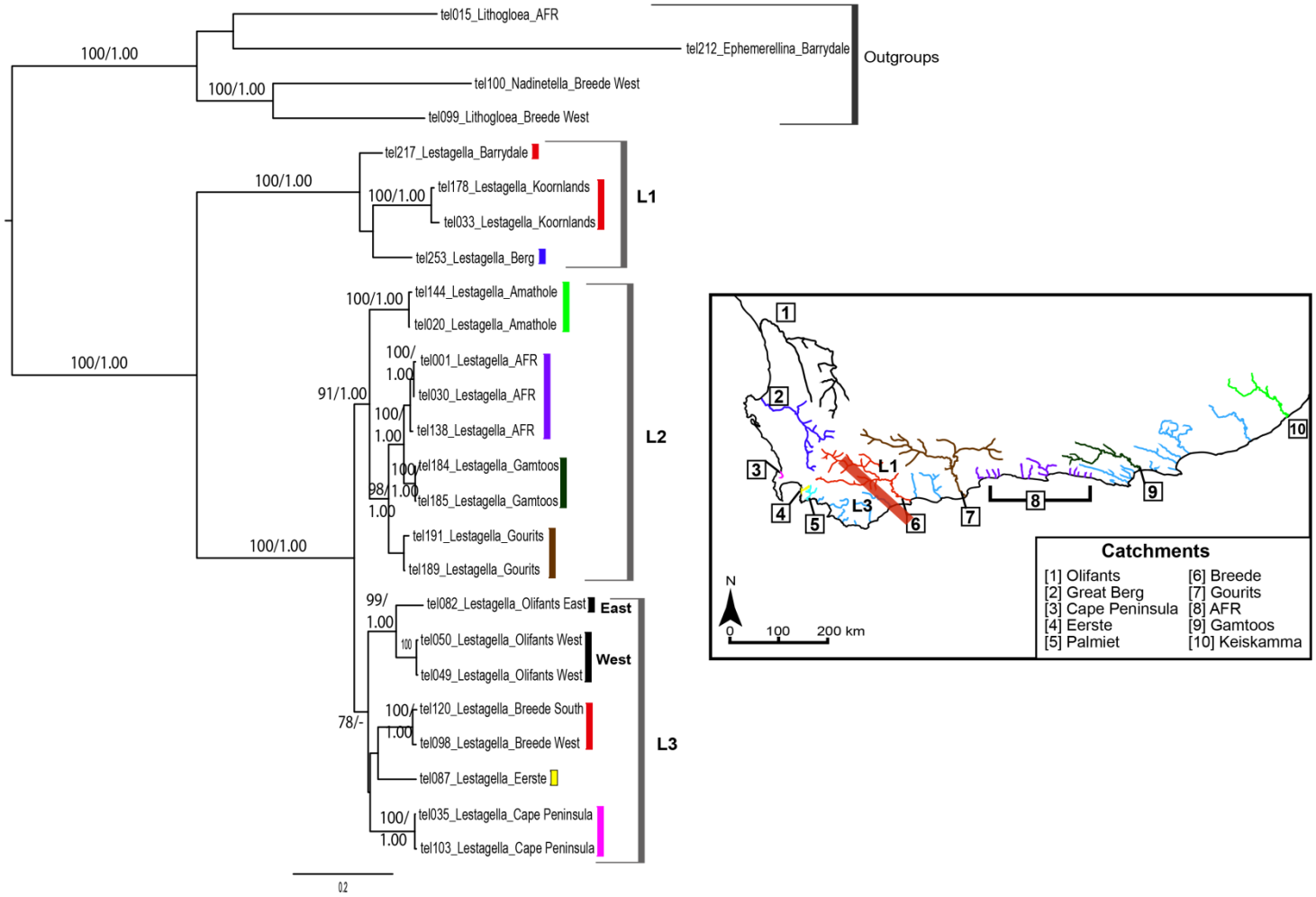


Figure 4.12. "Lest Clade", ML best scoring tree with ML bootstrap support followed by BI posterior support. Well-supported clades are shown with colour-coded bars, which correspond to the coloured catchments on the included map of the sample area. The red bar on the map between L1 and L3 represents a genetic barrier observed between the two groups

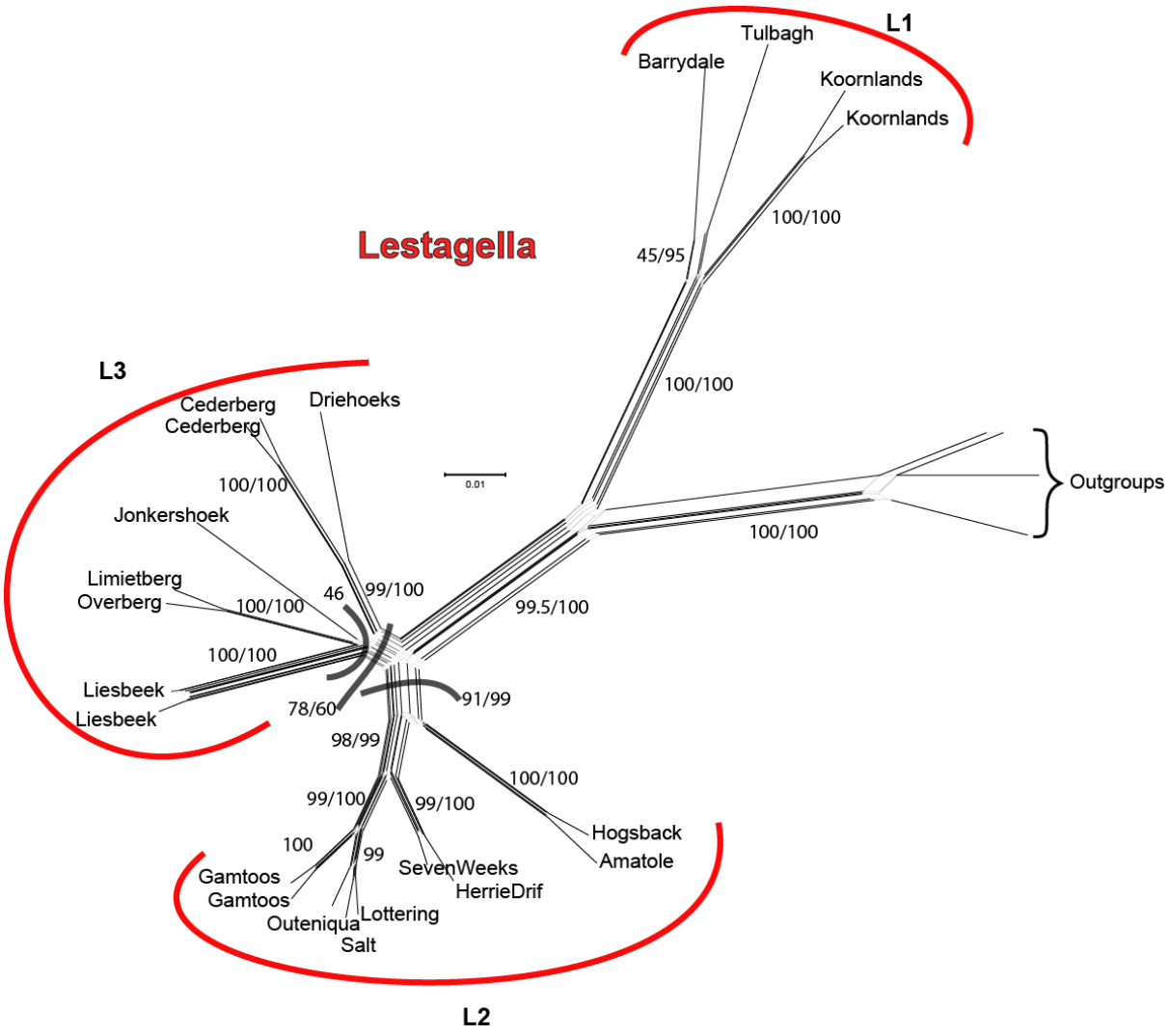


Figure 4.13. Implicit NN network with ML bootstrap consensus of the "Lest Clade". Both the bootstrap from ML tree and the bootstrap result from NN distance analysis presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch"). Long edges represent good support.

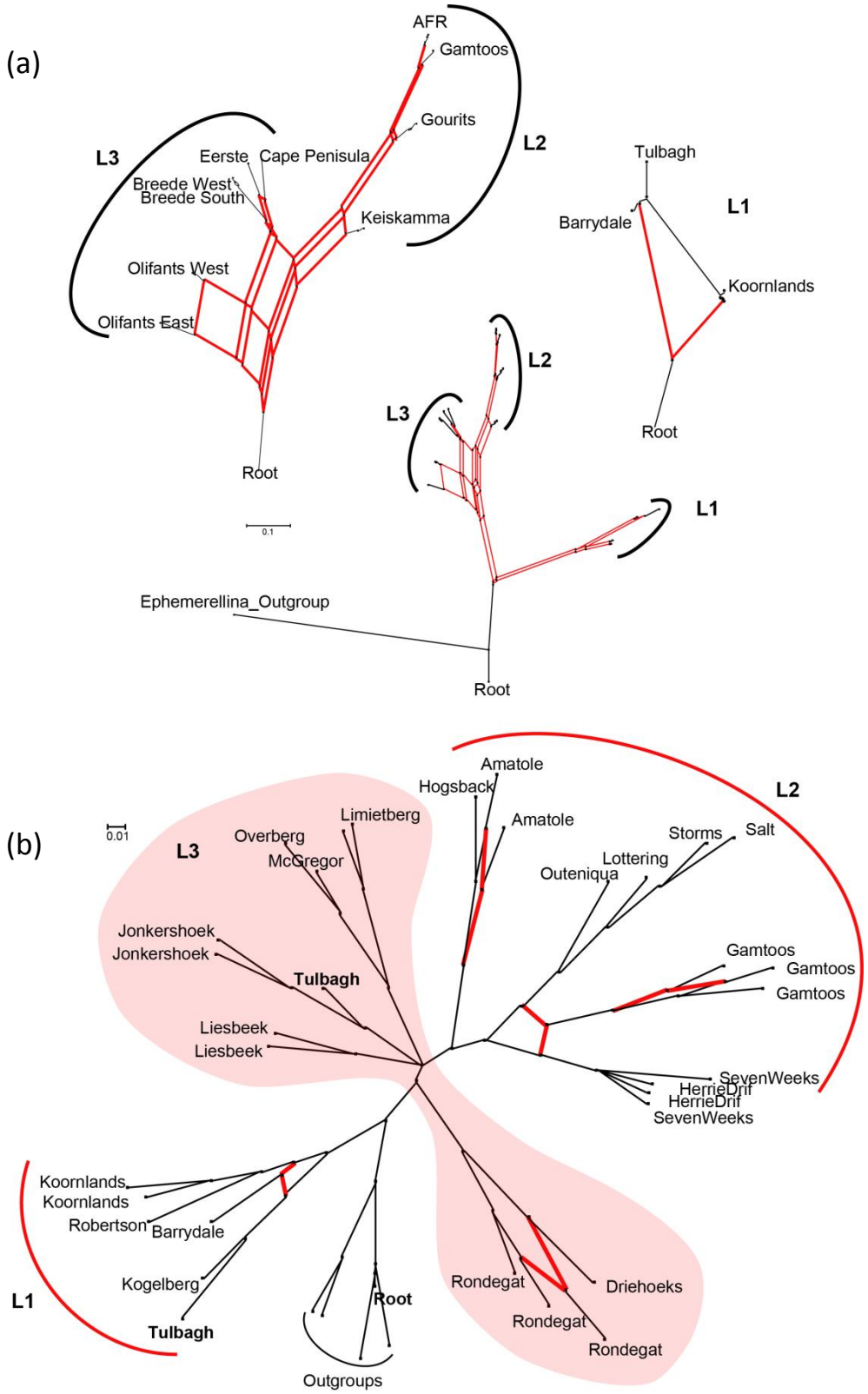


Figure 4.14. "Lest Clade" (a) Reticulation network based on consensus network of ML gene trees and b) simplified Hybridisation network based on 50 majority rule of ML gene trees.

4.3.2.6 *Nadinetella*/*Lithogloea Clade* ("Nad/Lith Clade")

The "Nad/Lith Clade" shows the relationship between and within the genera *Lithogloea* and *Nadinetella* (Figures 4.15 – 4.17). *Lithogloea* forms a well-supported monophyletic clade and covers a range of catchments; Breede, Olifants, Eerste and the Palmiet catchment. It is difficult to make any conclusions for the relationships within the genus due to fairly poor resolution at the tips of the branches (Figure 4.15).

The *Nadinetella* genus phylogeny recovers the two currently described species, *N. brincki* and *N.crassi* (Figures 4.15 – 4.17). These however seem to form complexes of divergent species and/or populations that overlap in rivers and catchments. There are at least four well-supported clades within the *N.crassi* complex (Figure 4.15), where *TEL110* and *TEL112* from Kogelberg (Palmiet) are distinct with high support.

There are at least five well-supported clades within the *N. brincki* complex (Figure 4.15). Sample distributions overlap throughout the genus (Figure 4.15, highlighted corresponding to map). There is one distinctive clade that forms a sister to the *N. brincki* complex, found only in Breede-Robertson area, which are sympatric with the Breede-Robertson population in the *N. brincki* complex, which is also found within the same habitat (*Wardia* moss on rocks in fast current).

The NN network for *Lithogloea* forms an untidy cluster, with base conflicting signal (Figure 4.16). The Olifants cluster forms a distinct group with fairly good support from the ML (BS = 77) and high support from NN analysis (BS = 96), while the other taxa do not have much resolution.

The *Nadinetella* NN network clusters follows a similar pattern to the phylogenetic tree analyses (Figure 4.16). There is ambiguous signal mostly in the *N. brincki* complex excluding the Olifants/Berg cluster. There is good support that clusters Gourits and Breede-East/South together, AFR clusters with good support, as does Palmiet and Jonkershoek together. The placement of Molenaars (Breede-West) however, is ambiguous.

Nadintella crassi complex in the NN network analysis (Figure 4.16) is well-supported, tree-like and follows similar patterns to the phylogenetic tree analyses (Figure 4.15). The *N.* 'Robertson' is well separated from the other *Nadinetella* clusters with high support.

The consensus reticulation network (Figure 4.17a) shows much conflicting signal and could not be simplified based on the ML trees used. The "supernetwork" reticulation network using 50% Majority-rule trees provided a much simpler network where only two reticualtion events were found. The Olifants-East has a reticulate link between Olifants-West and whole-clade *Lithogloea* common ancestors (Figure 4.17b), and the other in the *N. crassi* complex where Breede-South has a reticulate link between Breede-West and the whole clade's common ancestor (Figure 4.17).

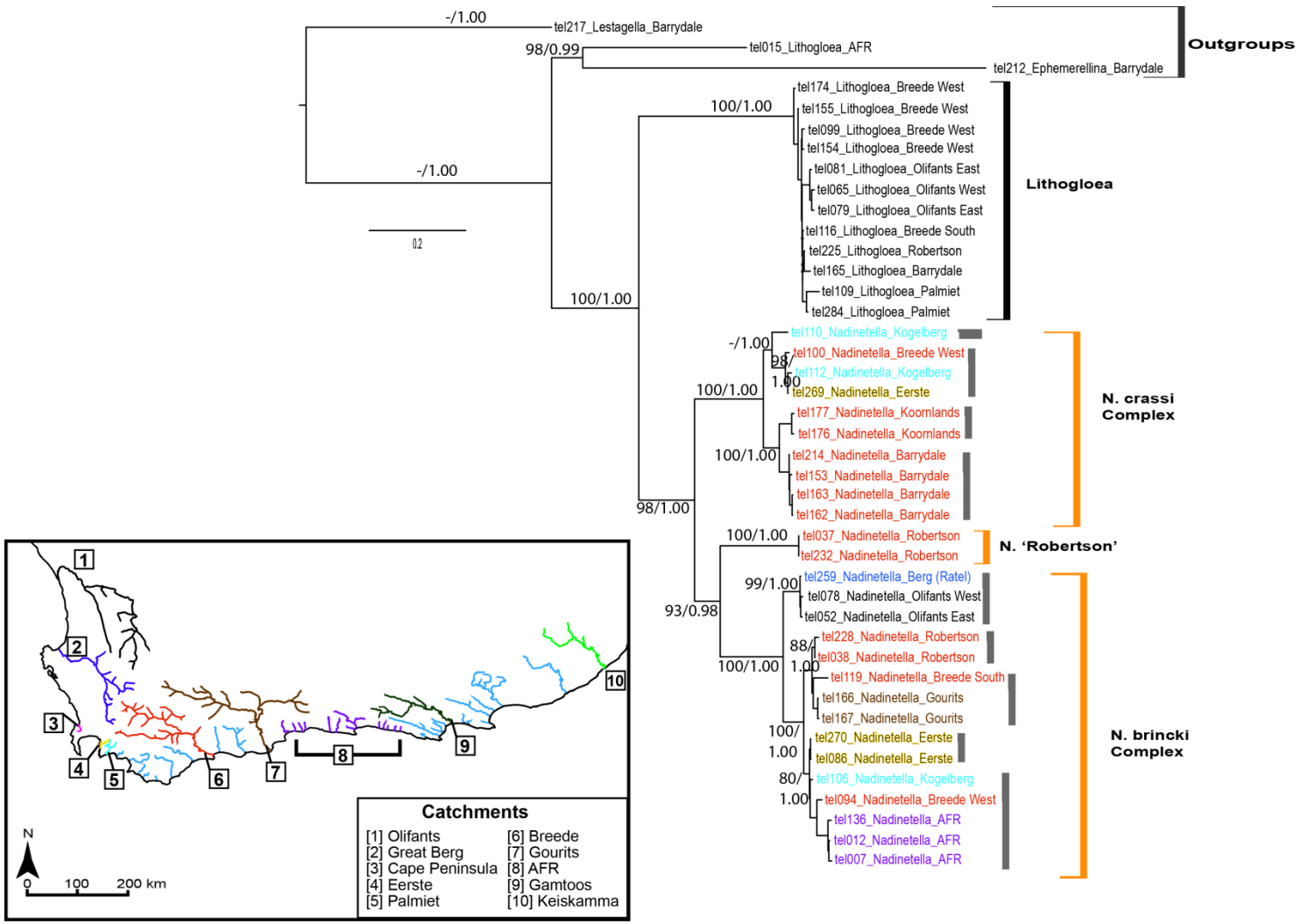


Figure 4.15. "Nad/Lith Clade". ML best scoring tree with ML bootstrap support followed by BI posterior support. Tip labels for *Nadinetella* are colour-coded corresponding to the included map. Grey bars depict well-supported clades.

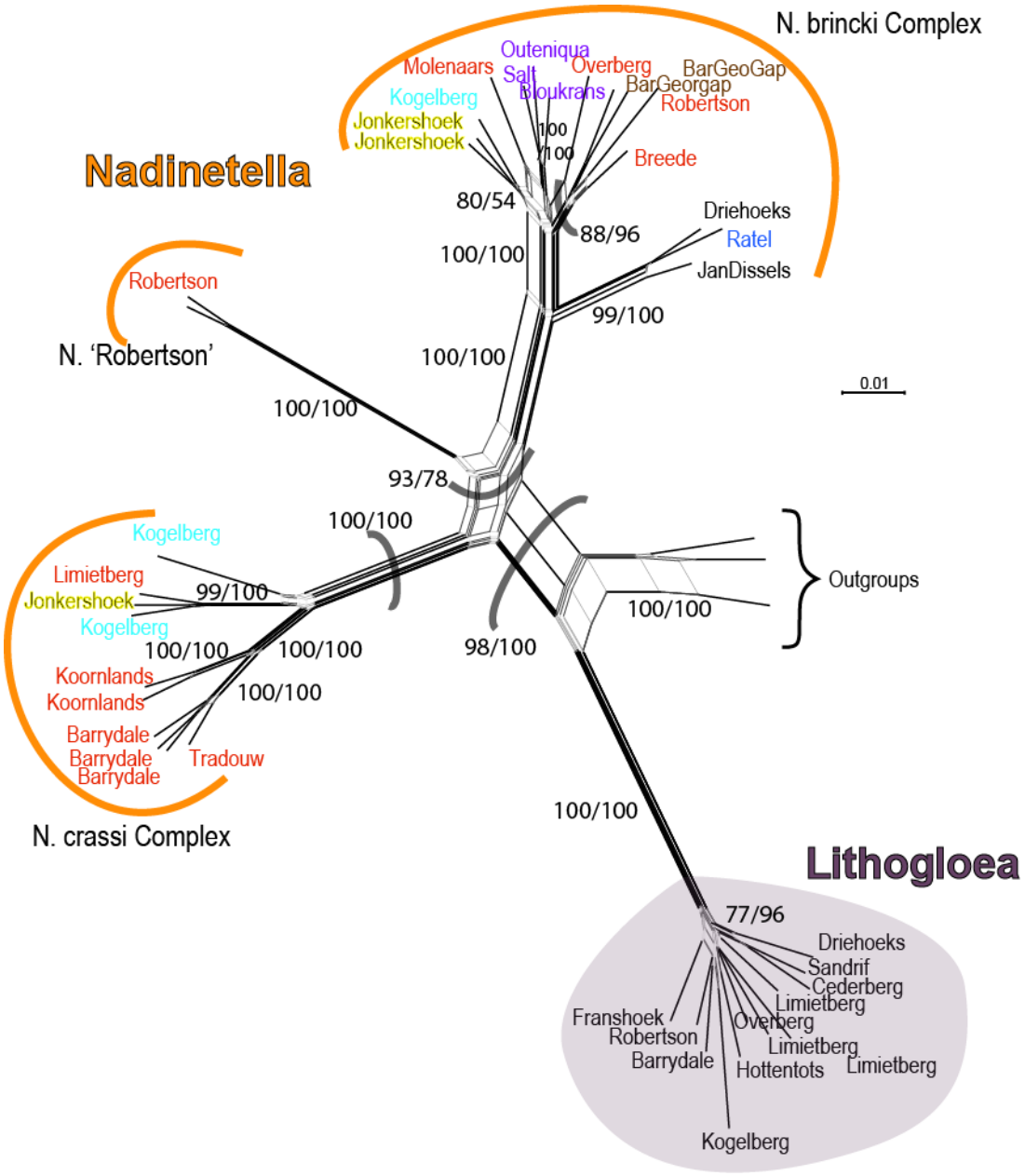


Figure 4.16. Implicit NN network with ML bootstrap consensus of the "Nad/Lith Clade". Both the bootstrap from ML tree and the bootstrap result from NN distance analysis are presented. The NN network edges confidence intervals are highlighted by shadows, the darker the line, the higher the confidence of that edge (or "branch") Long edges represent good support.

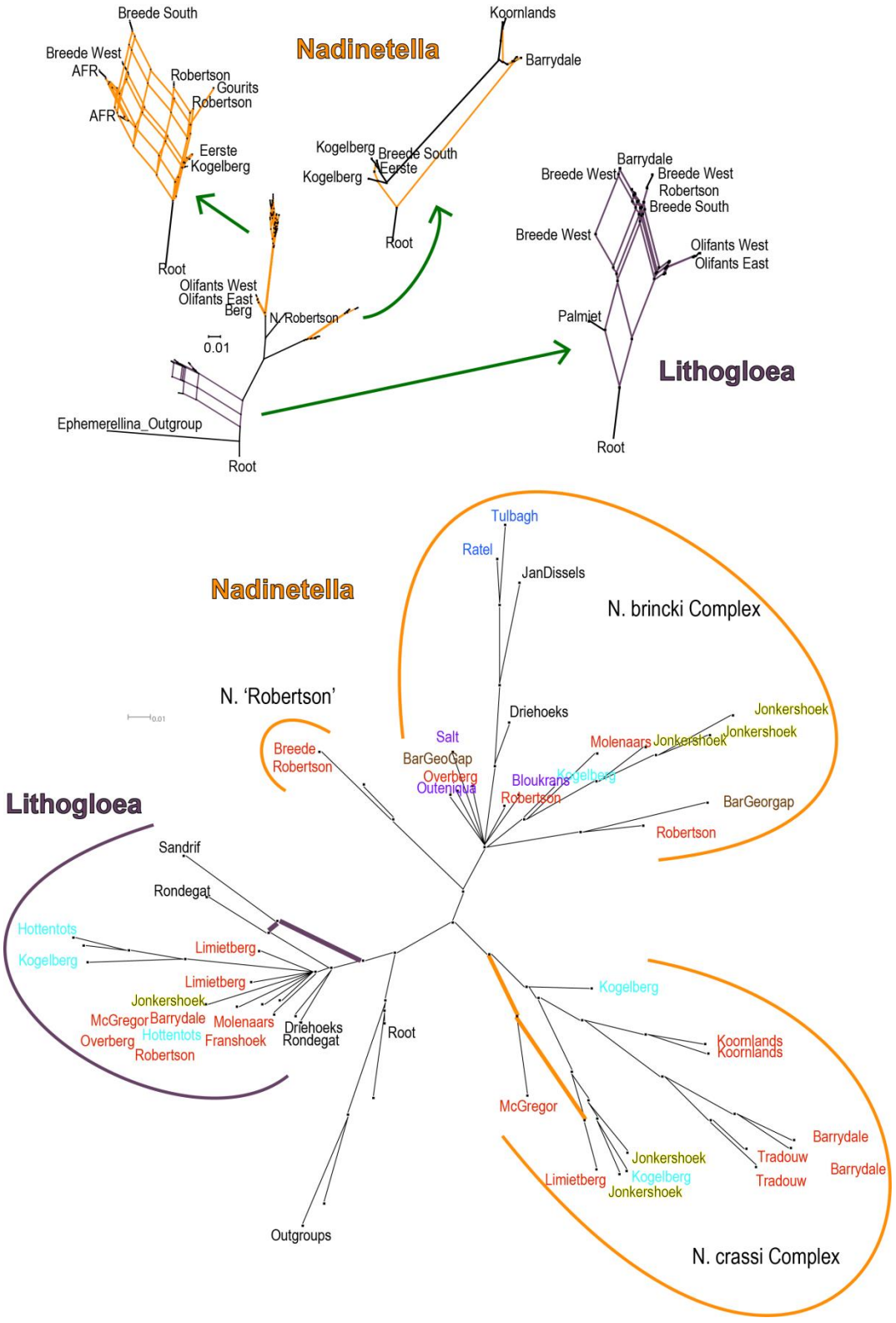


Figure 4.17. "Nad/Lith Clade" (a) Reticulation network based on consensus network of ML gene trees and b) simplified reticulation network based on 50 majority rule of ML gene trees. Coloured labels correspond to catchments (see Figure 4.15).

4.3.3 Species Tree Estimation and Rosenberg's Probability

A species tree for the three mitochondrial genes are presented in Figure 4.18, this dataset was selected because it had a much larger sample size (n = 115) than those with nuclear genes included. Where group assignments were uncertain, they were left as a single taxon. SVD quartet analyses were run on all the gene combination datasets (Table 4.2) and these results are presented in Appendix 4C. All species tree estimations gave similar results, and are discussed in the text where any trends or discrepancies were found.

In Rosenberg's test (2007) the null hypothesis is that monophyly is a chance outcome of random branching. Significant (under the 95 % confidence interval), distinct taxonomic groups are superimposed onto the SVDquartet estimated Species Trees (Figure 4.18). Red circles/squares represent significant nodes found in the combined mitochondrial (COI/16S/12S) dataset and blue circles/squares represent significant nodes from the COI-only dataset (more samples in the COI dataset allowed for better sampling of groups and therefore recovered more reciprocal monophyletic groups).

There is strong evidence that the majority of the species recovered from the SVDquartet analysis in the species trees were taxonomically distinct according to Rosenberg's test (Figure 4.18). The groups supported by the SVD quartet species trees that were found not to be significant in Rosenberg's test are indicated in Figure 4.18 by elongated blocks effectively "joining" the terminal taxon groups into a single group.

The *Lestagella* generic relationship as sister to the non-fringed Teloganodidae is not well-resolved in the mitochondrial tree (Figure 4.18), however in the "Afr/SAm Group" (which includes the 28S gene), there is very high support that *Lestagella* forms a sister clade to the non-fringed clade (BS = 99; Appendix 4Ci). Within the *Lestagella* group, three distinct, highly supported clades are present (L1, L2 and L3), which are similarly supported in all species tree analyses (Appendix 4C). Within L1, not all taxa were found to show reciprocal monophyly in Rosenberg's test. Within L2, all groups assigned had high support in the SVD analyses (Figure 4.18 and Appendix 4C), and showed reciprocal monophyly according to Rosenberg's test, meaning that each clade is true. Clade L2 seems to be restricted to the eastern side of the teloganodid distribution range, occurring from the Gourits to Keiskamma catchments. In L3, separation of the Olifants-East and West catchment clades is well supported by the SVD analyses (Figure 4.18 and Appendix 4C) and by Rosenberg's test (COI

analysis). Although found to show reciprocal monophyly, the *Lestagella penicillata* (Cape Peninsula), Eerste and Breede-West/South populations had low support in the SVD analysis on the mitochondrial data (Figure 4.18). However when the Histone 3 (H3) nuclear gene is included, these three groups have high support (Appendix 4C), but the relationship between the Olifants catchment and the remainder of the group is not well resolved.

Within the non-fringed teloganodids, the "Ephem Clade" (Species A, Species B, Species C and *Ephemerellina* E1 and E2) is a well-supported sister clade to the "Nad/Lith Clade" (*Nadinetella* and *Lithogloea*) (Figure 4.18). The high support can also be seen in "Afr/SAm Group" which includes the nuclear 28S gene (BS = 91), but only moderate to low support in the other species trees (Appendix 4.C). The support is weakened when *Manohyphella* is included in the analysis, possibly due to the reticulation with this group (Figure 4.8).

The placement of Species A as sister to the rest of "Ephem Clade" (Species B, Species C and *Ephemerellina*) has low support in the mitochondrial analysis, there is also low support for the sister relationship between Species B and Species C (Figure 4.18). Species A clade is clearly split into three groups with high support (BS ≥ 89), this is apparent in all species trees (Appendix 4C), however Rosenberg's test did not find A2 and A3 to show reciprocal monophyly (Figure 4.18). The species tree relationship between Species B and Species C is also not well resolved in any of the analyses (Figure 4.18, Appendix 4C) however the Rosenberg test for these two groups is significant. With the addition of either nuclear gene, Species B and Species C (combined) is supported sister clade to *Ephemerellina* and the support for the relationship between the two is moderate in the "Afr/Mad Group" (Appendix 4Cii).

Ephemerellina is well supported for the species tree groups for both bootstrap (BS ≥ 87) and Rosenberg test (Figure 4.18), this is apparent in all species tree estimates (Appendix 4C). Within *Ephemerellina* E1 forms a distinct clade, E2 is divided into three sub-groups (Figure 4.18) with *Ephemerellina* from Breede Robertson and Olifants forming a reciprocally monophyletic clade that is sympatric with the Berg/Olifants E1 group. The subgroup for *E. barnardi* forms a clade that primarily occurs in the western part of the distribution area, while another eastern group (*Ephemerellina* "East") forms a separate distinct clade with high support. *Ephemerellina* "East" includes Breede catchment areas "Barrydale" and "Koornlands" (see map in Figure 4.1) and catchments East of this (namely Gourits and AFR);

while *E. barnardi* includes the Breede-West areas (Figure 4.1), Palmiet and Eerste catchments.

Lithogloea forms a moderate to well-supported sister clade to the *Nadinetella* clade within the different species trees, better supported with the inclusion of 28S (Figure 4.18 and Appendix 4Ci). Within the *Lithogloea* clade, there are two well supported subgroups (BS = 97) which are significant in Rosenberg's test for reciprocal monophyly. The Olifants catchment population and *L. harrisoni* combined is supported by species tree bootstrap and Rosenberg. Breede and Palmiet specimens have low support as separate clades by the SVD analysis, but are found to show reciprocal monophyly for the Rosenberg test on COI data only.

Nadinetella shows similar support for nodes in all the species tree estimations (Figure 4.18, Appendix 4C). The clade has high support for the split of the *N. crassi* complex and *N. brincki* complex, also found to be significant in Rosenberg's test.

Within the *N. crassi* complex, one sample from the Kogelberg (Palmiet catchment, TEL110, see Appendix 4A) is distinct from the TEL112 specimen also from Kogelberg (Palmiet). The SVD species tree does not support the split of the Breede-South group (from McGregor, see Appendix 4A), which implies that it groups with the Kogelberg TEL110 sample, which is also supported by the Rosenberg test. Rosenberg's test combines the *N. crassi* and Kogelberg TEL112 group (blue) and includes the Breede West group when considering all mitochondrial markers (Figure 4.18).

The relationship of the *N.* 'Robertson' group to the *N. crassi* complex and *N. brincki* complex is not well resolved, although reciprocal monophyly for the *N. brincki* complex is significant. *N. crassi* complex is sister to *N. brincki* complex + *N.* 'Robertson' clades with high support from the mitochondrial tree (Appendix 4Ciii), although the node supporting *N.* 'Robertson' as sister lineage to *N. brincki* complex is moderately supported (BS = 73; Appendix 4Ciii). In the trees, with 28S nuclear genes included, *N.*' Robertson' forms a clade with the *N. crassi* complex (with low support separating the two as separate species) and *N. brincki* complex is sister to the clade. The *N. brincki* complex separates into six distinct clades with high support according to the SVD analysis (Figure 4.18). The Breede-South group (from Gobos River, a tributary of the Breede River) forms a well-supported clade (significant in Rosenberg's test on the COI dataset) that is sister to the Gourits and *N. brincki* complex Breede Robertson

group (different to *N.* 'Robertson" but occurs sympatrically in Robertson rivers). This Gourits/Breede Robertson group is not well supported by the SVD quartet analysis, but does show reciprocal monophyly.

The higher taxon relationships including families considered to be closely related to Teloganodidae, and other non-African teloganodids, are represented in Appendix 4C(i-ii); these include Melanemerellidae (South America), *Manohyphella* (Madagascar), Ephemerythidae (African) and *Dudgeodes* (Asia). Another SVD quartet analysis was run on the mitochondrial dataset, this time including the higher taxa (Appendix 4Ciii). They were removed from the analysis shown in Figure 4.18 because they caused weak deep-node support in the species tree. The other datasets (Afr/SAm, Afr/Mad) had fewer sample numbers, but included nuclear genes and the higher taxa (Appendix 4Ci-ii).

In the mitochondrial species tree, *Manohyphella* and Ephemerythidae are grouped with the non-fringed teloganodids as a supported sister to the *Lestagella* clade (Appendix 4Ciii); however sister status with the non-fringed clade was not resolved in the mitochondrial tree. In the "Afr/SAm Group", *Dudgeodes ulmeri* is sister to the South African teloganodids, Melanemerellidae and Ephemerythidae with very high support (BS= 96). Melanemerellidae and Ephemerythidae are also sister groups to the South African Teloganodidae, but the relationship is unresolved, possibly due to insufficient sampling. *Manohyphella* in "Afr/Mad Group" is a well-supported sister lineage to the non-fringed teloganodids (BS = 89; Appendix 4Cii).

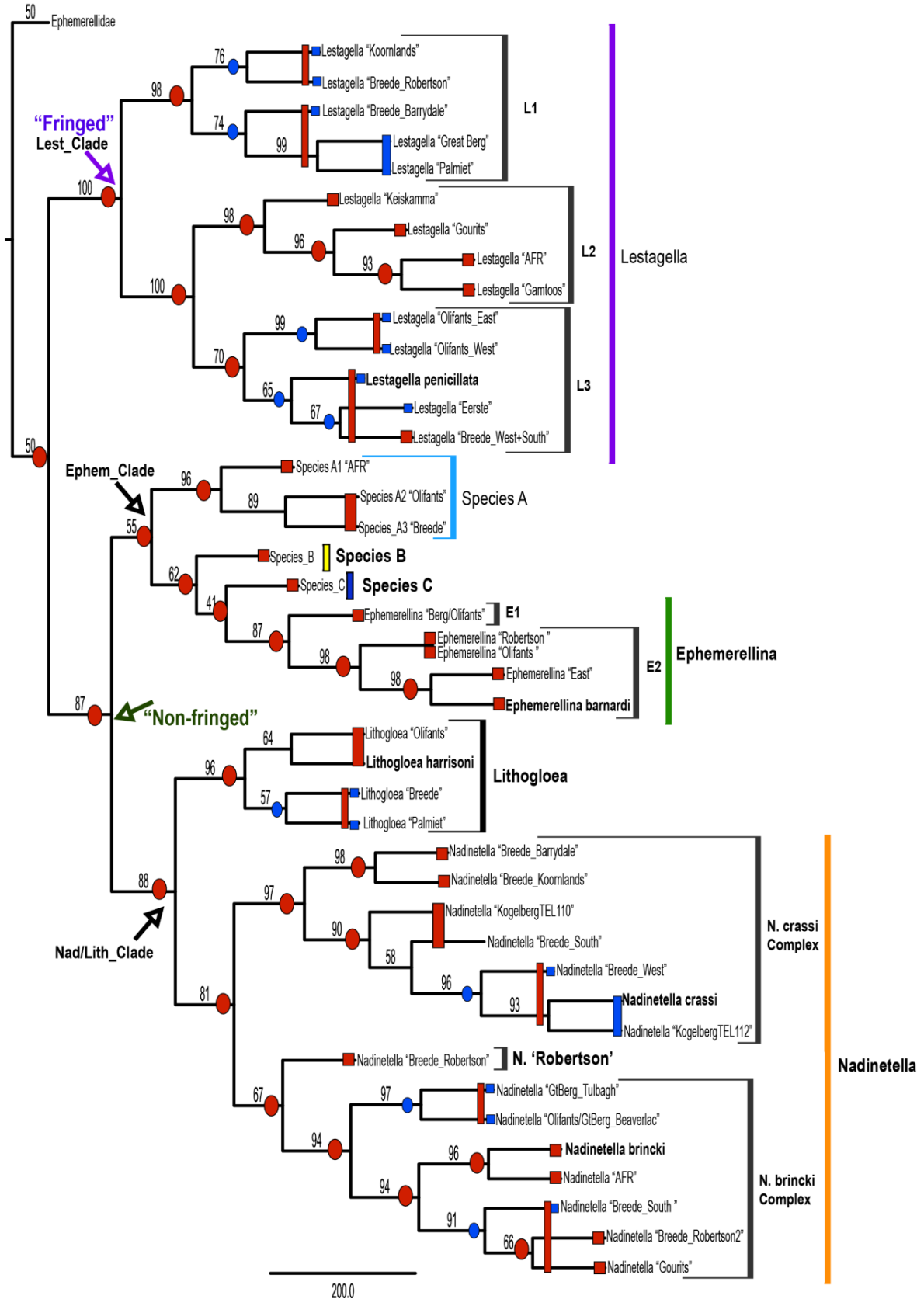


Figure 4.18. See legend overleaf.

Figure 4.18. Species Tree estimation (a) using only mitochondrial genes COI/16S/12S, analysis done using 115 taxa (represented within the terminal taxon assignment groups above). Significance from Rosenberg analysis indicated as red circles/squares (COI/16S/12S) and blue circles/squares (COI only, when there were not enough samples) at the nodes. Rosenberg single representatives were removed. Rosenberg's probability of reciprocal monophyly, circled nodes significant to alpha < 0.05. Described species are shown in bold.

4.3.4 Landscape genetics

Considering only currently described species, isolation by distance was not a factor in the population structure of most of the groups (Table 4.4). The IBD was calculated for the species defined as prior to this study (all monospecific except for *Nadinetella* which has two species) and is summarised in Table 4.4.

Table 4.4. The contribution of isolation by distance (IBD) for described species of Teloganodidae. Using log (genetic similarity (M)) and log (geographic distance). Bold values indicate a significant effect (alpha < 0.05). Please note that there were too few specimens of Species C and *N.* 'Robertson' to conduct an IBD test. Sample numbers used for IBD analyses can be found in Appendix 4E.

Species	**R^2**	**r**	***P***
Ephemerellina barnardi	0.0047	-0.0685	0.3090
Lestagella penicillata	0.0252	-0.1588	0.1050
Lithogloea harrisoni	0.0790	-0.2811	**0.0150**
Nadinetella brincki complex	0.320	-0.5656	0.0800
Nadinetella crassi complex	0.0543	-0.2331	0.0970
Species A	0.0450	-0.2121	0.2270
Species B	0.4970	-0.7050	0.3250

There was no evidence of isolation by distance for the *E. barnardi*, *L. penicillata*, *N. crassi* complex, *N. brincki* complex, Species A or Species B populations. There was however evidence of small, but significant isolation by distance in *L. harrisoni*.

The barrier analysis highlights important genetic distinctions within the genera, thicker lines on the maps indicate higher support (Figures 4.19 – 4.23) while bootstrap support results can be seen in Appendix 4D. Several similar trends can be seen between the genera, possibly due to changes in the environment which have isolated certain coexisting populations. However, dispersal abilities of different species would affect the distributions considerably.

There is a general trend that isolates the Robertson area populations from the rest of the Breede catchment and mostly all other catchments completely (*Ephemerellina*, Figure 4.19; *Lestagella*, Figure 4.20; *N. brincki*, Figure 4.23), however in *Lithogloea* this trend had low support (Figure 4.21, Appendix 4D).

The strongest trend in the Breede catchment when considering all genera, is a general East / West barrier within the catchment. However, there appears to be various levels of separation/barriers between east, west and southern areas of the catchment. This ranges from seemingly no genetic barriers throughout the catchment (*Lithogloea*, Figure 4.21) to a highly divided catchment in the *N. brincki* complex (Figure 4.23). In the eastern Breede areas, Koornlands and Barrydale appear to be separate from the rest of the catchment (*Ephemerellina*, *Lestagella*, Species A and *N. crassi*), and in some for some genera these areas show considerable barriers to gene flow (*Lestagella* with high BS = 100% and *Ephemerellina* with moderate support, BS = 69 %; Appendix 4D).

The western Breede (Figure 4.1, Appendix 4A) areas seem to have little support for any genetic barriers. There are strong barriers between the southern and western Breede areas for *N. brincki* complex and *N. crassi* complex, and a moderately supported barrier for *Lithogloea* (BS = 72, Appendix 4D). There is also little gene flow between the Breede catchment and the northern catchments (Berg and Olifants) for any of the genera.

Another trend is the separation of the Palmiet and/or Eerste catchments from the rest of the catchments, this barrier is present in all genera (in *N. crassi* complex, the Palmiet is isolated and Eerste appears to not have a genetic barrier, Figure 4.23). In some genera, the Palmiet is distinct from the Eerste (*Lestagella*, *Lithogloea*, *N. brincki* complex and *N. crassi* complex).

There does not appear to be much gene flow between the AFR and Gourits River populations (and between the remainder of the catchments) for most of the genera (that have distributions in these areas), aside from *Lestagella* (Figure 4.20).

The Gourits catchment is generally separated from the western catchments (high support in *Lestagella* and *N. brincki* complex, low support in Ephemerellina) except for Species A, where there is no barrier between Barrydale and Gourits, however there is a strong barrier between Barrydale and the western localities (Figure 4.1, Figure 4.22).

There is also a prominent genetic barrier between the Olifants and the Berg catchments for *Ephemerellina* (Figure 4.19) and *Lestagella* (Figure 4.20) and a prominent barrier between two areas within the Berg catchment for the *N. brincki* complex (Figure 4.23).

Other barriers within the genera include a fairly strong separation between Olifants-East and Olifants-West populations of *Lestagella* (BS = 75%). It is interesting to note here that the two sample sites representing eastward-flowing Olifants-East and westward-flowing Olifants-West were 13 km apart with a relief of ca. 300 m. There is also a strong barrier between the Keiskamma catchment and the rest of the *Lestagella* distribution, and no apparent genetic barrier between Gamtoos, AFR and Gourits catchments (Figure 4.20).

Species A shows strong genetic barriers within the AFR, between rivers that are situated very close together, yet show moderate support (BS = 71%) between Breede-South and Palmiet catchments (Figure 4.22).

The *N. crassi* complex shows no genetic barrier between the Palmiet and Breede-South (Appendix 4D). Yet there is a strong barrier within the Palmiet (BS = 98), where there is gene flow towards the Eerste river for some samples, and no gene flow for other samples within the Palmiet catchment (Figure 4.23).

The interesting patterns show that some geographically close sample localities have strong genetic barriers for some groups and not others. Conversely, some groups that are geographically farther apart have little support for genetic barriers, which may be due to the extent and direction of mountain ranges between these sites. The data however gives the overall impression that species within the Teloganodidae are generally poor dispersers and are prone to population isolation between and within catchments.

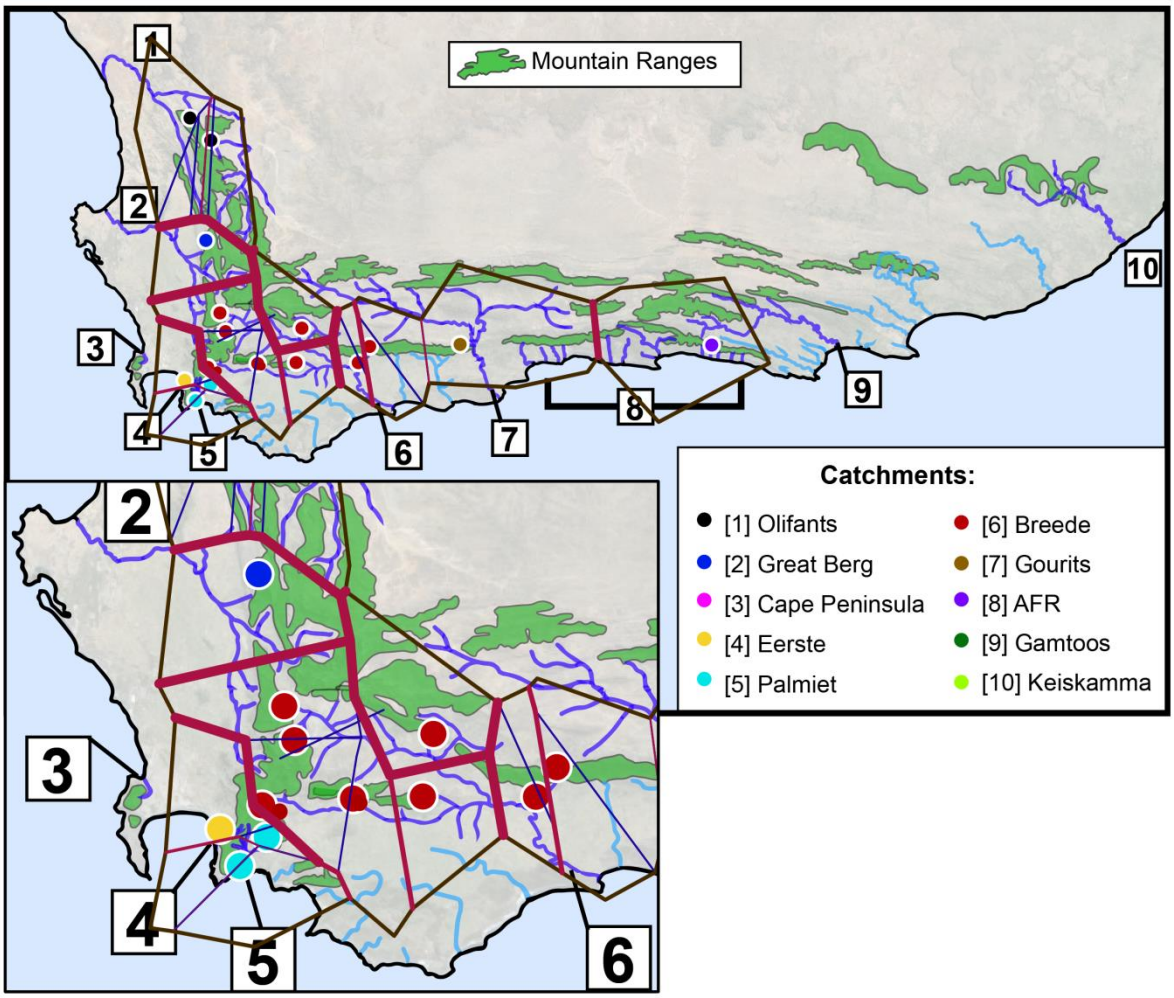


Figure 4.19. Barrier Analysis for the *Ephemerellina* clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier.

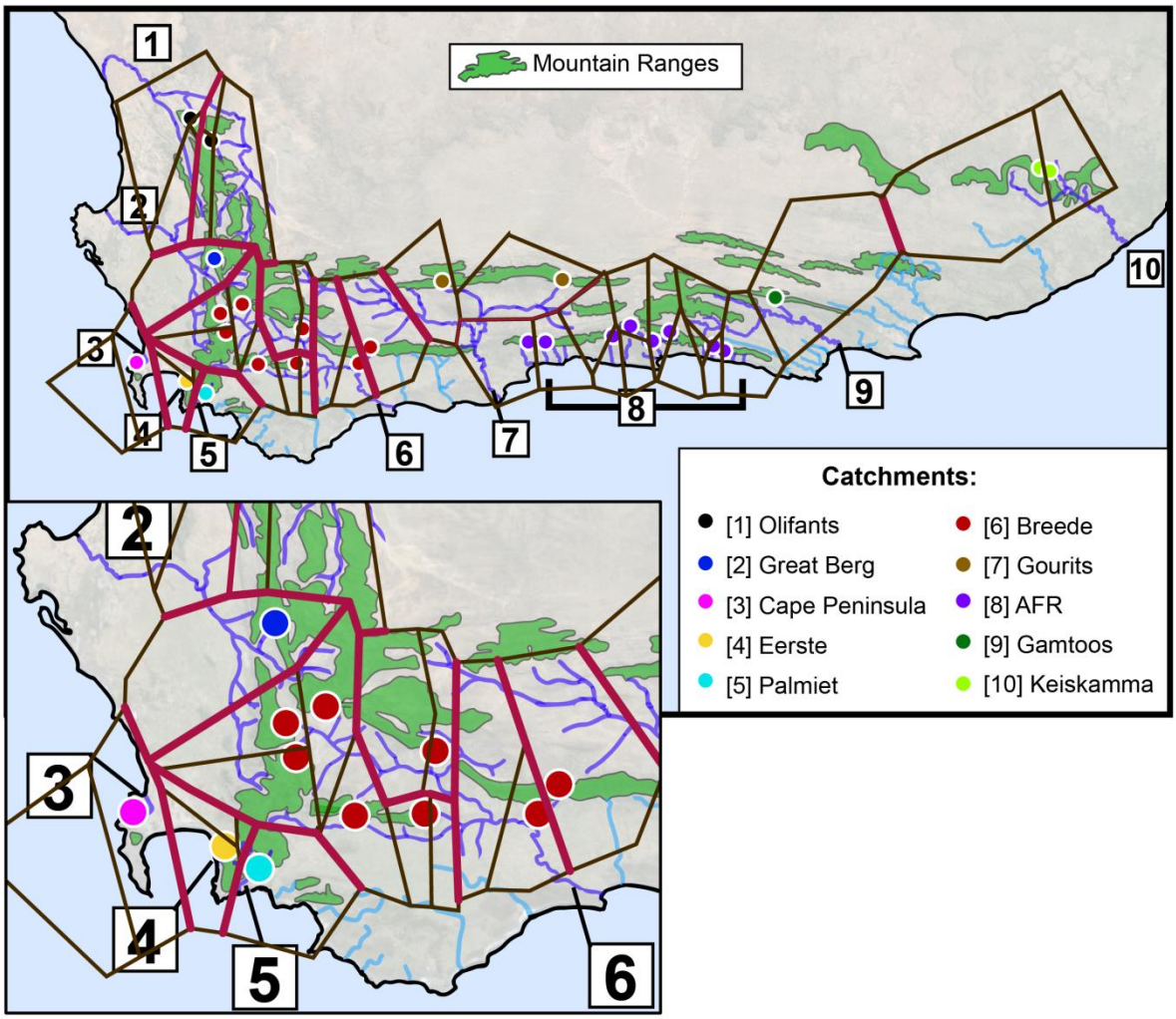


Figure 4.20. Barrier Analysis for the *Lestagella* clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier.

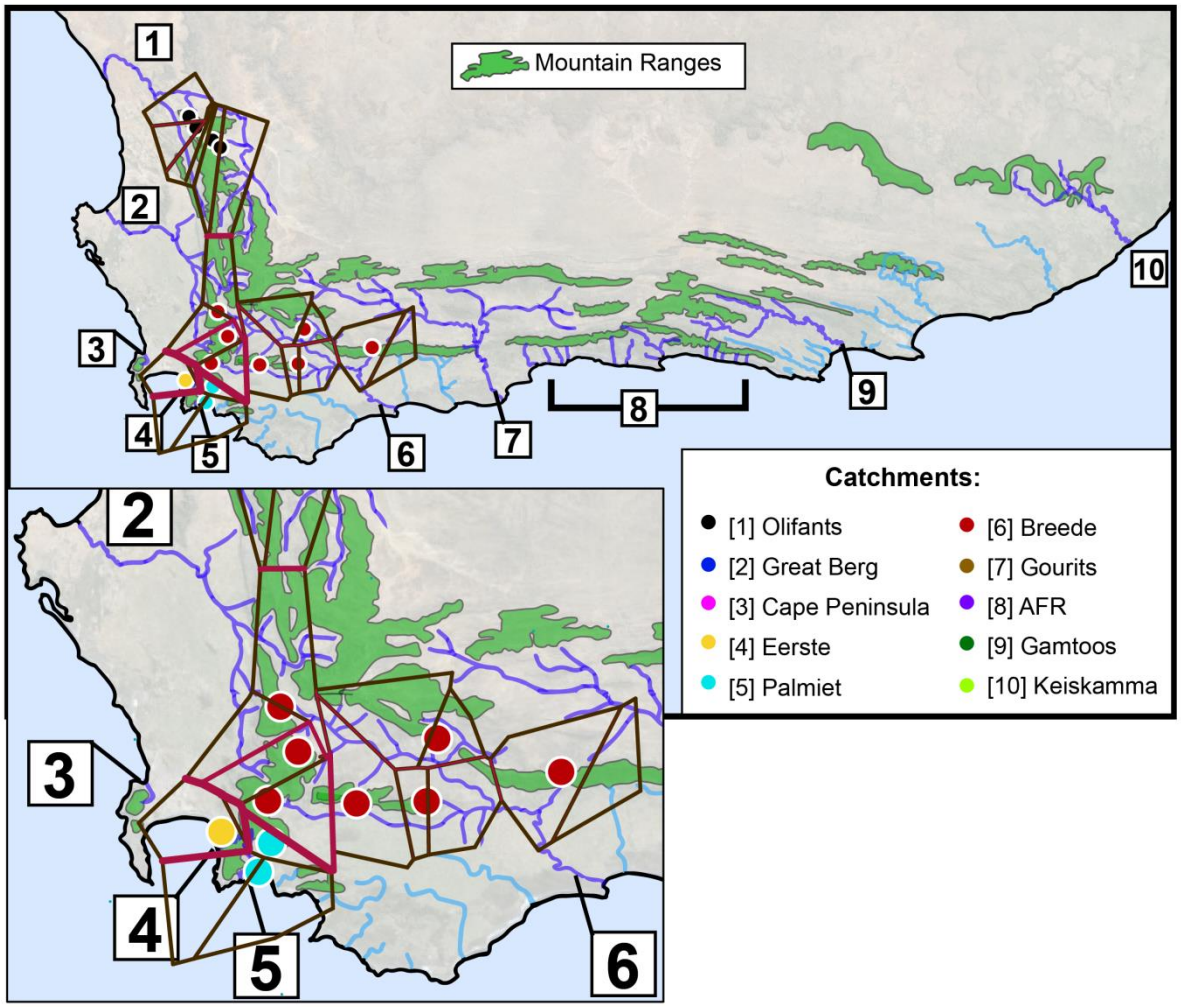


Figure 4.21. Barrier Analysis Lithogloea clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier.

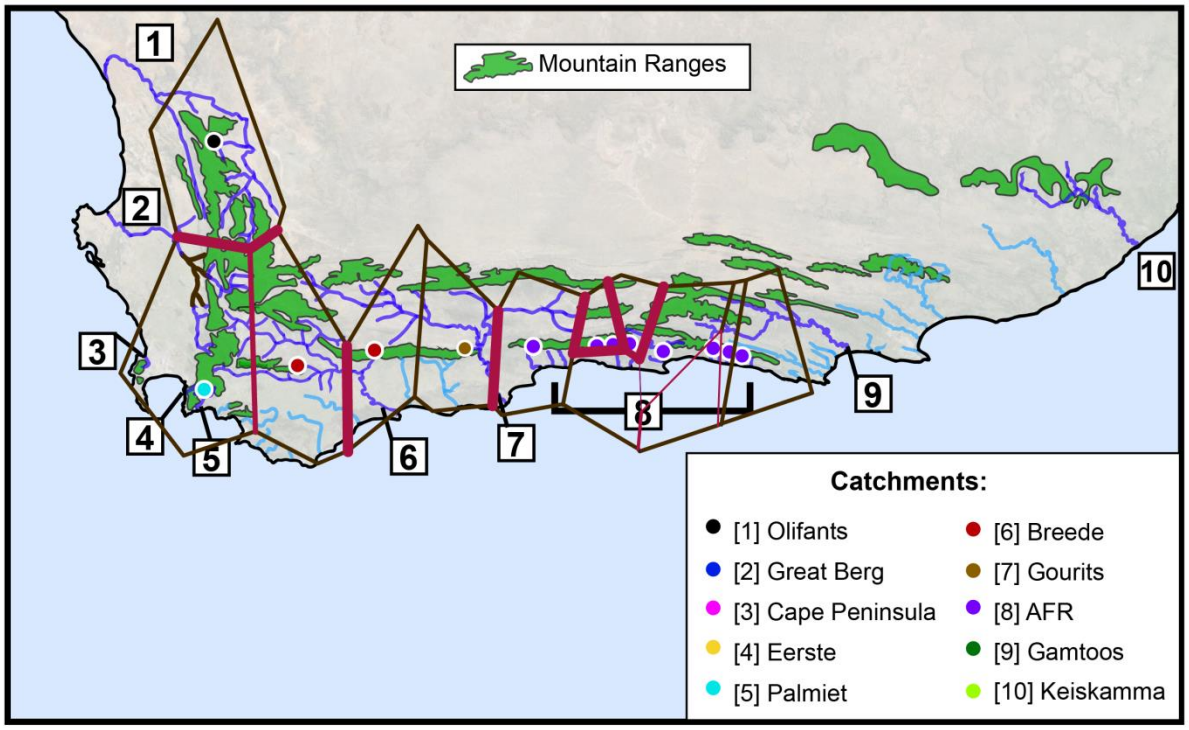


Figure 4.22. Barrier Analysis for the Species A clade, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier.

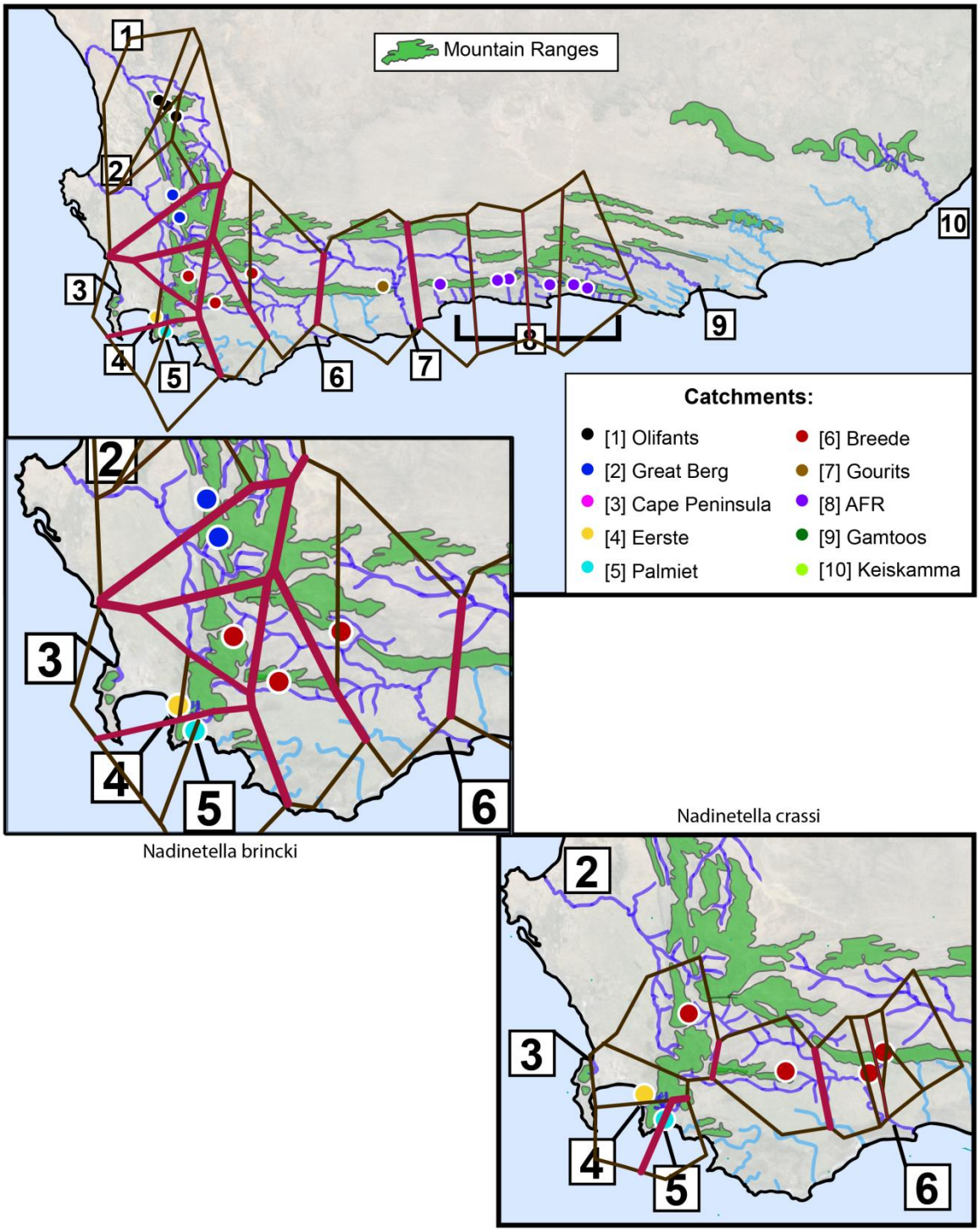


Figure 4.23. Barrier Analysis for the *N. brincki* and *N. crassi* complexes, sampling localities are colour-coded according to catchment. Thickness of the barrier (red bars) represents strength of support for the genetic barrier.

In Summary:

In the L1 clade within *Lestagella*, the species tree recovers five species, while Rosenberg's (Rosenberg, 2007) test (3-gene mitochondrial dataset) supports two of these species, and Rosenberg (COI dataset) supports four of these species. Populations from Robertson and Koornlands form one or two of these species (depending on analysis), Robertson shows clear, strong genetic barriers, suggesting that there is no gene flow between these areas. The Breede-Barrydale and Berg + Palmiet groups form the other species. The Berg and Palmiet lineage split was not significant in the species tree or either of Rosenberg's tests. The reticulation network confirms that there is a shared reticulation event between the Breede-Barrydale and Berg + Palmiet catchment populations, no barrier was calculated separating Barrydale from the Berg or Palmiet catchments (Appendix 4D).

The species tree L2 clade recovered four distinct species, all of which are supported by Rosenberg 3-gene analysis. The Keiskamma species possesses a genetic barrier with 100% support. The reticulation network showed that the Gamtoos ancestor and Gourits + AFR ancestor share a reticulation, and there is little or no support for these three being separated by genetic barriers, so these populations are currently experiencing gene flow.

The species tree for L3 clade recovered three species with good support, all of which were supported by the Rosenberg test (two species for 3-gene test, five species in total for the COI test). Although there is a distinctive difference between Olifants-East and Olifants-West, there is only moderate evidence of a genetic barrier and so it is doubtful that they are separate species. *Lestagella penicillata* (Table Mountain), Eerste and Breede-West and South were not well supported by the species tree. There is evidence of reticulation of ancestors of the Breede-West+South and Table Mountain populations to form the ancestor of the Jonkershoek population, however these three groups have 100 % support for a genetic barrier, so gene flow between these groups has become limited.

Species A is a well-supported sister to Species B/ Species C/ *Ephemerellina* clade in the gene trees, but this is not evident in the species trees, however reciprocal monophyly is significant. The reticulation network suggests that Species C was the result of a reticulation between common ancestors of Species A and Species C, which may explain the lack of resolution at these nodes. This has also affected the species tree estimations for the relationship between Species B and Species C, however reciprocal monophyly is also significant for these groups.

Species B and Species C are likely to belong to the same genus as they have numerous morphological similarities including general body shape, gill structure, tubercle structure, striped caudal filaments and enlarged mandibles.

Ephemerellina E1 and E2 are distinctly separated and highly supported in every analysis. E1 is a highly supported (by species tree and the 3-gene Rosenberg test) sister group to E2, with sympatric occurrence in the Olifants system. Within E2, there are three distinct clades supported by the species tree and Rosenberg (3-gene test supports four clades, i.e. the split of Robertson and Olifants populations). One of which is the already described as *E. barnardi*, and the other two are from Olifants/Robertson and an eastern catchment group (excluding Breede Robertson and including Breede Barrydale, Breede Koornlands, Gourits and AFR).

The reticulation networks indicates a reticulate event between *E. barnardi* (from western catchments) and the ancestral base group (including *E. barnardi*, Breede Koornlands, AFR, Gourits and Breede Barrydale) and the ancestor of the Breede-Koornlands population. The Robertson and Olifants populations were separate from this reticulation event, which may have caused the two populations to group together (superficially) in the analyses. The more complex reticulation network shows a possible situation of homoplasy (convergent evolution) between these two populations.

There is moderate support for a genetic barrier between AFR and Gourits, and little support for a barrier between Barrydale and Gourits, which suggests gene flow in the eastern catchment group ("*Ephemerellina* East"). Robertson is isolated from the entire Olifants catchment (100 % genetic barrier), which is reflected in the Rosenberg test, suggesting that this relationship needs investigation.

Within the *E. barnardi* group, the Palmiet and Eerste catchments seem to be isolated from the rest in the barrier analysis, which is not reflected in any of the other analyses, possibly indicating a recent divergence or early stages of speciation. With examination of the combined evidence, *Ephemerellina* E2 consists of four distinct clades when reciprocal monophyly and genetic barriers are considered.

The *Lithogloea* species tree and Rosenberg's test recovered two of the four assigned groups, with low support for separation of *L. harrisoni* (Eerste catchment) for the Olifants population, and low support for separation of Breede and Palmiet catchments. The reticulation networks show much conflicting signal, and the simplified reticulation network shows only a

reticulation within Olifants catchment, and a polychotomy of the other catchments. The Barrier analysis shows strong barriers between Eerste and Palmiet, and between these two catchments and all other catchments.

There are no distinct barriers within the Breede catchment, and moderate support separating the Olifants from the Breede. While no *Lithogloea* samples were collected from the Berg catchment for this study, it is suggested that this river may be the connection between the closely related Eerste and Olifants populations (both of these catchments are on the western side of the mountain range), as these appear to be one species (*L. harrisoni*). There is fairly good support for a genetic barrier between the Breede and Palmiet Rivers, possibly a recent event leading to early stages of speciation. *Lithogloea* has at least two distinct species clades (Figure 4.18).

The *N. crassi* complex recovers six clades in the species tree, four of which are support by Rosenberg (3-gene) and five by Rosenberg (COI data) test. Breede-Barrydale and Breede-Koornlands form two distinct clades; however the barrier analysis shows no distinct genetic barriers between Barrydale and Koornlands areas, which suggests there is gene flow between these two areas. The Breede-South and one Kogelberg sample (TEL110, Palmiet River – Palmiet catchment) seem to group together with support from Rosenberg and SVD quartets, there is also no genetic barrier calculated between these two sites. Upon morphological comparison, these two samples seem to share similar characters not seen in the other *N. crassi* specimens (namely the "half-stripe" occurring on the first lamellate gill on segment II) which suggests these two populations are closely related.

The Kogelberg sample (TEL112, Dwars tributary – Palmiet catchment) appears distinct from the TEL110 sample (Palmiet River) and is recovered in the species tree analysis and Rosenberg (COI test) to form a monophyletic group with *N. crassi* (Eerste catchment). The Breede-West population is supported by Rosenberg's COI test, however barrier analysis shows no genetic barrier between *N. crassi* and Kogelberg TEL112 (Palmiet). The reticulation network shows a link between Breede-South (McGregor) and the ancestors of Breede-West + base ancestor of the whole *N. crassi* complex group. It is possible that the Kogelberg TEL 110 sample dispersed to the Palmiet from the Breede-South population, while the other sample (TEL112) dispersed from elsewhere (possibly the Eerste catchment). This would need further investigation to get a clearer indication.

Although the *N.* 'Robertson' group has only been found in the Robertson area, the group itself is well supported and significant in Rosenberg's 3-gene test. However the relationship between *N.* 'Robertson' and the two complex groups (*N. brincki* and *N. crassi*) is not certain. According to the reticulation networks, *N.* 'Robertson' is distinct from the other species, suggesting that when it diverged from the other groups, it was possibly isolated for some time. A population from the *N. brincki* complex occurs in the same river, and (possibly due to a wider distribution) may have recolonized the Robertson area from the Gourits population (just east of the Robertson area).

The species tree recovered six clades in the *N. brincki* complex which were supported by Rosenberg's (COI) test, and five were supported by Rosenberg's (3-gene) test. The Berg (Ratel River only) and Olifants clade is well supported by all analyses, however there appears to be a strong barrier (100 % support) between the two rivers sampled in the Berg (Ratel River and Tulbagh). A possible explanation is that the Tulbagh population has recently become isolated from the Ratel River population, but the reticulation network does not indicate any ambiguities between these populations.

There are only moderate barriers between the Palmiet, Breede-West and Eerste catchments in the *N. brincki* clade, suggesting gene flow between these areas, the AFR is isolated and separated by strong barrier (100%). The Breede-South region appears to be distinct with strong barriers preventing gene flow with other catchments, while the *N. brincki* complex Roberston population appears to form a monophyletic clade with the Gourits catchment (not supported by the SVD quartet analysis), even though there is a strong barrier between the two catchments. The consensus reticulation network shows complex ambiguities within the *N. brincki* complex (possibly due to a fair amount of homoplasy), while the simplified "supernetwork" reticulation network shows no reticulation events within this group.

4.4 Discussion

South African Teloganodidae lineage

Under the unified species concept (de Queiroz, 2007; see Chapter One) species are seen as segments of separately evolving metapopulation lineages. Delimitation of species requires at least one of the properties from earlier species concepts (e.g. genealogical, mate recognition, phenology or isolation). This chapter has explored isolation, phylogenetic monophyly and

phylogenetic genealogical aspects of species delimitation, in terms of geographic patterns and how they contribute to the diversity within the Teloganodidae. South African Teloganodidae species diversity has increased from four genera and five species to a potential of six genera and 27 species.

The most clear-cut lineages in the analyses are highly supported by all analyses. These include the three lineages of *Lestagella* (L1, L2 and L3), *Ephemerellina* (E1 and E2), *N. brincki* complex, *N.* 'Robertson', *N. crassi* complex, Species A, Species B, Species C and *Lithogloea*. Within these groups, the relationships become more clustered and network-like. Note however, with regards to the genera, unless strong morphological evidence is provided, we cannot exclude the possibility that Species A, Species B and Species C belong to *Ephemerellina*. These four taxa were shown to form a monophyletic clade, and support was generally low when found non-monophyletic in some analyses. To what extent these 27 genetically distinct species will be described in due course is likely to be slightly more conservative, especially when the morphology (adult and nymphal stage) is taken into account. However, these results show a considerable underestimation of biodiversity within this group previously. The application of molecular techniques has increased biodiversity considerably on a local and global scale, this has been seen in many organisms particularly in the GCFR, showing distinct new species (fynbos flora: Linder, 2003; Barraclough, 2006; Decapoda, Potamonautidae: Daniels *et al.* 2001) and cryptic species (Baetidae, *Baetis harrisoni*: Pereira-da-Conceicoa *et al.* 2012; Onycophora, *Peripatopsis*: Daniels *et al.* 2009; Isopoda, Phreatoicidaea: Gouws *et al.* 2004; Chameleon, *Furcifer*: Florio *et al.* 2012; Cicadidae, *Platypleura*: Price *et al.* 2007). Even on a global scale, in the last decade many distinct Ephemeroptera taxa have been and are still being discovered; (Indian Teloganodidae: Selvakumar *et al.*, 2014; Teloganellidae, *Teloganella*: Kluge et al. 2015; Heptageniidae: Vuataz *et al.* 2013) and cryptic (Baetidae, *Baetis rhodani*: Williams et al. 2006; *Baetis vernus*, Ståhls and Savolainen, 2008).

The lack of evidence for isolation by distance (IBD) for all populations (except *Lithogloea*) is not surprising as the genetic variation is quite substantial for some populations as seen in the phylogenetic analyses in this chapter, further indicating that there are more species within these monospecific genera. For isolation by barrier (IBB), the results suggest that the mountain ranges have most likely caused an "island effect" as teloganodids are short-lived and poor fliers, therefore becoming genetically isolated, especially during phases of high sea

levels (discussed in Chapter Five). This "island effect", where catchments and their corresponding watersheds structure the genetic history of the organisms is well documented (Atyidae shrimps, Hughes *et al.* 1996) in southern Africa (Cicadidae, Price *et al.* 2007, Price *et al.* 2010; Cyprinidae: *Pseudobarbus*, Swartz *et al.* 2008). However, the effect of different catchments on invertebrates with aquatic stages is dependent on the dispersal ability of the organism (Wishart, 2000), within South African Teloganodidae, *Lestagella* is more affected by the catchment effect than the other genera, possibly due to a poorer dispersal ability compared to the other genera (smallest body and wing size, see Chapter Six).

Each genus shows intricate patterns with regards to diversity and distribution; however they also share a few common trends. The Breede catchment populations show the greatest diversity between its rivers, indicating that these lineages have gone through numerous dispersal and vicariant events throughout its history. There are a number of possible explanations for this diversity; 1) the catchment is surrounded by mountain ranges that form an almost circular "bowl" shape, with a southern opening directed towards the coast; 2) leading on from this, there are various mountain range orientations present (running North-South, East-West and North-West / South-East), which may experience different environmental conditions; 3) the southern opening of the "bowl" is pointed towards the coast and therefore was likely affected by sea levels during glacial cycles (discussed in Chapter Five), therefore cutting off the eastern rivers from the western rivers and; 4) the Breede catchment experiences three types of climatic rainfall zones, winter mesic, aseasonal mesic and aseasonal arid (Colville *et al.*, 2014) that roughly corresponds to Breede-East, Breede-South and Breede-West respectively. This may affect emergence times of the various populations, resulting in delayed mating periods or dispersal limitation to other zones due to unfavourable conditions. The Breede-Robertson region (from the Breede-East group) appears to have been isolated at some time, whereby species diverged and represent distinct lineages now. In terms of rainfall, the Robertson area is on the cusp of 'aseasonal arid' and 'aseasonal mesic', which may have contributed to the extensive divergence, also this area may have been cut off during sea level transgressions during glacial cycles (see Chapter Five).

Populations from the other catchments seem to be fairly consistently distinct from one another across the genera, with some overlap existing which may be due to favourable dispersal conditions in the past (Chapter Five). On the other extreme, geographically close catchments (for example, the Palmiet and Eerste catchments) show no evidence of gene flow

for some genera. Factors possibly contributing to this include the orientation of the mountain ranges and direction in which the rivers are flowing (this is also seen for *Lestagella* between Olifants-East and Olifants-West). The genus with the widest distribution range is *Lestagella*, extending farthest South West on the Cape Peninsula and farthest North East in the Keiskamma (Amathole Mountains). This group also shows a great amount of phylogenetic structure, where lineages are genetically distinct between river and catchment populations.

Higher Taxon Relationships

In terms of the higher classification addressed in this chapter, the lack of a sufficient number of samples for the Oriental Teloganodidae, Melanemerellidae and Ephemerythidae make reconstructions and conclusions more speculative. With the current state of knowledge, it is not possible to conclude that the Oriental lineage is monophyletic. The reconstructions lack important Oriental taxa (see Table 1.1 for a list of Oriental Teloganodidae) and the OTU in the analyses appear at the base of the phylogenies, therefore making the lineage paraphyletic if linked with any other clade. That is, Melanemerellidae and Ephemerythidae share a close connection with the Teloganodidae, forming a monophyletic group that includes the Asian Teloganodidae (*Dudgeodes ulmeri*) as a sister group at the tree base, therefore rendering the Teloganodidae paraphyletic as currently defined. The results obtained for the higher taxon relationships, although more speculative, will be briefly discussed here.

Based on the "supernetwork" reticulation network, Ephemerythidae and Melanemerellidae share a reticulation event between common ancestors of Zimbabwean Ephemerythidae and Melanemerellidae + Zambian Ephemerythidae. The link between Melanemerellidae and Ephemerythidae has been picked up before by Gillies (1960) who referred to the wing venation similarities of the two taxa (they both belonged to the family Tricorythidae at the time), unfortunately the male genitalia cannot be compared as they are still unknown for Melanemerellidae. The Asian Teloganodidae and *Teloganella* (Teloganellidae) have a shared reticulation. There may be scope for examining this relationship in detail in future studies (with many more samples), especially as *Teloganella*, recently found in India was mistaken for Teloganodidae (Selvakumar *et al.*, 2014; Kluge *et al.*, 2015).

The presence of reticulation violates assumptions that underlie tree-based methods which produce hierarchical and bifurcating trees (Huson and Bryant, 2005), which could be the reason for low bootstrap support for *Manohyphella*, and placement of the species is not

confidently resolved in the phylogenetic analyses. More data is needed to resolve the South African/Madagascan teloganodid complex. From these analyses however, it appears that Madagascan *Manohyphella* shares a closer link to South African teloganodids than it does to the Asian lineage, represented by *Dudgeodes*. Instead it appears that *Manohyphella* (also lacking a head-fringe) is more closely related to the South African Teloganodidae (particularly to the "non-fringed" clade), a trend not unusual in mayflies (McCafferty, 1999; Monaghan *et al.* 2005; Sartori *et al.* 2000, Vuataz *et al.* 2013), and known for other insects too (Goodman and Benstead, 2005; Coleoptera: Cicindelidae, Cassola, 2003; Odonata, Donnelly *et al.* 2003).

Using cladistics, *Manohyphella* was shown to represent a basal branch of a clade that includes all Teloganodidae except *Ephemerellina*, and the Madagascan genus is considered most closely related to *Nadinetella* (McCafferty and Benstead, 2002). This was not the case in this study, where *Manohyphella* is more closely aligned with the "non-fringed" South African Teloganodidae, and *Lestagella* forms the basal branch that includes all Afrotropical teloganodids.

Many Madagascan endemics originate either from transoceanic dispersal (e.g. lemurs, Goodman and Benstead, 2005; Apidae, Chenoweth and Schwarz, 2011; Isoptera, Fungus growing termites, Nobre *et al.* 2010; Heptageniidae, Vuataz *et al.* 2013; Canthonini dung beetles, Wirta *et al.* 2010) or a result of ancient continental separations (Ephemeroptera: Edmunds, 1972; Edmunds, 1975; Ichneumonidae: Gauld and Walsh, 2002). The results from this study imply a single origin for Afrotropical Teloganodidae (South Africa and Madagascar), supporting previous biogeographical and cladistics studies which suggest a Gondwanan origin for Teloganodidae (Craw, 1988; Edmunds, 1972; 1975; McCafferty and Wang; 1997; McCafferty, 1999; Sartori *et al.*, 2008; Selvakumar *et al.*, 2014). This will be examined in the following chapter.

4.5 Conclusions

All analyses conducted have shown similar, underlying patterns. From these results it can be seen that the relationship between Ephemerythidae and Melanemerellidae should be further investigated, as well as the relationship between the African and Asian Teloganodidae. Based on the results presented in this chapter, one option would be to sink Ephemerythidae and

Melanemerellidae to subfamilies within Teloganodidae and elevate the Asian teloganodid lineage to their own subfamily within Teloganodidae. However more samples are needed from these taxa to make any confident conclusions with regards to the taxonomy.

Prior to this study, the species level diversity of this family in South Africa was greatly underestimated. This work has resulted in the discovery of new genera previously unknown and many allopatric populations separated clearly by geographic / catchment location, which probably represent new species. These results have significant effects on the conservation prioritisation of certain areas within the GCFR. For example, Species C which is only recorded in the Breede catchment and certain *Lestagella* clades which are restricted to isolated localities. The biodiversity of the Teloganodidae is much richer than expected or portrayed from previous literature. As the Cape Floristic Region is a recognised biodiversity hotspot, the trends shown in the western and southern Cape Teloganodidae are probably not unique, suggesting much undiscovered biodiversity in this area. These complex diversity trends seen in the data for each genus/group are plausibly fine-tuned to the historical biogeographical patterns associated with the landscape and climates throughout millions of years. These patterns are investigated in the following chapter and go further in explaining the rich biodiversity present today.

Chapter Five

Ancestral Reconstruction and Historical Biogeography

"We will now discuss in a little more detail the struggle for existence"

~Charles Darwin

SUMMARY

Molecular dating analyses using the Africa/Lemuria tectonic split as a calibration point for the Madagascar/South Africa (SA) Teloganodidae node resulted in a divergence rate estimate of 0.5 % My^{-1} (for genes 16S/COI). Three ancestral reconstruction methods were used to investigate ancestral ranges of deep-time lineages, including other closely related families, and for SA Teloganodidae. Based on available evidence, a preliminary hypothesis for deep-time reconstruction is presented. The Madagascar/SA teloganodid lineage is thought to have originated in SA, and the ancestor may have occurred in the main palaeo-drainage system of the Orange River in the Cretaceous. The lineage may then have dispersed northwards to Madagascar (*Manohyphella* ancestor) during the humid Early Cretaceous. In the Late Cretaceous, a dry interior possibly caused migration of the SA lineage, via the palaeo-Orange River, to the wetter west coast. Erosion of the African landscape and retreat of the Great Escarpment facilitated dispersal and migration of the *Lestagella* and Species A lineages to the south east coast during the Late Cretaceous/Early Tertiary. The confluence of the Orange/Olifants/Berg Rivers (during regressions) enabled the ancestral lineage to move into the Cape Fold Belt. From the Early Tertiary to the Pleistocene; widespread forests facilitated dispersal through the GCFR, which became fragmented and forced into montane regions as a result of sea-level transgressions, regressions, tectonic uplift events and underwent gradual transformation to fire-adapted vegetation (fynbos). These events greatly affected dispersal and vicariance of teloganodid lineages within and between catchments. Teloganodidae are associated with the ancient relictual forests and are thought to have used them as refugia during unfavourable conditions. Association with aquatic vegetation habitats is thought to have limited the dispersal ability of some lineages (*Nadinetella* and *Ephemerellina*), as these are not as widespread as those occurring under stones in current (*Lestagella*). These results underline the complex history of many fauna in the GCFR. Close association and allopatric isolation of lineages between and within catchments emphasises the considerable biodiversity of the region. As other still unstudied fauna probably exhibit similar patterns, this accentuates the need for this knowledge for conservation purposes.

5.1 Introduction

The Greater Cape Floristic Region (GCFR) is an ancient landscape, approximately 2.6 billion years old, and has experienced many geological events, although is said to have been relatively stable since the fragmentation of western Gondwana (from ca. 140 Ma; Partridge and Maud, 1987). The climate, drainage and vegetation however has transformed considerably, leading to the diversity of flora and fauna that we see today.

Afrotemperate forests were widespread in the early to mid-Tertiary (Hendey, 1983, Partridge and Maud, 1987, Reyment and Dingle, 1987). These forests have been fragmented due to climatic changes and tectonic uplift (Hendey, 1983; Partridge and Maud, 1987; Reyment and Dingle, 1987; Siesser and Dingle, 1981). Furthermore, the gradual transformation of the GCFR vegetation to fire-adapted flora fragmented forests to the current palaeogenic zones in montane regions (Allsop *et al.*, 2014). The Afromontane forests of South Africa currently consist of ca. 900 patches totalling approximately 600 km^2 (Geldenhuys, 1993). The largest fragment (ca. 568 km^2) is the Knysna–Tsitsikamma forest (the area in which the Afromontane Forest Rivers (AFR) are located as defined in Chapter Two), which is home to a number of isolated endemic forest specialists (Carruthers and Robinson 1977; Mucina and Rutherford 2006; Tolley and Burger 2006; McDonald and Daniels 2012).

This chapter aims to reconstruct a working hypothesis for the deeper phylogenetic history of the Gondwanan Teloganodidae ancestors, followed by an investigation into the more recent biogeography of the southern African group. The distributions and phylogenies of modern taxa are used in a comparative phylogeographic approach, looking at possible ancestral ranges in conjunction with known palaeoclimates, vegetation and tectonics to determine evolution of each species over time. Comparisons with other organismal groups are made in an attempt to better understand the complex biodiversity of the GCFR and its possible origins.

A summary of the most important events (tectonic, marine, freshwater, climatic and vegetation) and their possible effects on ancestral Teloganodidae (Table 5.1) helps contextualise the complex environment throughout their evolutionary history. In addition to this summary, a pictorial reconstruction of the palaeo-drainage systems, marine regressions and transgressions, possible catchment connections (for migration and dispersal) and important uplift events from the Late Cretaceous to Plio-Pleistocene is provided (Figure 5.1).

Table 5.1. Summary of key events and effects on Africa from the Late Jurassic until the Holocene, with focus on southern Africa.

FP – Falklands Plateau; AB – Agulhas Bank; S Atlantic – South Atlantic; SA – South Africa; temp – temperature; LGM – Last Glacial Maximum; CFR – Cape Floristic Region; CFB – Cape Fold Belt; csl – current sea level

Age	Physical event and effect [12,13,14]	Sea level Regression/ Transgression [3,4,12,17]	Climate, Vegetation and fresh water bodies [4,12,13,14,17]	Possible effect on Teloganodidae Ancestors
Late Jurassic 160 – 145 Ma	▪ Separation of Madagascar from Gondwana, opening up to Indian Ocean ▪ Desert across central Africa towards Antarctica [15] ▪ FP still attached to South Africa ▪ Sea advances from East onto FP creating elongate basins (on AB) ▪ S Atlantic rifting from South to North caused volcanism on West Coast ▪ Fragmentation of Gondwanaland, formed new base levels, rapid erosion [11]	▪ Sea level below csl	▪ Humid in South eastern Africa ▪ Woodlands around lakes ▪ Large lakes develop in rupture zones in East Africa (North-South direction) ▪ Massive fluvial fans from Limpopo and Zambezi Rivers	▪ Dispersal limited from North West to East (India) due to lakes and Madagascar splitting ▪ Dispersal to Madagascar from SA via Antarctic corridor ▪ Desert and volcanism limited North-South dispersal
Early Cretaceous 145 – 112 Ma	▪ FP starts separating from Africa and S Atlantic opens from the South ▪ Shallow water barrier across FP causes anoxic conditions in deep S Atlantic basin ▪ Parana-Etendeka volcanism North of SA on West Coast[10] ▪ Continental separation caused local reorganisation of current patterns	▪ Trangression along east coast and southern Cape (Agulhas Bank)	▪ Humid ▪ Woodlands, inland well vegetated ▪ Large Orange River delta flowed towards anoxic basin ▪ Limpopo and Zambezi fluvial fans present	▪ Volcanism on West Coast limiting North-South dispersal ▪ Reorganisation of currents and separation of FP cause climate change, may have affected rivers especially on South West Coast ▪ Madagascar and South African ancestor disperses to Madagascar, and separates with tectonic separation.

Late Cretaceous 112 – 65 Ma	▪ Significant ocean changes in S Atlantic ▪ Further FP separation breaks shallow water barrier, causing deep water circulation ▪ South America-Africa connection causing northern barrier in S Atlantic, still anoxic in North ▪ Start of African erosional phase until Miocene, causing large scale planation of African surface (at different levels above and below escarpment), deep weathering on erosion surface [11] ▪ Great Escarpment relatively close to coastal margins ▪ Land bridge connecting Cape Peninsula to HH mountains starts eroding ▪ Uplift and tilting at 112 Ma [2] ▪ Final separation of Africa from S. America (100 Ma) ▪ Major uplift at 100 – 80 Ma[1] ▪ Coastal current in North-eastern direction (opposite to present day) ▪ K-T boundary	▪ Sea transgression mostly in West, regression seen in South and East (99 Ma) ▪ Followed by sea regression evident on AB (93 Ma) ▪ Transgression (85 Ma) ▪ Transgression (70 Ma) up to 367 m above (current) seal level on South East Coast ▪ Severe regression (65 Ma) up to 1350 m below current sea level	▪ Warm and dryish toward end of Cretaceous (relative to previous tropical climate with forest and >700 mm/ yr rainfall) ▪ End of epoch: tree forests with lianas, epiphytes, tree ferns and mosses ▪ Low diversity of Proteaceae, Ericaceae and Restionaceae ▪ Drop in water temp across boundary ▪ Uplift causes western drainage of Orange River ▪ Orange, Olifants and Berg Rivers connected	▪ Dry inland of South Africa caused migration of South African lineage to the wetter west coast ▪ Severe regression allowed Orange/Olifants/Berg Rivers dispersal ▪ Migration of *Lestagella* and Species A from Orange tributary to Keiskamma region and AFR (Late Cretaceous to Early Tertiary)
Paleocene 65 – 55 Ma	▪ No major physical changes known	▪ Transgression from below current sea level to above	▪ Warm and wet ▪ Forested lowlands, fynbos in mountains ▪ Olifants and Berg Rivers connected ▪ Late: Thermal maximum	▪ Orange tributary geographically close to AFR and other coastal drainage ▪ Possible watershed migration to AFR/Keiskamma via scarp retreat

Eocene **55 – 33 Ma**	▪ No major physical changes known	▪ Continued transgression to ca. 200 – 300 m above csl ▪ Possible smaller regression (to just below csl) and transgression from mid to late Eocene	▪ Climatic optimum ▪ Eocene climate largely unknown ▪ Eocene – Oligocene boundary: rapid drop (5 °C) of sub-Antarctic ocean (largest climatic change of Cenozoic)	▪ **Early to mid-Tertiary:** corridor of migration and dispersal for *Nadinetella*, Species B, Species C and *Ephemerellina*
Oligocene **33 – 23 Ma**	▪ Cape Canyon incision (of deeper water portion at severe low sea-level) [3,4] ▪ Advanced stage of planation	▪ Major regression to ca. 400 - 500 m below csl (in west and south west)	▪ Cooler and drier than previous climate ▪ Antarctic snow and ice accumulation ▪ Paleogene Orange River discharge indicates dry interior ▪ Upper Orange-Vaal system enters S Atlantic via Cape canyon [10] ▪ Berg River changed course from north-westerly to westerly direction (no longer joins Olifants River)	▪ Separation of Berg and Olifants Rivers split populations ▪ **End of Oligocene:** Orange River captured, mouth exit further North (similar to present)
Early Miocene **23 – 15 Ma**	▪ Mild tectonic uplift triggered erosion of surface silcrete layer ▪ Rejuvenation of erosional cycles had important impact on drainage systems ▪ Uplift: ca. 200 m on western margin ▪ Interruption (from uplift) of erosional phase, westward tilt of African surface	▪ Trangression (below csl to similar csl)	▪ Warm and wet (tropical to sub-tropical) ▪ Climatic optimum reach late to middle Miocene [5] ▪ Forests now widespread on coastal lowlands ▪ Fynbos vegetation establishes ▪ Widespread fluvial activity	▪ Uplift isolated Keiskamma River from the rest of southern Cape (possibly from Orange as well because of westward tilt and scarp retreat)

Middle Miocene 15 – 11 Ma	▪ Major Antarctic ice cap (permanent feature) ▪ Another uplift event, causing seaward tilt of western margin – only affected western margin [3] ▪ **Late Tertiary**: Land bridge connecting Cape Peninsula and CFB erodes away, isolating Cape Peninsula	▪ Transgression (to ca. 150 - 300 m above csl)	▪ Dramatic climatic and environmental deterioration ▪ Global cooling continued throughout Miocene ▪ Cooler, more arid, temperate ▪ Cape flora diversity evolves with recent fire regime [7,8,9] ▪ Extinction of mesic forest flora	▪ Many river systems would have been drowned ▪ Breede-East lower reached would have been cut off, isolating populations ▪ *Lestagella* on Cape Peninsula isolated from mainland ▪ Some population isolated from forest fragmentation
Late Miocene 11 – 5 Ma	▪ Benguella upwelling establishment ▪ Commencement of uplift of margins	▪ Regression from high sea levels to below csl	▪ Summer-dry climate may have evolved at ca. 10 Ma ▪ Gradient increasing summer rainfall to East ▪ Vegetation drastic change – similar to present day ▪ Increased aridification = decrese in river flow ▪ Diversion of upper Orange River to present course	▪ Major cladogenesis within generic subgroups, possible consequence of earlier trangression
Pliocene 5 – 2 Ma	▪ Uplift lead to elevation of Great Escarpment ▪ Max uplift = 900 m, in the East ▪ Uplift not as extreme in West ▪ Eastern CFR = windward slop of escarpment mountains (more rain) ▪ Western (Namaqualand and Succulent Karroo) = Leeward side (more arid) ▪ Cape canyon incision of upper reaches [3] ▪ Uplift major: asymmetric uplift of continent, westward tilting	▪ Early: Transgression from ca. 100m below csl to above csl ▪ Mid: Regression ▪ Late: Regression to below csl followed by minor rise to above csl, followed closely by another minor regression	▪ Low temps and low rainfall, ▪ Summer-dry / winter-wet rainfall regime ▪ Cycles of Antarctic warming and cooling ▪ Uplift increased rainfall gradient (eastern CFR wetter, western more summer arid) ▪ Forests confined to well-watered mountain slopes and river valleys ▪ Cape Canyon upper reaches cut by Olifants River during regression [3]	▪ Biota evidence still contained relicts from previous, more subtropical climate in Miocene ▪ Some of these became extinct, others adapted and evolved (similar to present day biota) ▪ Changes in river courses affected dispersal/vicariance ▪ Uplift isolated forests, fauna confined to high-lying relict areas [6]

Pleistocene 2 – 0.01 Ma	▪ Incision of gorges ▪ Marine benches, coastal dunes, river terraces until Holocene	▪ Transgressions (interglacial) and regressions (glacial) occurring every 100 ka, reaching ca. <10 m above csl, and 120 m below csl (West coast)	▪ Cool-dry glacial and warm-wet interglacial cycles every ca. 100 ka ▪ LGM 18 000 years ago ▪ Western GCFR wetter during glacial periods than eastern GCFR ▪ 45 km westward bank exposed during regression	▪ Climate cycles caused drastic changes in fauna, distinct from one cycle to the next ▪ Species distribution and diversity influenced ▪ Promoted significant cyclical migration (geographical and elevational) causing extinction and speciation
Holocene 0.01 – 0 Ma	▪ South western Cape tectonically stable	▪ Current sea level transgression	▪ Present interglacial	▪ Current species – effects of changing climates to be seen in the future. Already changes in emergence times of other aquatic taxa noted over the last few decades [12,13]

Information in table from Hendey 1983, Allsop *et al.*, 2014, (1) McMillan, 2003 (2) Brown *et al.*, 1990 (3) Franceschini, 2003 (4) Dingle and Hendey, 1984 (5) Zachos *et al.*, 2001 (6) King, 1978 (7) Linder *et al.*, 1992 (8) Cowling and Richardson, 1995 (9) Mucina and Rutherford, 2006 (10) Goudie, 2005 (11) Moon and Dardis, 1988 (12) Doi, 2008 (13) Jonsson *et al.*, 2015

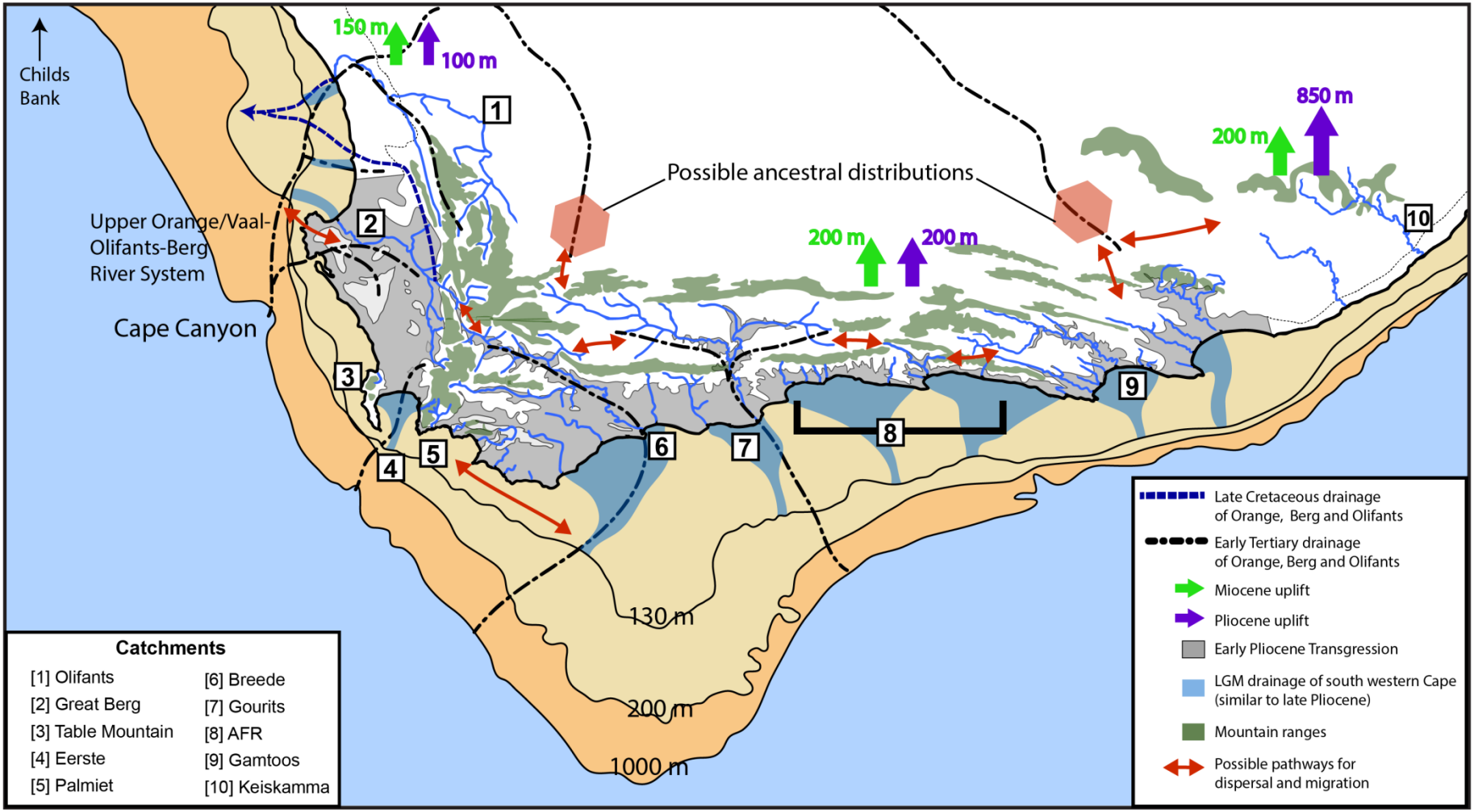


Figure 5.1. Pictorial reconstruction showing isobath up to 1000m below current sea level, and palaeo-drainage patterns from Late Cretaceous to LGM. Inland Early Tertiary drainage flows towards the Orange River. Data from sources: Partridge and Maud, 1987; Swartz *et al.*, 2008; Chakona *et al.*, 2013; Franceschini, 2003; Hendey, 1983; Allsop *et al.*, 2014.

5.2 Methods and Materials

5.2.1 Molecular dating using BEAST

Due to the lack of fossil evidence for the Teloganodide, calibrations were made using vicariant, tectonic events; namely the Madagascar-Africa split at ~150 Ma (Barron, 1987; Rabinowitz *et al.*, 1983; Ali and Aitchison, 2008; Scotese, 2009) and the Africa-South America split at ~110 Ma (Ogg *et al.,* 2004; Nishihara *et al.,* 2009; Scotese, 2009). The method of using tectonic events provides a reliable, minimum age for divergence (Heads, 2009). For comparative interest, a molecular clock was also used on datasets following Papadopoulou *et al.,* (2010) who used a well-established biogeographic barrier in the Aegean archipelago to estimate COI and 16S substitution rates for coleopteran family, Tenebrionidae.

Dataset alignments for genus and species levels (see Chapter Four, Table 4.2) were imported and prepared in BEAUti v.2 (Bouckaert *et al.,* 2014) and analysed using BEAST v.2.2.0 (Bouckaert *et al.,* 2014). Analyses were set up under a single model (see Chapter Four, Table 4.1), an uncorrelated lognormal (UCLN) relaxed clock and a Yule speciation prior. For the Africa/Madagascar split, the age on the node for the group including Madagascan *Manohyphella* and the *Ephemerellina* clade (excluding *Lestagella*) was set using a Log Normal prior (M = 5.0 and S = 0.03), specifying a distribution centred at ~148 Ma with a standard deviation of about 10 Ma, resulting in the 95 % probability range of 140 – 157 Ma, encompassing some of the various estimates of the timing of this tectonic event (Barron, 1987; Rabinowitz *et al.*, 1983; Ali and Aitchison, 2008; Scotese, 2009). Following this analsyis, the estimated clock rate for the combined COI/16S genes was used to calibrate the dating analyses for the species clade datasets.

The Africa/South America split was set on the node for African Ephemerythidae and South American Melanemerellidae (*Melanemerella brasiliana*), based on the assumption that these two shared a common Gondwanan ancestor. This node was set using a Log Normal prior (M = 4.7 and S = 0.045), specifying a distribution with median at 110 mya and a standard deviation of around 10 mya. The 95 % probability range covers 101 – 120 mya to include any tectonic time discrepancies (Ogg *et al.,* 2004; Nishihara *et al.,* 2009; Scotese, 2009). After running an initial analysis, 28S was found to have substantial rate heterogeneity among lineages (ulcd.stdev for 28S > 2), therefore all subsequent analyses excluded the 28S gene.

The comparative BEAST analyses were then conducted using the published divergence rate of 2.69 % My^{-1} for COI and 16S combined (or 3.54 % My^{-1} for COI gene alone for datasets without the 16S gene), under the same priors as mentioned above.

These analyses were submitted to the CIPRES Science Gateway (Miller *et al.,* 2010), each with one run of 20 – 50 million generations with tree-logging adjusted to obtain a final total of 10 000 trees. Stationarity and the estimation of the effective sample size (ESS) in the analyses were examined using Tracer v1.5.0 (Rambaut and Drummond, 2007). Trees were annotated in TreeAnnotator v.2.2.0 (part of BEAST2 package) with a 10 % burn-in and viewed using FigTree v.1.3.1 (available at http://tree.bio.ed.ac.uk/software).

For interest, the clock rate estimated from the analysis calibrated using the Madagascar/Africa split (~ 148 Ma) was run on the Afr/SAm dataset. This was done to assess the estimated time for the split between Ephemerythidae and Melanemerellidae according to the Africa-Madagascar split clock rate, which was to ascertain whether the age of the split in this analysis validated the 110 Ma calibration point.

5.2.2 Ancestral Area Reconstruction

Three event-based methods were implemented in RASP (Reconstruct Ancestral State in Phylogenies; Yu *et al.,* 2015) to estimate ancestral areas: 1) Statistical dispersal-vicariance analysis (S-DIVA; Nylander *et al.,* 2008, Yu *et al.,* 2010); 2) Bayes-Lagrange or statistical dispersal-extinction cladogenesis (S-DEC; Ree *et al.,* 2005; Yu *et al.,* 2010) and 3) Bayesian binary MCMC (BBM; Yu *et al.,* 2010).

S-DIVA is parsimony-based (Ronquist, 1997), where vicariance is assumed and dispersal events are assigned a cost. The assumption of vicariance in the Dispersal-Vicariance Analysis (DIVA) may bias reconstructions (e.g. Lamm and Redelings, 2009), and so a model-based Bayesian reconstruction analysis was also used. BBM infers binary states (for geographic ranges) at the ancestral nodes using a Bayesian approach (Yu *et al.,* 2010). The S-DEC method estimates ancestral areas uses maximum likelihood and inferring biogeographic speciation parameters for dispersal and extinction (Ree *et al.,* 2005). All three methods use a statistical approach to account for uncertainty (calculating ancestral area probabilities over the Bayesian posterior tree topologies from the BEAST analyses). This statistical approach

allows the calculation of probabilities and therefore incorporates phylogenetic uncertainty, which in turn reduces uncertainty in the ancestral reconstruction (Nylander *et al.,* 2008).

All three methods were run on the genus and species-level BEAST results, 10 000 binary trees were imported into the RASP program, 1000 trees discarded as burn-in and 1000 random trees used for each analysis. The BEAST consensus tree and a distribution CSV file (user defined areas, see Table 5.2) were also imported into RASP.

The ancestral ranges in all analyses were set to contain a maximum of two areas. S-DIVA analyses were optimized with 1000 maximum reconstructions for the final tree. Two types of analyses for S-DEC were performed: firstly, a general analysis allowing all dispersal routes equal probability and secondly, constrained analyses for the corresponding dataset which inhibits any dispersal to or from Madagascar after 150 Ma or South America after 110 Ma due to the separation of these continents. Ancestral ranges were set to include no more than two areas to simplify interpretation, as seen in other studies (Moyle *et al.,* 2012; Ali *et al.,* 2013; Rodriguez *et al.,* 2015;). For the BBM analyses, two independent runs of 10 chains with a temperature of 0.1 were run for one million generations and sampled every 100 generations. Convergence between runs was confirmed by a distance < 0.01 (Moyle *et al.,* 2012).

A time-events graph was calculated for all analyses (to display all dispersal, vicariance and extinction events over time). This is an algorithm that treats events at each node as having a modified Gaussian distribution (Yu *et al.,* 2015).

Table 5.2. Ancestral range areas used in the RASP analyses, each area is coded with an alphabetic letter, codes are shown for each of the RASP analyses performed on the various datasets.

Dataset	Area	Code	Dataset	Area	Code	Dataset	Area	Code
"Afr/SAm Group"	North East Africa	A	**"Afr/Mad Group"**	North East Africa	A			
	South America	B		Madagascar	B			
	Indonesia	C		Keiskamma	C			
	Keiskamma	D		Western Cape	D			
	Western Cape	E						
***Ephemerellina* Clade**	Afromontane Rivers	A	***Lestagella* Clade**	Keiskamma	A	***Nadinetella* Clade**	Afromontane Rivers	A
	Gourits	B		Gamtoos	B		Gourits	B
	Palmiet	C		Afromontane Rivers	C		Eerste	C
	Berg	D		Gourits	D		Palmiet	D
	Olifants	E		Eerste	E		Berg	E
	Breede-West	F		Liesbeek	F		Olifants	F
	Breede-Barrydale	G		Berg	G		Bereede West	G
	Breede-Koornlands	H		Olifants	H		Breede-Barrydale	H
	Breede-Robertson	I		Breede-West	I		Breede-Koornlands	I
				Breede-Barrydale	J		Breede-Robertson	J
				Breede-Koornlands	K			

5.3 Results

The divergence rate calculated for the Teloganodidae COI/16S gene combination was 0.5 % per million years. The clock rate estimated from the analysis calibrated using the Madagascar/Africa split (~ 148 Ma) was run on the Afr/SAm dataset to investigate whether or not the clock rate returned a similar age for the Melanemerellidae/Ephemerythidae split. This analysis yielded very similar results to those obtained by calibrating the Ephemerythidae /Melanemerellidae split to 110 Ma based on the Africa – South America landmass split during the breakup of Gondwanaland (data not shown), however the error bars were much larger for this analysis and so there results are not presented.

The comparative BEAST analyses conducted using the divergence rate from tectonic calibration can be seen in Appendix 5B and the published divergence rate of 2.69 % My^{-1} for COI and 16S combined (or 3.54 % My^{-1} for COI gene alone for datasets without the 16S gene), can be seen in Appendix 5C. Ages estimated from the published molecular clock yielded slightly younger, yet comparable estimates (when considering the error bars) for each lineage when *Manohyphella*, Ephemerythidae and Melanemerellidae were included, and considerably younger estimates when only South African Teloganodidae were included in the analyses (Appendix 5B and 5C).

For the BBM analyses, the two concurrent runs reached convergence for every analysis (i.e. the distances between runs one and two were always <0.01). The constrained and unconstrained S-DEC analyses yielded identical results, therefore only the constrained results are presented.

Results for key nodes showing dispersal, vicariance or extinction events can be found in Appendix 5A, with the proposed ancestral ranges event routes with support values corresponding to Table 5.2. Note that not all nodes have dispersal and/or vicariance events; probability for these nodes is not shown.

5.3.1 Deeper time reconstruction

For the three RASP analyses with “Afr/SAm Group” and “Afr/Mad Group”, the results for S-DEC and S-DIVA were similar for both groups, while results for BBM were not (Appendix 5A). Key nodes containing dispersal, vicariance or extinction events with a probability > 0.70 are discussed here, otherwise other key node event routes can be found in Appendix 5A.

The results from the BEAST and RASP analyses for the "Afr/SAm Group" and "Afr/Mad Group" are combined into Figure 5.2, along with palaeo-distributions corresponding to the RASP analysis. There was low support in the Bayesian BEAST phylogenetic tree for the relationship between the common ancestor of the Asian teloganodid as sister to the South African teloganodids ("Afr/SAm Group") and so there is little resolution at this node. In the "Afr/SAm Group", the Ephemerythidae/Melanemerellidae clade forms sister to the Asian and African groups, however this relationship is not well supported in the "Afr/Mad Group". In the RASP reconstructions, the BBM analyses calculated the single most probable area of origin, while the S-DEC and S-DIVA analyses calculated the origin with a maximum of three or four areas per node (to give an idea of potential distributions of common ancestors). The "Afr/SAm Group" and "Afr/Mad Group" are mostly congruent with their respective reconstructions (the deeper nodes have overlapping error bars, see Appendix 5B for the BEAST result trees).

"Afr/SAm Group" (Figure 5.2, Table 5.2, Appendix 5A):

Node 1 shows low probability (p) for event routes (ER) found by all three analyses, the highest for S-DIVA with two dispersal and one vicariance events (ER: BC->ABCE->AB|CE; p = 0.2543; node 1), which implies an ancestral range of South America (B) and Asia (C), before dispersal to A, B, C and E. This could be an indication that the ancestral clade was widespread throughout Gondwana. There is high probability (S-DIVA, p = 1.00) that there was a vicariance event between South American Melanemerellidae/North East African Ephemerythidae when South America separated from Africa.

All three analyses support a vicariance event for the Asian Teloganodidae / Western Cape Teloganodidae split. S-DEC and S-DIVA give a strong probability ($p > 0.722$) for one dispersal event from Western Cape to Keiskamma, followed by all three analyses with strong support for a vicariance event splitting the Western Cape / Keiskamma groups ($p > 0.939$).

"Afr/Mad Group" (Figure 5.2, Table 5.2, Appendix 5A):

There is some probability (S-DIVA: p = 0.5239) that there were two dispersal events from North East Africa to Western Cape and then to Madagascar, with suggestion of a Western Cape/NE Africa group and a Western Cape/ Madagascan group. The connection of NE Africa and Western Cape is based on a weakly supported branch from the BEAST analysis (posterior = 0.55) that places Ephemerythidae and *Lestagella* into the same clade, this grouping is inconsistent with other well-supported analyses, therefore it is more likely that

there was a NE Africa split from Western Cape/Madagascar group. All three analyses support the vicariant event between Madagascan and Western Cape groups ($p > 0.8448$), while BBM also shows a high probability of dispersal from Western Cape to Madagascar before the split.

There is high probability of a vicariance event between Ephemerythidae and Western Cape Teloganodidae, while BBM also supports dispersal to North East Africa first before this vicariance event. S-DEC and S-DIVA support a dispersal event from Western Cape to Keiskamma (as seen in the "Afr/SAm Group"), followed by high support from all analyses for the vicariant event between Western Cape and Keiskamma.

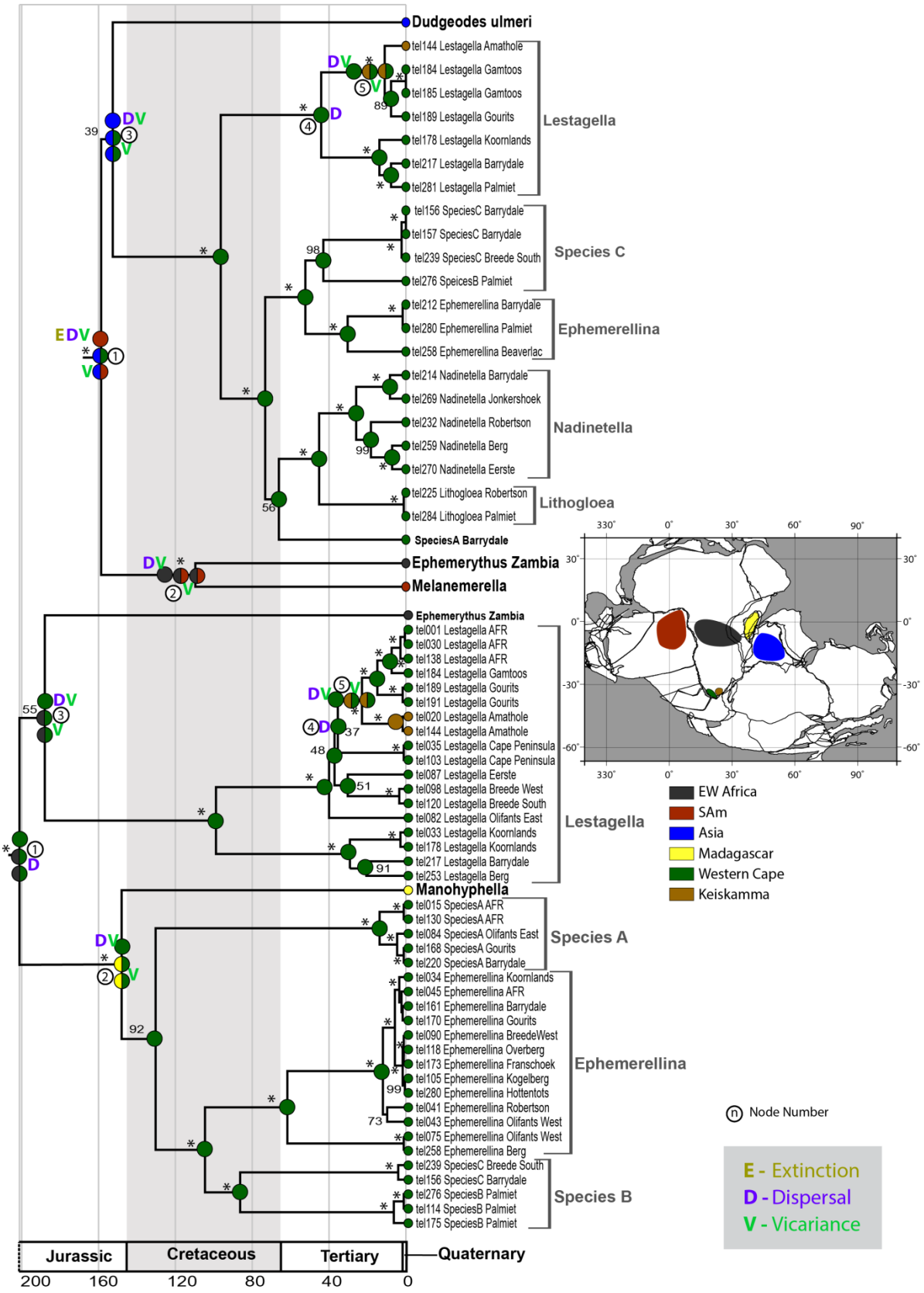


Figure 5.2. See legend overleaf

Figure 5.2. "Afr/SAm Group" and "Afr/Mad Group" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Nodes with three circles represent the BBM, S-DEC and S-DIVA analyses in this order (to be read from left-to-right or top-to-bottom). Nodes with only one circle indicate that all three analyses returned identical results. Numbered nodes are discussed in the text for possible dispersal and vicariant events. Asterisks indicate 100 % support.

The analyses suggest that the ancestral lineage of this group (Ephemerythidae, Melanemerellidae and Teloganodidae) occurred on Pangea in the early to middle Jurassic and was possibly widespread, with various dispersal and vicariance events splitting lineages. Notably, (especially in the "Afr/SAm Group") a vicariant event splits North East Africa and South America from Asia/ Madagascar and South Africa in the mid-Jurassic is indicated (evident in both analyses, Figure 5.2). The "Afr/Mad Group" indicates a Western Cape (South African) origin with dispersal northwards into North East Africa.

The Asian group seems to have become isolated somewhere from mid- to late Jurassic, though the resolution is low. Later, towards the late Jurassic, the analyses suggest that there was dispersal from the Western Cape region (this lineage was possibly more widespread in South Africa at this stage) to Madagascar before the tectonic split, which was then followed by the vicariant tectonic event when Gondwana began to separate. These results suggest that the Asian and Madagascan ancestors were widely spread distinct lineages before the separation of the India-Madagascar landmass (Lemuria; van Steenis, 1962). There appears to be evidence linking Madagascar to the non-fringed Teloganodidae (see Chapter Four), and it would appear that the ancestral lineage of the non-fringed Teloganodidae dispersed to Madagascar (Figure 5.2).

The East/West African and South American group (common ancestor of Ephemerythidae and Melanemerellidae, respectively) split when South America moved away from Africa in the mid Cretaceous (ca. 110 ma) according to the S-DEC and S-DIVA analyses.

The remainder of the reconstruction in all three RASP analyses show South African Teloganodidae with an ancestral range in the Western Cape, with a dispersal event within the genus *Lestagella*, from the Western Cape through to the Keiskamma catchment (in the Amathole Mountains, Eastern Cape), which was followed by a vicariant event which isolated this catchment.

5.3.2 **"Ephem Clade"** (Figure 5.3, Table 5.2, Appendix 5A)

The "Afr/SAm Group" gave low branch support for the inclusion of Species A within the "Ephem Clade" and suggests that the "Ephem Clade" excluding Species A (*Ephemerellina*, Species B and Species C) diverged from the rest of the group in the late Cretaceous. In contrast, the "Afr/Mad Group" (Figure 5.2) favours the mid Cretaceous for this split, which also gives high branch support for Species A, which split from the "Ephem Clade" in the Early Cretaceous. *Ephemerellina* diverged from Species B and Species C in the Early Tertiary according to the Afr/SAm results and mid to late Cretaceous in the "Afr/Mad Group". Species B and Species C diverged toward mid Tertiary in Afr/SAm analysis and late Cretaceous in the Afr/Mad analysis. In the detailed "Ephem Clade" analysis (Figure 5.3), the *Ephemerellina*, Species B, Species C clade diverged from Species A in the mid-Cretaceous (as seen in the "Afr/Mad Group").

Dispersal, vicariance and/or extinction events for Node 1 and 2 are not well resolved in any of the analyses with probability $p < 0.15$. Node 3 shows low support from the S-DIVA analysis ($p=0.52$) for one dispersal and one vicariance event for the dispersal from a Palmiet-Breede-Barrydale distribution to Breede-West, followed by vicariant split between Palmiet and Breede-Barrydale+Breede-West lineages in the late Cretaceous, forming the lineage that today is represented by Species B and Species C. Node 4 has a high probability for one vicariant event between Breede-Barrydale and Breede-West within the Species C group in the Pliocene.

There is no vicariant event for the *Ephemerellina* E1/E2 split (early Eocene), and a proposal of two dispersal events from Olifants to Berg and Breede-West with low probability. There is high probability of one vicariance event for the Olifants/Berg split within E1 in the Late Plio-Pleistocene.

There was also sea level transgression at this time which may have caused the vicariant event that separated Species A from the rest of the clade in the Breede catchment, which probably cut off what is now the Barrydale area from the rest of the Breede catchment during the transgression (Figure 5.1).

All three analyses show support for a vicariant event between Breede-Robertson and Olifants populations within E2 (S-DIVA; highest $p = 1.00$; node 8) in the late Miocene. Node 10 has a high probability of a vicariant event between the Palmiet and Breede-West E2 populations in

the Late Plio-Pleistocene. BBM shows low probability ($p = 0.54$) of two dispersal and one vicariant event at node 11, E2 populations dispersing from Breede-Koornlands to the AFR in the middle to late Pliocene (other analyses suggest an involvement of Barrydale and Gourits which are found in between the Koornlands and AFR catchments). There is a high probability of one vicariant event at node 13, splitting the E2 populations of Barrydale and Gourits in the early Pleistocene.

Node 14 is not well-resolved, Node 15 has low probability ($p = 0.36$) from the BBM analysis, for two dispersal and one vicariance events for a Species A population dispersing from the AFR to Olifants and then diverging as a result of a vicariant event in the middle Miocene. Node 16 shows some probability for at least one dispersal event (BBM shows two dispersal events) and one vicariant event within the Species A group. Dispersal from Olifants to Gourits (and possibly to Breede-Barrydale according to the S-DEC result), may have occurred before the Olifants and Gourits (+Barrydale) clades split by a vicariant event in the early Pliocene. There is good probability for one dispersal event at node 17 (for S-DEC and S-DIVA; $p \leq 0.83$) within the Barrydale / Gourits lineage, implying that a population within the Gourits became isolated, however this node is poorly supported in the BEAST analysis. There is strong indication that a vicariant event (node 18, high probability from all analyses) split the Barrydale / Gourits populations of Species A in the Pleistocene.

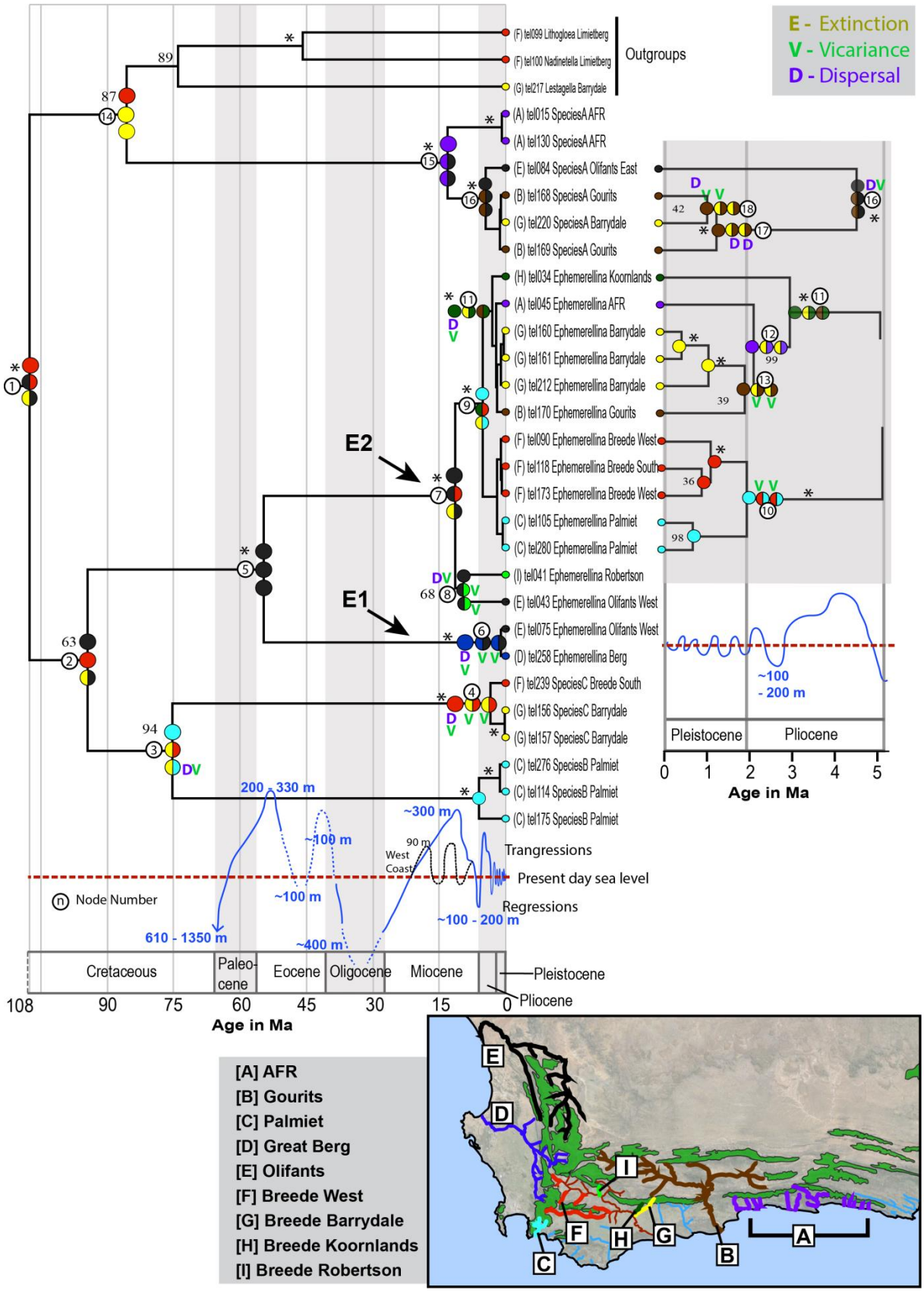


Figure 5.3. See Legend overleaf

Figure 5.3. "Ephem Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast).

5.3.3 **"Lest Clade"** (Figure 5.4, Table 5.2, Appendix 5A)

According to the "Afr/SAm Group" analysis (Figure 5.2), the *Lestagella* clade ("Lest Clade") separated from the non-fringed teloganodids in the mid Cretaceous. This is however unconfirmed due to low branch support in the "Afr/Mad Group". The "Lest Clade" analysis shows a similar result (Figure 5.4).

The split between L1 and L2+L3 within the "Lest Clade" occurred in the late Cretaceous. Dispersal, vicariance and/or extinction events for Node 1 and 2 (divergence between L1 and L2/L3) are not well resolved in any of the analyses with probability $p < 0.12$. Node 3 (L1) has low support ($p<0.5$) for one dispersal and one vicariant event, with dispersal from a Barrydale/Koornlands population to the Berg catchment followed by a vicariant event splitting Koornlands from the Berg/Barrydale population in the early to mid-Miocene. There is support for a vicariant event separating the L1 Barrydale and Berg populations during the middle to late Miocene (node 4; S-DIVA $p=1$; BBM/S-DEC $p>0.45$).

Towards the end of the cool, dry Oligocene, L2 and L3 diverged. Dispersal and vicariance events are not well-resolved for Node 5 (ancestral range in Olifants and Keiskamma). BBM shows low support for node 6 (L2 group) for two dispersal and one vicariant events, dispersal from Olifants southwards to the Cape Peninsula. The other analyses suggest a Breede-West element, which is one of the catchments found between Olifants and the Cape Peninsula, and therefore a possible track through north-south running mountain ranges. This is followed by a vicariant event separating Olifants and Cape Peninsula in the early to mid-Miocene. S-DIVA shows low support for L2 (node 7; $p = 0.495$) for one dispersal event from a Cape Peninsula/ Breede-West population to the Eerste catchment, during lower sea levels, the land bridge connecting Cape Peninsula and mainland was still intact (see Discussion section). This was followed by one vicariant event splitting the Eerste/Breede-West population from the Cape

Peninsula in the early to mid-Miocene (possibly due to Cape Peninsula being separated from the mainland during sea level transgression). Within the L2 clade (node 8; p = 1.00) has one vicariant event splitting the Eerste / Breede-West population during the middle Miocene. Sea level transgression may have pushed populations into higher river reaches within the mountains, protected within the forested ravines. The Olifants-East and Olifants-West populations show a well-supported and distinct divergence in the late Miocene.

There is low BBM support for L3 clade (Node 9; p = 0.50) for two dispersal and one vicariant events between catchments, dispersal to Keiskamma and then from the Keiskamma to the Gourits. Widespread forests, favourable climate, river piracies and captures (via scarp retreat) may have aided this dispersal to Gourits (see Discussion section). This was followed by a vicariant event separating the Gourits and Keiskamma catchments in the early to middle Miocene. Node 10 shows moderate probability support from S-DIVA (p = 0.5) for one dispersal and one vicariant event, dispersal from a AFR/ Gourits population to the Gamtoos catchment with a vicariant event splitting Gourits from the AFR/Gamtoos population during the late Miocene. Node 11 shows high probability support from the S-DEC and S-DIVA analyses (p > 0.9804) for one vicariant event separating the Gamtoos and AFR populations in the Pliocene.

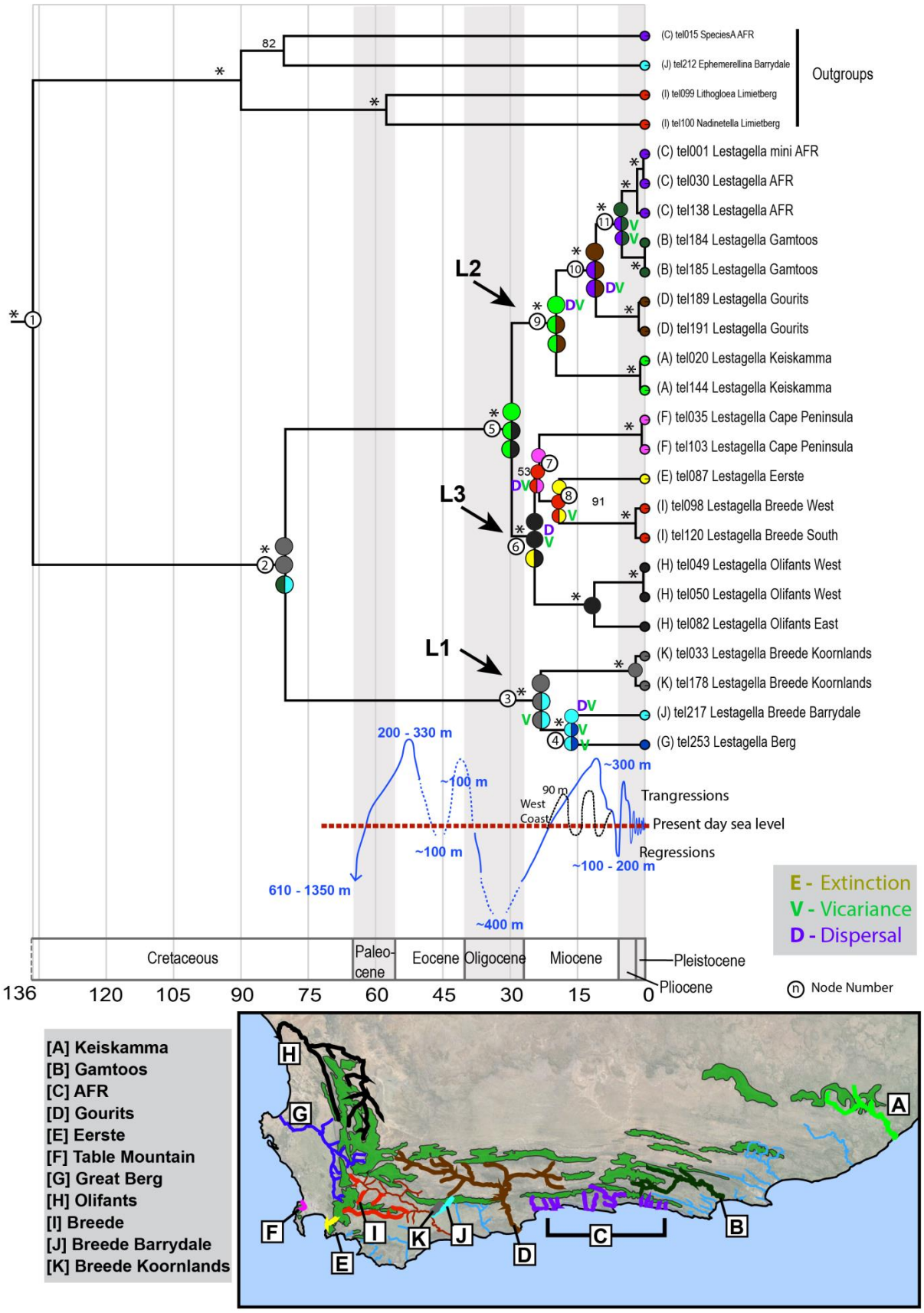


Figure 5.4. See legend overleaf

Figure 5.4. "Lest Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast).

5.3.4 **"Nad/Lith Clade"** (Figure 5.5, Table 5.2, Appendix 5A)

Lithogloea diverged from *Nadinetella* in the early to mid-Tertiary in the "Afr/SAm Group" analysis (Figure 5.2). In the detailed "Nad/Lith Clade" analysis (Figure 5.5), Lithogloea diverged from Nadinetella in the warm and wet Paleocene, possibly as sea levels transgressed, the Olifants+Breede-West was cut off from Breede-East+South by the increasing sea level (cutting off linked rivers and/or pushing populations into mountain refugia where there is less chance of dispersal).

Dispersal, vicariance and/or extinction events for Node 1 (divergence between *Lithogloea* and *Nadinetella*) are not well resolved in any of the analyses with probability $p < 0.13$. Node 2 (*Lithogloea*) has low support in the BBM analysis ($p = 0.51$) of two dispersal events to Olifants, and from Olifants to Breede-West. This was followed by one vicariant event splitting Olifants and Breede-West populations in the Early Pliocene. Node 4 has low support from the BBM analysis ($p = 0.58$) for two dispersal and one vicariant events, with dispersal to Breede-West and from Breede-West to the Palmiet catchment, followed by a vicariant event separating the two populations during the late Pliocene. S-DIVA shows low probability support for Node 5 ($p = 0.50$) for one dispersal event from Breede-West/Breede-Robertson to the Breede-Barrydale area, followed by a vicariant event separating the Breede-West population from the Breede-Barrydale/Robertson population in the late Pliocene. Node 6 shows high support from both S-DEC and S-DIVA ($p > 0.92$) for the vicariant event separating the Barrydale and Robertson populations during the mid to late Pleistocene.

Divergence within *Nadinetella* began during the late Eocene early Oligocene. There is a lack of ancestral range resolution for this node (Node 7; $p < 0.13$). The *N. crassi* complex then diverged in the mid-Miocene, however the ancestral range for this node has low probability ($p \leq 0.2$). BBM and S-DEC show support for Node 9 (*N. crassi* complex clade) for a vicariant

event between Barrydale and Koornlands during the late Miocene. There is also moderate support for node 10 (S-DEC; p = 0.53), of a dispersal event from the Palmiet catchment to the Breede-West during the middle to late Miocene. The Palmiet sample (TEL110) from the Palmiet River (flowing on the east side of the Hottentots Holland Mountain range, closest to the Breede catchment) diverged during the late Miocene transgression which may have drowned any river connection between the Palmiet and Breede-West. As sea levels regressed, the previously isolated Breede population was able to disperse again to the Palmiet. There is good support for Node 11(S-DEC; p = 0.88) for at least one dispersal event from the Palmiet/Breede-West to the Eerste catchment. There is also good support for a vicariant event separating Breede-West from the Palmiet/Eerste populations during the late Pliocene (possibly due to the cyclical climatic changes). One vicariant event has a high probability (BBM, S-DEC; Node 12; $p \geq 0.75$), splitting the Palmiet and Eerste populations during the Pleistocene.

The unusual (*N.* 'Robertson' clade) diverged from the *N. brincki* complex and became isolated in the Robertson area during the dry, cooler Oligocene. As the Robertson area is slightly inland, it may have experienced more arid conditions as it was shielded from wetter coastal conditions. These arid conditions may have restricted the distribution of the vegetation associated with the clade (*Wardia* moss), which also may have led to this split. The ancestral ranges for nodes 13 and 14 are not well-resolved. The *N. brincki* complex Olifants/Berg lineage diverged from the rest of the *N. brincki* complex in the mid to late Miocene (Node 14). Node 15 of the *N. brincki* complex has a high probability in the S-DEC and S-DIVA analyses ($p > 0.98$) for a vicariant event splitting the Berg and Olifants populations. The ancestral range for Node 16 is not clear, however the node is well-supported by the BEAST analysis and divergence is dated Mio-Pliocene.

Node 17 has low support from S-DIVA (p = 0.50) for one dispersal event from Breede-West/Breede-Robertson regions to the Gourits catchment and a subsequent vicariant event splitting the Breede-West region from Gourits/Breede-Robertson in the middle Pliocene (therefore the *N. brincki* complex-Robertson population re-enters the area where the *N.* 'Robertson' population has been established since late Oligocene). Node 18 has moderate to high probability for at least one vicariant event separating the Gourits and Robertson catchment populations during the late Pliocene.

Ancestral ranges for nodes 19 and 20 are not well-resolved in the analysis. The AFR population diverged in the early Pliocene (Node 20; possibly at the time of major uplift), then towards the late Plio-Pleistocene there were multiple divergences between the Palmiet, Breede-West and Eerste populations. Moderate to high support was found at Node 21 for at least one vicariant event separating Eerste and Breede-West populations in the Pleistocene.

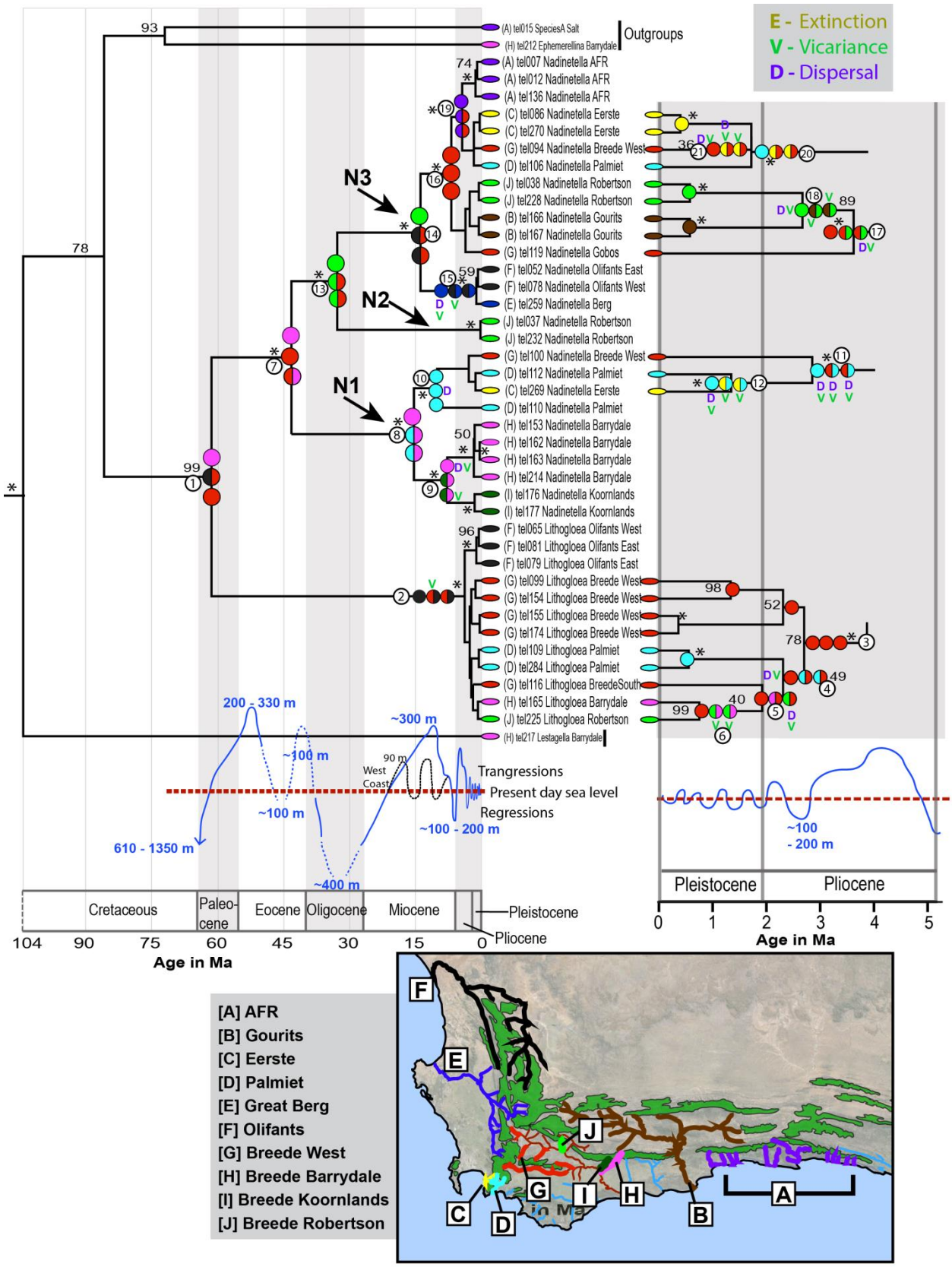


Figure 5.5. See legend overleaf

Figure 5.5. "Nad/Lith Clade" RASP analyses, coloured circles at nodes show ancestral ranges, with colours corresponding to the map inset. Sea level changes shown at bottom of phylogeny (modified from Siesser and Dingle, 1981). Curve maxima on sea level changes represent elevation in metres compared to current sea levels (i.e. uplift in not factored into these values). Differences in sea level changes between West and East coasts are depicted by an added curve in black (representing west coast).

5.3.5 Dispersal, Vicariance and Extinction Time-Events (Figure 5.6)

The analysis of time events give similar dispersal, vicariance and extinction results for the three analyses (BBM, S-DEC, S-DIVA) for each dataset. This graph shows the overall dispersal, vicariance and extinction events in all analyses, without considering the probability of the event.

The highest curves for dispersal and vicariance coincide with the minor Miocene uplift, and the major Pliocene uplift (green arrows; Figure 5.6). The late Cretaceous had various transgressions and regressions of sea levels (Table 5.1) resulting in vicariance and dispersal events. In addition, scarp retreat is also a contributing factor (see Discussion) to migration and dispersal. The "Ephem Clade" and "Lest Clade" show similar patterns, with two large events causing most of the diversification. "Ephem Clade" appears to have been more affected by the Pliocene uplift, whereas "Lest Clade" was more affected by the Miocene uplift.

The "Nad/Lith Clade" events are more spread out, possibly more responsive to changes in its associated vegetation habitat (*Wardia* moss) distribution and evolution. Dispersal seems broadly related to sea level transgressions, warmer and wetter conditions during low transgressions may have facilitated dispersal of *Wardia* moss, and ultimately dispersal of *Nadinetella* populations.

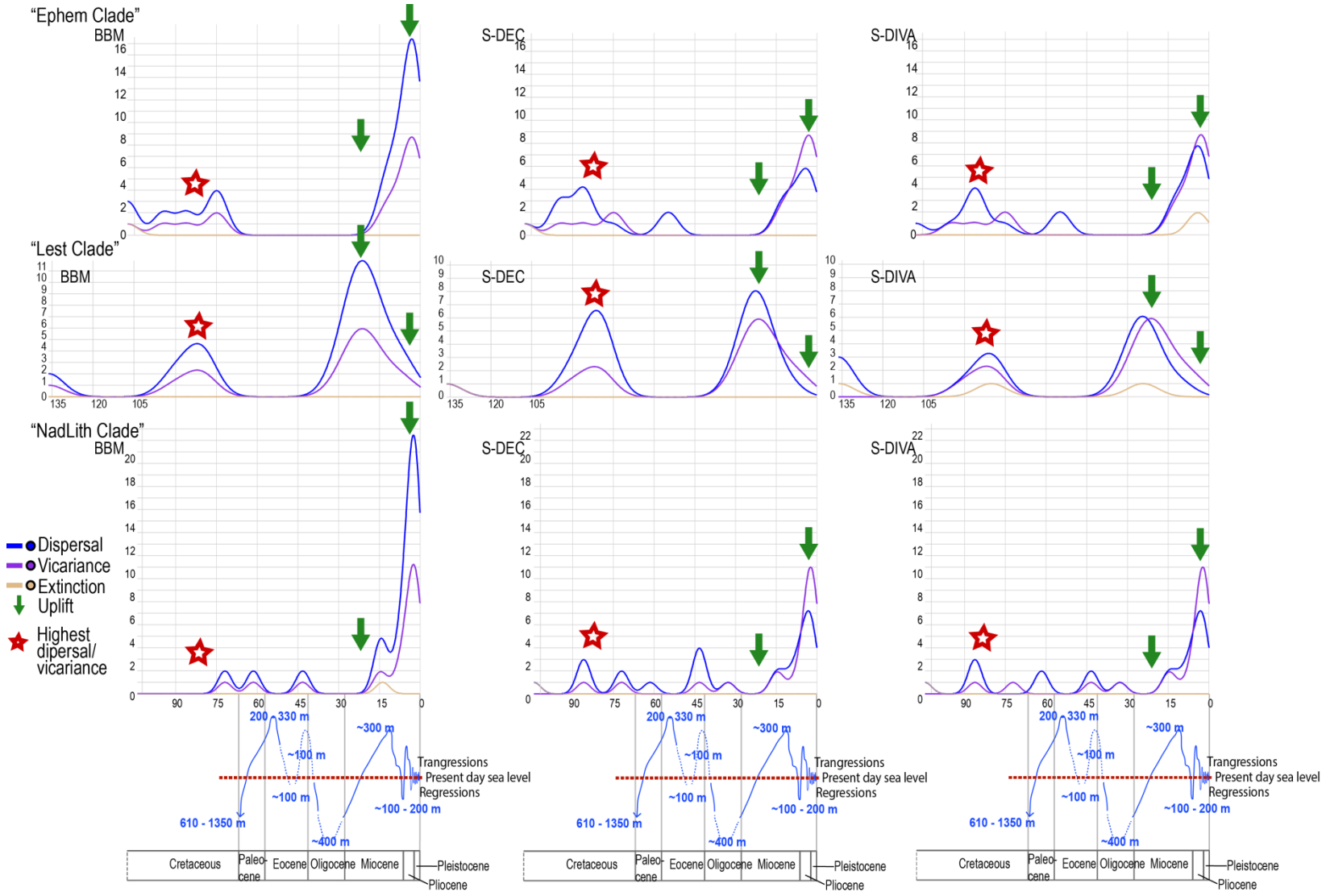


Figure 5.6. See legend overleaf.

Figure 5.6. Time-events algorithm graphs showing dispersal, vicariance and extinction events from all analyses (BBM, S-DEC and S-DIVA) between the different clade analyses. Timing and magnitude of sea level changes are depicted at the bottom of the graphs (modified from Siesser and Dingle, 1981). Green arrows represent timing of uplift events in the Miocene and Pliocene. Red star represents timing of earliest and highest number of dispersal and vicariant events. Trends are similar between analyses for each clade. Note that these graphs included all events estimated in the analyses, regardless of probability.

5.4 Discussion

Due to a lack of fossil evidence, this study used only a single calibration point based on a reliable date of continental fragmentation, therefore these results warrant some caution against using any widespread conclusions derived from the divergence time estimations as there are potential pitfalls of using single calibrations points (Rutschmann, 2006).

The results presented here mirror that of other studies in terms of distribution and lineage dating, for example, there is a clear biogeographic trend between the Teloganodidae (particularly *Lestagella*) and the velvet worm, Onycopohora (Daniels *et al.*, 2009, McDonald and Daniels, 2012) following remnant Afrotemperate forests throughout the GCFR. This may be a result of the relictual, wet montane-forest habitat that would have provided a good buffer against drier conditions for numerous other nemophilistic organisms, as will be explained in more detail in section 5.4.2.

Comparison with the BEAST analysis using the molecular clock based on divergence rates retrieved from the literature (COI: 3.54 %, 16S/COI: 2.69 %; Papadopoulou *et al.*, 2010) gives a younger age for the teloganodid lineages (Appendix 5C). This is a common outcome with this method, particularly for older lineages. Global molecular clocks can problematic in terms of accuracy because of the differences in rate variation between branches which is not considered (Rambaut and Bromham, 1998; Buckley *et al.*, 2001; Heads, 2005). In this study, the published molecular clock favours a rapid radiation of South African teloganodids during the Plio-Pleistocene, rather than a slower, long-term isolation of Miocene origin as indicated using the tectonic split calibration.

Other comparative phylogeographic research implies that early Miocene events (climatic and tectonic) have been major cladogenic drivers for species diversification among a wide range of South African taxa based on inferences made from phylogenies and biogeography (Skelton, 1994; Matthee & Flemming, 2002; Gouws *et al.*,, 2004) and molecular clocks, and/or fossils (Daniels *et al.*, 2006, 2007; Tolley *et al.*,, 2006, 2008). The invertebrates show a trend towards long-term population isolation, with the initial lineage divergences reported from the middle Miocene, using continental fragmentation calibration points between Africa and Australia (Daniels *et al.*, 2009) and molecular clocks (de Jager and Ellis, 2013); and Early Pliocene, inferred using molecular clocks (Daniels *et al.*, 2001; Daniels, 2003; Daniels *et al.*, 2006; Pereira-da-Conceicoa *et al.*, 2012). Within-population cladogenesis has been reported from the Plio-Peistocene to late Pleistocene, mainly in response to climatic glacial and interglacial cycles, inferred from fossil evidence and/or molecular clocks (Price *et al.*, 2007, 2010; Pereira-da-Conceicoa *et al.*, 2012; McDonald and Daniels 2012) and inferred using biogeographic and phylogenetic patterns compared with continental separation (Liu *et al.*, 2013).

Comparing the results based on the published molecular clock calibration (16S/COI: 2.69 %), many of the cladogenic events within the South African Teloganodidae can be aligned with events in the Plio-Pleistocene period, giving younger ages of speciation. Rapid radiation could have occurred due to the series of glacial and interglacial cycles and a shift in the ecological niches (‘empty’ niche hypothesis; Goldblatt, 1978) during the change to winter rainfall. If these results were accurate, the Madagascan connection could then possibly be explained by a long-distance dispersal event (Waters *et al.*, 2013) during the Paleogene period (Appendix 5C). As there is only one genus occurring in Madagascar, this is possible that a single dispersal event of the *Manohyphella* ancestor (or other lineages may have since gone extinct). This dispersal would have had to cover the 430 km distance between the African mainland and Madagascar. Strong, sporadic surface currents that occurred during the Paleogene period (due to storms) from the northeast of Mozambique and Tanzania towards Madagascar (Ali and Huber, 2010) may have transported the *Manohyphella* ancestor the long distance to Madagascar, as has been suggested for water beetles (Jackson, 1956) and Heptageniidae (Vuataz *et al.* 2013). Flying (without aid of wind currents) and rafting (exposure to saline environment combined with the mayfly’s short lifespan) are unlikely scenarios for dispersal.

However, the results presented in this chapter favour the long-term isolation scenario, which has been reported in other aquatic invertebrates (Skelton, 1994; Matthee & Flemming, 2002; Gouws *et al.*, 2004; Daniels *et al.*, 2006, 2007; Tolley *et al.*, 2006, 2008), with cladogenesis commencing in the Late Cretaceous /early Tertiary, and major cladogenesis in the Miocene and Plio-Pleistocene. Motivation for the older divergence estimates are supported mostly by the *Lestagella* clade ("Lest Clade"), where the ancestral range suggests a close, early Tertiary link between the distant catchments, Olifants and Keiskamma. This link could be explained by an ancient inland connection with the Orange River palaeo-drainage system which only occurred at this time. Geographically isolated distributions of *Lestagella* are strongly correlated with fragmented, relict Afrotemperate forests (where fragmentation occurred during Miocene uplift, isolating the Keiskamma area from the southwestern Cape), and this strong correlation would have unlikely been the case in a younger lineage, as the forests would already have been fragmented. There are studies on other organisms showing that the Amathole Mountains (location of the Keiskamma catchment) acted as a region for lineage isolation during the Miocene uplift (Skelton, 1994; Stevens, 2008; Daniels *et al.*, 2009).

Inferences regarding the ancestral reconstruction of the deeper nodes should be made with caution, while BBM infers binary states, hence the single geographic range estimates, inferred ancestral states from S-DIVA analyses can become unreliable if there have been extinctions, local speciations or the formation of widespread species within the group (Nylander *et al.* 2008). While S-DEC has similar assumptions to S-DIVA, it allows peripatric speciation which results in one descendant occupying the area of speciation (usually peripheral to the main population) whereas the other inherits the entire range and (unlike S-DIVA) does not permit vicariance scenarios in which each daughter occupies more than one area (Ronquist and Sanmartin, 2011).

5.4.1 Deep time reconstruction

Many of the current river courses of Africa reached their present routes from the Miocene to Pleistocene (Goudie, 2005). Prior to this, drainage systems had undergone numerous changes as a result of various events of uplift and climatic changes through time, which would have allowed the faunal components of these systems opportunity for migration, dispersal,

vicariance and extinction. Following these palaeo-drainage courses through time provides insight to possible paths taken by different fauna.

Knowledge of changes in climate and vegetation are imperative to this goal, and while some conditions would imply an "obvious" route for fauna, certain climatic events or vegetation could have impeded dispersal. It is therefore essential to incorporate as much information as is possible to create the most likely reconstruction. This gets progressively more difficult with deeper time reconstructions as the resolution decreases considerably due to less information that is available. It is still possible to attempt to hypothesize and describe plausible reconstructions with the data at hand.

A proposed reconstruction for the deep-time nodes summarising the results from the "Afr/SAm Group" and "Afr/Mad Group" (Figure 5.2) is presented in Figure 5.7 (a – f). After the P-T extinction, life began to rediversify in the early Triassic (Meyen, 1987; Eskov, 2002, Rasnitsyn and Quicke, 2002). The ancestral clade of the Teloganodidae, Ephemerythidae and Melanemerellidae occurred on Pangea, possibly widespread (Figure 5.7 a). In the early to mid-Jurassic, a large band of hot, arid desert extended from South America to Antarctica (including the region of which is now Madagascar). This created a barrier between northern Africa/ northern South America lineage and the southern Africa lineage, possibly extending through southern Antarctica and up to India on the outskirts of the desert (Figure 5.7 b).

Towards the late Jurassic (ca. 160 ma, as Lemuria began to separate from Africa), the Asian population may have become isolated from the South African lineage. Palaeoclimates (Scotese, 2009) indicate that eastern South Africa experienced a dry period which may be the cause of this barrier, leading to the separation of Asian and South African lineages (Figure 5.7 c). In the early Cretaceous, a more temperate to humid climate returned to the eastern South African region, possibly due to the Indian Ocean opening up from north to south of Lemuria (on the west coast of Madagascar), as it was separating from Africa ca. 140 Ma. The South African lineage would have then been able to disperse north easterly (possibly via the western rim of what is now Antarctica towards Madagascar (then warm and with a wet climate); or via a longer route on the outer, eastern edge of the desert in Antarctica; Figure 5.7 d). *Manohyphella* today is only found in remnant mountainous forests on the West Coast of Madagascar. After the *Manohyphella* ancestor colonised Madagascar, some 140 Ma, glacial periods would have resulted in severe arid conditions at lower elevations, driving the teloganodid ancestor and many other species to move to higher elevations towards more

humid areas (the Watershed hypothesis, Wilme *et al.* 2006). Other species distributed in lower elevation watersheds would have become trapped in arid pockets, and adapted and diversified in isolation. The *Manohyphella* ancestor may have also followed the Montane Endemism hypothesis (Wollenberg *et al.* 2008), where some populations of species that were broadly distributed during glacial periods became isolated on mountain tops during warmer interglacials. This also ties in with the Riverine Boundary hypothesis (Goodman & Ganzhorn, 2004) proposing that the river systems in Madagascar restricted gene flow therefore causing isolation between populations. *Manohyphella* consists only of three species and has apparently not undergone significant species radiations, unlike the South African lineage which radiated considerably in the GCFR. Many other taxa have thrived in Madagascar, radiating into numerous endemic taxa (e.g. lemurs: Goodman and Benstead, 2005; Apidae: Chenoweth and Schwarz, 2011; Isoptera, Fungus growing termites: Nobre *et al.* 2010; Heptageniidae: Vuataz *et al.* 2013; Canthonini dung beetles: Wirta *et al.* 2010). However there are also reports of taxa colonising Madagascar without significant species radiations (Lepidoptera, *Colotis*: Nazari *et al.* 2011; Nephilidae: Kuntner and Agnarsson, 2011; Dytiscidae: Bukontaite *et al.* 2015).

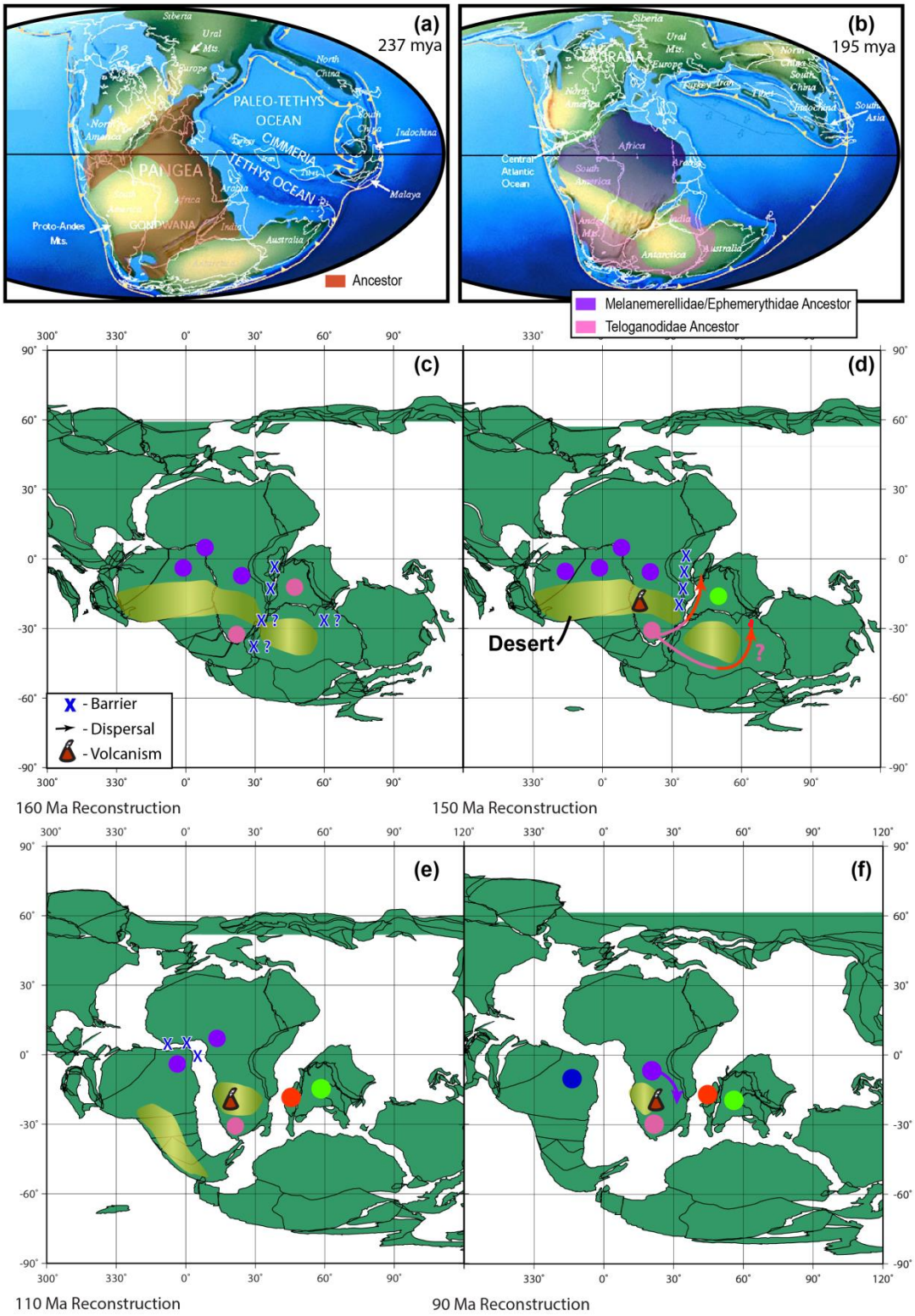
When Lemuria detached from the main gondwanan landmass, the lineages continued to diversify (Figure 5.7 e). The South American and northern African lineages would have separated when South America separated from Africa in the mid-Cretaceous (ca. 110 Ma), at which time there was considerable volcanism to the south, preventing a dispersal of the Ephemerythidae ancestral clade to the southern African clade and vice versa (Figure 5.7 e).

From ca. 90 Ma when Gondwana was well separated, the ancestral lineage of Melanemerellidae remained on South America, with an Ephemerythidae ancestor in northern Africa. Meanwhile Madagascan and Asian Teloganodidae ancestors remained on the Indo-Madagascan subcontinent and South African Teloganodidae in South Africa (Figure 5.7 f).

Although the exact distributions of Ephemerythidae in Africa are largely unknown due to poor sampling, it appears that the family occurs from West to East Africa through Central and southern Africa, with a South African lineage occurring in the more tropical north eastern area of South Africa. Crass (1947) indicates an Ephemerythidae population as far south as the Kwazulu Natal Province, although there are no specimens available to confirm this report, and specimens have not been found on subsequent sampling expeditions (Chapter Two, Figure 2.1, 2.2). East, western-Central and West Africa share closely related Odonata taxa

(Clausnitzer, 2003), possibly due to continuous forest that stretched across Africa between the Oligocene and Miocene (Lovett, 1993; Roy *et al.*, 1997; Burgess *et al.*, 1998). It is possible that the northern Afrotropical African Ephemerythidae lineage dispersed south on the eastern side of the continent after the break-up of Gondwana, once the period of desertification and volcanism subsided, and following the widespread tropical forest (Figure 5.7f).

The higher level reconstruction is based on the evidence available. Important lineages for the ancestral state analyses are inadequately represented by only one member each, making these deeper nodes ambiguous, and the inferences somewhat speculative. Ephemerythidae, Melanemerellidae and particularly the Asian Teloganodidae, where material from only one representative for the diverse Oriental Teloganodidae was available, may explain the apparent lack of affinity between the Malagasy and Indian faunas. Including more samples of Indian and South East Asian Teloganodidae may help to put the relationship between Madagascar and the Oriental realm. Possibly the Indian fauna may prove to have a closer affinity to Madagascar than the African fauna, but it is not possible to make any assumptions about the Asian connection here as there is not enough information available.



*****Purple dot = Melanemerellidae/Ephemerythidae ancestor; Pink dot = Teloganodidae ancestor; Green Dot = Asian Teloganodidae ancestor; Orange dot = Madagascan Teloganodidae ancestor. Map (a) and (b) derived from Scotese (2009) and (c-f) from ODSN Plate Tectonic Reconstruction Service website

Figure 5.7. See legend overleaf

Figure 5.7. Possible hypothesis for the Jurassic/Cretaceous distributions and divergences of the common ancestors of Teloganodidae, Melanemerellidae and Ephemerythidae; based on available evidence, Possible ancestral ranges during (a) the Triassic; (b) Jurassic, where growing desert band prevents north-south dispersal; (c) Late Jurassic, desert band and Lemuria separation causes barrier, isolating Asia and northern Afrotropical Africa; (d) Late Jurassic/Early Cretaceous, volcanism prevents north south dispersal, apparent dispersal from South Africa to Madagascar, possibly via the north western rim of Antarctica, (e) Cretaceous, Ephemerythidae/Melanemerellidae diverge as South America separates from Africa, volcanism prevents north-south dispersal, Madagascan and Asian lineages distinct and continue diverging, (f) after Mid Cretaceous, northern Afrotropical African Ephemerythidae lineage disperses towards the south as desert recedes and forests become widespread. See text for more detailed explanations.

5.4.2 Southern African Teloganodidae Reconstruction

As a consequence of tectonic events, even the most subtle faulting can magnify effects on rivers and lakes, and subtle changes in relief can cause major changes to aquatic habitats (Cotterill and de Wit, 2011). It has been suggested that the break-up of Gondwana led to an up-warped continental margin of southern Africa (de Swardt and Bennett, 1974). This created two main drainage systems, a short and vigorous coastal system, and a more gentle sloping interior drainage – each operating on opposing sides of the retreating escarpment (Figure 5.8a). During the Cretaceous, the escarpment was relatively close to the coast, with the African erosional phase beginning in the Late Cretaceous, and over a period of 100 Ma, to the Miocene (Moon and Dardis, 1998). This ultimately resulted in large scale planation of the African surface and scarp retreat causing the inland retreat of the Great Escarpment (Partridge and Maud, 1987; Moon and Dardis, 1988) (Figure 5.8a). The shorter and better-watered coastal drainage progressively captured more of the inland drainage at an uneven pace, as erosion was dependent on structural weaknesses in the escarpment (e.g. escarpment remnant Amathole Mountain range, Figure 5.8a). The erosional phase in the southern Cape is more complicated with the Cape Fold Belt (CFB), where individual folds determined the direction of drainage. Uplift in the Miocene warped the African surface (and interrupted this erosional phase), giving the margins a slight westerly tilt, which rejuvenated westerly flowing drainage of the interior (Dingle and Hendey, 1984).

In general, sea level transgressions were believed to be gradual, while regressions more rapid, indicating that there would have been a rapid transition in environmental conditions from warm and wet to harsher cool, dry conditions (Vail *et al.*, 1977). The regressions possibly would have had a dramatic effect on the flora and fauna (Hendey 1983), and so, palaegenic zones (such as isolated pockets of montane forests) would have provided a necessary sanctuary for organisms.

Lower sea levels during regressions expanded wide areas of the coastline, especially on the Agulhas Bank (to the south of the Overberg) and Outeniqua regions (Hendey, 1983). The mouth of the present Breede River extended from 50 to 200 km during the regression of the last glacial maximum (LGM) when the sea level dropped to ca. 120 m below its current level (Dingle and Rogers, 1972). The magnitude of most sea level regressions during the tertiary is largely unknown, with some evidence suggesting that the most substantial drop during the Cenozoic was the Oligocene regression which was several hundred metres below current seal level (Siesser and Dingle, 1981), increasing the coastal lowlands by 300 % or more (Hendey, 1983).

Sea level fluctuations also caused changes in river courses throughout the Cenozoic. It has been suggested, for example, that there was a progressive northward shift in the lower course of the Berg River between the Oligocene and Pleistocene (Figure 5.1).

Southern African Teloganodidae have a South African origin from the middle to late Cretaceous (Table 5.1), which seems to emanate from the Olifants catchment. Divergence between the main (generic) lineages of the Teloganodidae occurred throughout the Cretaceous (with the first split between fringed (*Lestagella*) and non-fringed lineages), with extant lineages established by the mid-Tertiary, and modern species distributions are a result of drainage evolution within the GCFR region. Various rifting and uplifting events were prevalent during the tertiary, in the Oligocene and especially during the Miocene (John, 1986) and resulted in many diversifications within the Teloganodidae, and has been reported in other aquatic groups (fish (Cyprinidae): Skelton, 1994; crabs (Potamonautidae): Phiri, 2014).

The analyses indicate that *Manohyphella* (Madagascar) and the Western Cape non-fringed teloganodids were linked before the separation of Madagascar from Africa. This implies that the ancestral clade may have occurred further north or had been more widespread throughout

southern Africa (south of the desert band indicated in the reconstruction shown in Figure 5.7). This would have allowed for dispersal to what is now Madagascar when the desert retreated, while it was still part of Gondwanaland.

This could be explained by a hypothesized Teloganodidae ancestral distribution at least in the palaeo-Orange River in the early Cretaceous, as it was one of the largest drainage basins of South Africa at the time (Reyment and Dingle, 1987). Although teloganodid mayflies have never been recorded from the Orange River or from the north-eastern parts of southern Africa, it is possible that the family migrated to the wetter west coast (to the Cape Fold Belt) via the Orange-Olifants River connection in the Late Cretaceous /early Tertiary during arid inland conditions. In the Late Cretaceous (ca. 95 MA), sea level transgressions were experienced in the West, while regressions were experienced in the South and East (Reyment and Dingle, 1987), which may have encouraged western migration. Separation of the Falklands Plateau would have caused drastic climate change, particularly affecting rivers on the south west coast. Increased volcanism in West and Central Africa (Goudie, 2005) would have prevented further north-south dispersal. The K-T extinction boundary would also have had a substantial effect on the biota of the region, the CFB possibly providing sanctuary from unfavourable conditions. The more southerly drainage of the paleao-Orange River at the time would have meant that the southwestern Cape would have been a substantial source of fresh water during the cool, dry regressive periods of the early Tertiary (Reyment and Dingle, 1987; Table 5.1), possibly facilitating the radiation of the Teloganodidae in the GCFR.

The possible occurrence of Teloganodidae within the palaeo-Orange River and its tributaries while connected to the Berg and Olifants rivers (during sea level regression) would explain the link between the Olifants and geographically distant Keiskamma/AFR catchments (Figure 5.1), and further explain why there is no evidence for an easterly dispersal through the Cape Fold Belt via the various catchments. The process of scarp retreat between the Late Cretaceous and the Oligo-Miocene would have allowed for watershed / drainage divide migration. This process did not necessarily cause a species range expansion, but is rather a situation where the drainage components have changed from one system to another (Figure 5.8b).

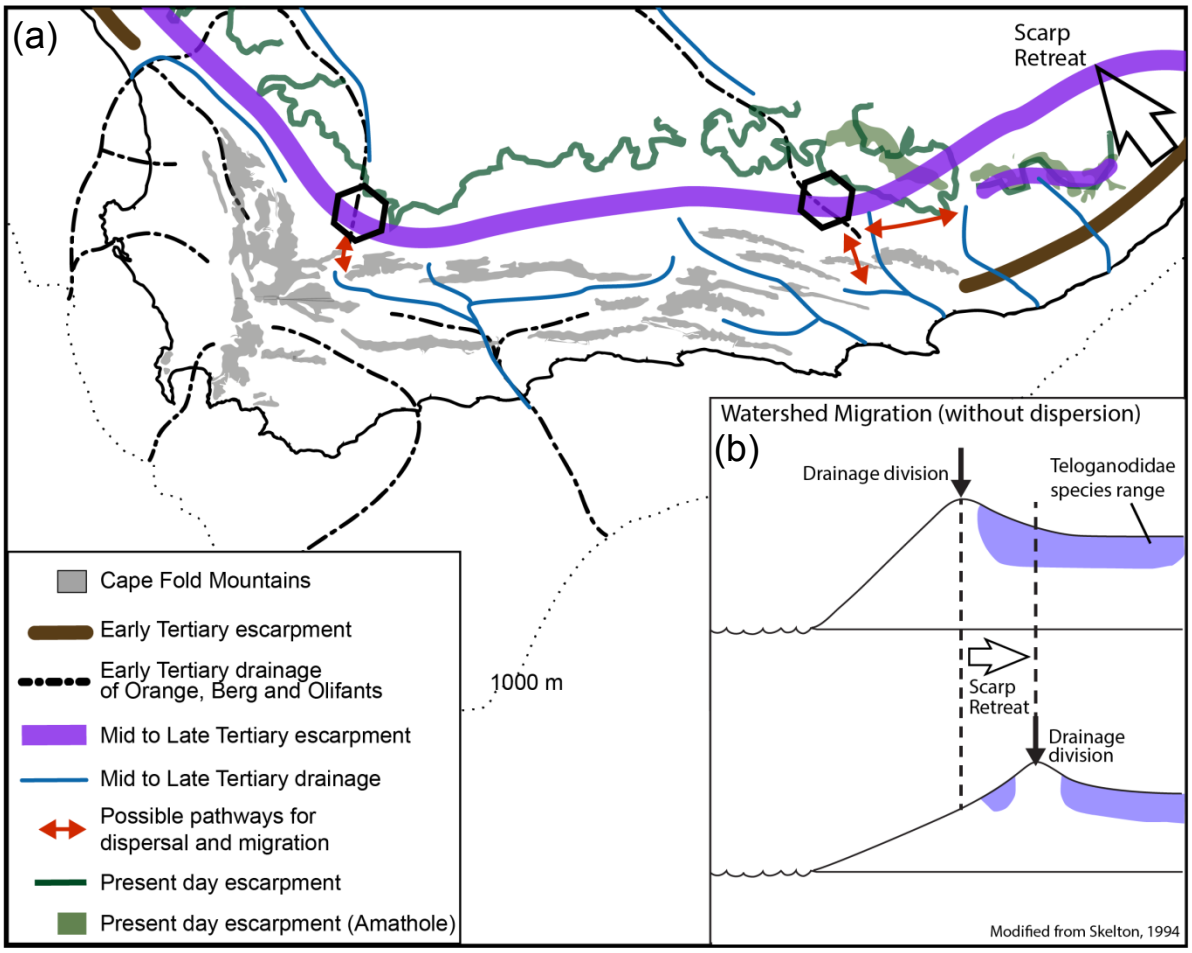


Figure 5.8. Escarpment retreat in the Tertiary and watershed migration, (a) Early Tertiary escarpment was located near the coast (brown line), and moved inland with erosion or "scarp retreat" (purple line), the Amathole Mountains are remnant of the Mid- to Late Tertiary. 1000 m isobaths illustrated by dotted line. Note that drainage on the coastal side increases as the escarpment retreats, hexagons and red arrows depict possible areas where watershed migration may have occurred, (b) diagram of watershed migration redrawn from Skelton (1994) [Permission received 09/01/2016]. Species transferred to opposing drainage system and fragmented by scarp retreat without dispersion (range expansion taking place). Extinction in this process could mean the complete transfer of species from one system to another (e.g. from palaeo-Orange tributary to coastal rivers).

This process may have been the cause for the ancestral range (RASP) results observed in *Lestagella* and Species A. Coastal rivers (in this case, the Keiskamma and AFR; Figure 5.1, red polygons) would have tapped into the inland Orange tributaries (which were also connected to the Olifants and Berg rivers during severe sea level regressions in the Late

Cretaceous and Oligocene; Figure 5.1) via scarp retreat (Figure 5.8a,b). The scarp retreat gradually eroded the escarpment further away from the coast, thereby shrinking the margins of the Orange River interior drainage, progressively transferring the head water drainage to the adjacent coastal rivers (Figure 5.8a). Geomorphological evidence places an Orange River tributary near the AFR/Keiskamma region in the early Tertiary (Partridge and Maud, 1987), which would have allowed the ancestors of *Lestagella* (L2/L3) and Species A to migrate to the Keiskamma and AFR respectively, yet keeping its link with the Olifants system. Planation of the African surface at this stage had transformed the land into a fairly flat landscape between 500 – 700 m asl (Linder, 2003). The lack of vicarious mountain ranges, combined with the widespread presence of forests and a humid climate may have facilitated this drainage divide migration (Figure 5.8) and dispersal (Partridge and Maud, 1987; Franceschini, 2003) of the Teloganodidae to the East (the Keiskamma and AFR areas).

In the Late Cretaceous, the Berg River flowed out north-westwards via a longitudinal valley that paralleled the Olifants (Figure 5.1) on the West Coast of South Africa. During the early Tertiary, the most significant drainage change affecting the fynbos region occurred, this being the southerly diversion of the Upper Orange/Vaal river system. This event caused drainage of most of the South African inland plateau to be directed into the southern Orange Basin off the Western Coast, the Olifants and Berg Rivers joined into a single river system that discharged into the Orange Basin south of Childs Bank (Figure 5.1; Dingle and Hendey, 1984).

This southern mouth of the Orange River remained for ca. 40 Ma until the end of the Oligocene (where the Upper Orange/Vaal system cut the Cape Canyon in the continental shelf off the southwestern Cape during severely low sea-levels; Figure 5.1). The Olifants and Berg rivers would have been tributaries of this system in the low sea-level Oligocene, with the confluence near the Cape Canyon, south of Childs Bank (Hendey 1983), which would have allowed dispersal between catchments. The *Lestagella* ancestor probably entered the Olifants and Berg Rivers through this (currently off-shore) confluence. The Berg River then shifted to a westerly direction in the early Miocene (Hendey, 1983), isolating this lineage (*Lestagella* L1). *Lestagella* L2/L3 were still linked in the Orange-Olifants connection, only disconnected in the mid to late Miocene. The Olifants River separated from the Orange when uplift caused the capture of the Olifants-Orange River northwards (Partridge and Maud, 1987), thereby separating the L2 and L3 lineages.

The *Ephemerellina* clade (*Ephemerellina*, Species A, Species B and Species C) and *Nadinetella/Lithogloea* clade entered the Cape Fold Belt via the Olifants system (seemingly from the Orange River). The ancestor of Species B and Species C dispersed to the Palmiet catchment via the Berg river on the west side of the mountain (Species B has been recorded from the Berg River, unfortunately there were no samples available for analysis) and to the Breede system on the east side of the mountain range to the Barrydale area. These two lineages split in the Late Cretaceous, possibly during a major transgression (ca. 70 Ma on south east coast, up to 367m above current sea level), where populations would have been isolated and forced deep into the mountains. *Ephemerellina* (E1/E2) appears to have been isolated in the Olifants system, E1 dispersed to the Berg River, possibly during the Oligocene when sea levels were low and Olifants / Berg catchments were connected. *Ephemerellina* E2, *Nadinetella* and *Lithogloea* followed the same route as the ancestor of Species C to the Breede catchment, which suggests a possible corridor for dispersal between the Olifants and Breede during the mid-Tertiary.

Extant *Nadinetella* is strongly associated with an endemic, southwestern Cape moss, *Wardia hygrometrica* (Wardiaceae). This moss is restricted to Afromontane forested streams (Mwafongo, 2003) much like the forested ravines which the Teloganodidae inhabit. It is possible that *Nadinetella* adapted with this vegetation habitat (or close ancestor), as *Wardia* moss is thought to have been widespread along with the ancient Afrotemperate forests in the southwestern Cape (Mwafongo, 2003). This Suggests either a Pleistocene origin for *Nadinetella* during the expansion and contraction of forests occurred during glacial cycles, or an older origin when forests were widespread in the early to mid-Tertiary. As the latter coincides with the origin of *Wardia*-living *Nadinetella*, it is likely that the moss diverged in the early to mid-Tertiary. The genus *Ephemerellina* is associated with endemic sedge *Isolepis digitata* (Cyperaceae) which is found in swift flowing currents, though the strength of this association is uncertain as the nymphs have (occasionally) been recorded on whatever vegetation type is attached to rocks in fast-flowing currents and waterfalls (in the southwestern Cape, this happens to be mostly only *Isolepis* and/or *Wardia*). *Isolepis digitata* is thought to have originated in the mid to late Miocene (Verboom *et al.*, 2009), which is too young for the E1/E2 *Ephemerellina* clade. The predatory behaviour of the E1/E2 clade nymphs could indicate that the exact type of vegetation is not critical, rather the general rheophilic vegetation containing prey items, although major cladogenesis of the catchment

lineages within the E2 clade initiates in the mid to late Miocene, possibly in response to dispersal of *I. digitata*.

River piracies and captures have been shown in the drainage evolution of the Cape Fold Mountains (Figure 5.1, denoted by red arrows) and using fish distributions (Barnard, 1936; Skelton, 1994). Examples of such piracy are seen between the Berg River and Breede River via the Tulbagh valley, where the Little Berg River (a tributary of the Great Berg River) has penetrated the Worcestor-Tulbagh valley (Barnard, 1936). Various captures of the Gourits headwaters by the Breede tributaries are evident (Barnard, 1936), providing opportunity for transferral and dispersal of aquatic organisms (this is seen in *Sandelia* and *Galaxias* fish (Skelton, 1994).

Lestagella L1 probably entered the Breede system via the Berg River during a marine transgression (wetter climate) in the Late Cretaceous/early Tertiary as a result of this river piracy. Table 5.1 shows that there were vast changes in the tectonics, climate and vegetation throughout this time, allowing for lineage divergence and probably extinctions. It is likely that the Cape Fold Belt (CFB) would have buffered these ancestral lineages from unfavourable environmental conditions (Picker and Samways, 1996).

A land bridge connecting the Cape Peninsula to the Hottentots Holland Mountain range is said to have existed from the Cretaceous, continuously being eroded away into the Tertiary (Harrison and Barnard, 1972; Wishart and Hughes, 2001, 2003). Only the genus *Lestagella* is found on the Cape Peninsula and appears to have become isolated here in the early to mid-Miocene. It is possible that with the Miocene marine transgression (on the West Coast, see Figure 5.4), the eroded land bridge was then submerged, therefore isolating the Cape Peninsula population from the mainland, which has subsequently not dispersed from this region. This distribution pattern seen in *Lestagella* has been observed in many of the taxa that are endemic to the Cape Peninsula (invertebrate fauna: Picker and Samways, 1996; Diptera, Blephariceridae: Wishart and Hughes, 2001, 2003; Plecoptera, Notonemouridae: Stevens, 2008; Onychophora, Peripatopsis: Daniels *et al.*, 2009; McDonald and Daniels, 2012; Megaloptera, *Platychauliodes*: Liu *et al.*, 2011; Megaloptera, *Taeniochauliodes*: Liu *et al.*, 2013; Decapoda, Potamonautidae: Phiri, 2014).

Prior to the proposed land-bridge submersion, there is evidence that the *Lestagella* clade dispersed from the Olifants to the Breede-West and Eerste catchments. None of the other

genera (non-fringed clade) had dispersed this far South by this time, which may account for the absence of these genera on the Cape Peninsula. The non-fringed teloganodids also have specific vegetation-associated habitats, which may have slowed the progress of dispersal (populations are limited by presence of vegetation habitat), while *Lestagella* occurs under rocks in riffles, thus making dispersal to new rivers less complex.

Changes in the river systems of the Overberg (Breede-South) and the Outeniqua (AFR) were probably less dramatic and it is thought that the large rivers in these areas (Breede and Gourits) were likely to have been confined to their present valleys throughout the Cenozoic. The Breede and Gourits rivers were not linked during off-shore during regressions, their courses were kept separate by the Agulhas Arch (Figure 5.1; Dingle et al 1983). Ultimately, the Cape Fold Belt was fairly stable in terms of rivers and watershed in the Cenozoic, which possibly led to the expansion of the teloganodids in this area. There was more water available and the mountains acted as an environmental buffer against any unfavourable conditions.

Forests are thought to have dominated southern Africa prior to the middle Miocene (Table 5.1), with a reduced forest distribution into the Pliocene in a response to global climate change to cooler and drier conditions (Linder *et al.*, 1992; Linder 2003). Uplift in the early Miocene and Pliocene are thought to have aided in forest fragmentation, and together with cooler climates pushed forests into montane ravines. The global glacial cycles in the Pleistocene would have had a dramatic effect on the distribution and abundance of forests (deMenocal, 1995; Eeley *et al.*, 1999; Partridge *et al.*, 1999; Fattorini, 2007).

Uplift and climate change consequently triggered allopatric speciation of forest biota (Jansson, 2003; Bellstedt and Edwards, 2004; Stevens, 2008; Daniels *et al.*, 2009). Speciation within various invertebrates are thought to have a strong correlation with these fragmented forests, which represent typical palaeogenic zones (upper-reach forested streams, riverine forests and caves), 52 % of taxa living in the Cape Peninsula forests are endemic (Picker and Samways, 1996). Mountains have been suggested as the most suitable topographical refugia for the preservation of relict fauna in periods of climatic change (Endrody-Younga, 1986).

Prior to the minor uplift of the early Miocene (Figure 5.1, Table 5.1), the continued scarp retreat would have opened up the area behind the Gourits catchment (Figure 5.1) by the mid-Tertiary, possibly facilitating dispersal to this catchment (Partridge and Maud, 1987; Skelton, 1994). The sub-tropical climate and widespread forests would have allowed *Lestagella* to

disperse west to the Gourits catchment (possibly during the Early Miocene climatic optimum; Zachos *et al.,* 2001). Any populations in the small, coastal AFR rivers would have been drowned during the Miocene sea level transgression. The Keiskamma lineage of *Lestagella* then appears to have been isolated after the minor uplift in the early Miocene (ca. 200 m, Figure 5.1), possibly due to fragmentation of forest due to the uplift, which has been seen in other organisms (aquatic organisms – Notonemouridae: Stevens, 2008; Anabantidae and Cyprinidae fish: Skelton, 1994; forest – Daniels *et al.,* 2009).

Certain areas along the south-east coast still have residuals of the original Escarpment (that were not as extensively eroded during scarp retreat), such as the Tandjesberg-Winterberg-Amathole range in the Eastern Cape (Wellington, 1928) which includes the Keiskamma catchment with one of the *Lestagella* lineages. According to Skelton 1994, these residual landscapes provided sanctuary for relict species of fish, *Sandelia bainsii* and *Barbus trevelyani*. This uplift event also seemed to cut off any connection with the Olifants River in Species A, which suggests that there was a possible link prior to uplift, between Species A AFR and Olifants populations, also seen in Onychophora (velvet worms: Daniels *et al.,* 2009; McDonald and Daniels, 2012). Daniels *et al.,* 2009 found a close phylogenetic connection between the Cederberg mountains (where the Olifants River is located) in the western Cape and the southern Cape (AFR area) and suggested that these Afrotemperate forest patches may have been linked. This is analogous with Species A, which shows the same close phylogenetic relationship. These authors also suggest a historical link between the southwestern Cape Afromontane forests and the Cape Peninsula Afromontane forests, forest patches that are currently allopatric. Their observed areas of endemicity align with that of the Teloganodidae which include the Cape Peninsula, Agulhas plain (Overberg region including Breede-South), Langeberg region (Breede-East) and the coastal forest areas of the southwestern Cape (AFR). These areas of endemicity are broadly similar to those detected for GCFR flora (Linder, 2003).

The Keurbooms River (AFR catchment) is the only system that has penetrated the coastal arc of the Outeniqua-Tsitsikamma ranges (Skelton, 1994). This connected with tributaries of the Gourits system and explains the presence of some Gourits *Lestagella* populations in the AFR region during the late Mio-Pliocene. The slender red fin minnow, *Pseudobarbus tenuis*, follows a similar trend (Skelton, 1994). Coastal and offshore connections between adjacent rivers would have further facilitated dispersal of the Teloganodidae between otherwise

isolated drainage basins, namely *Lestagella* populations within the AFR and to the Gamtoos catchment.

There is a remarkable distinction between the Olifants-East and West rivers in the *Lestagella* group, where divergence is suggested to be in the mid to late Miocene. The sampling sites of these two catchment areas are only 10 km apart and separated by a 300 m relief. This similar trend has been observed in the geographically close populations of fish *Pseudobarbus phlegethon* (Swartz *et al.*, 2004). They suggested that the difference between the two catchment areas lies in the variability of the Olifants-East catchment in terms of climatic changes (which may increase the likelihood of demographic instability). This system carries much less water than the rivers of the Olifants-West catchment area as it drains the rain shadow side of the Cederberg mountains and extends into the adjacent semi-arid region.

The late-Pliocene uplift, especially in the east, rejuvenated streams and modified the drainage network to conform largely to the modern arrangement (Partridge and Maud, 1987). These elevated areas have acted as ecological buffers against environmental changes; resulting in the survival of many relict floral and faunal communities (Skelton, 1986). This uplift and probably subsequent transgressing sea levels caused the isolation of *Lestagella* Gourits, AFR and Gamtoos populations. The AFR and Gourits populations of *Ephemerellina*, *Nadinetella* and Species A (Gourits only, not AFR) were isolated at this time, possibly remaining in relictual forests as a result of associated aquatic vegetation habitat restriction, which was also preserved in these forests. Subsequent to the Pliocene uplift, there may have been a river capture between Breede-East and the Gourits catchment, as *Ephemerellina*, *Nadinetella*, and Species A lineages show a link between these catchments around the Plio-Pleistocene.

The temperate acid waters of the Western Cape are a result of the geology, predominantly Table Mountain Sandstone, which produces acidic soils with low buffering capacity, also low in nutrient content (Allanson, 1990). The fynbos vegetation adds to the acidity by producing humic acids and other secondary compounds that are leached into rivers. At lower levels, the rivers are variously influenced by salts of marine origin, which increase the buffering capacity so that the pH may increase to neutrality (Allanson *et al.*, 1990). Fynbos vegetation established in the early Miocene, and a combination of uplift causing renewed erosion and increase in fynbos vegetation may have decreased the pH of rivers in the CFB from this time. If this increase was gradual, then fauna and flora would have had ample time to adapt

accordingly, which may be a factor contributing to the acid-tolerant endemic species that occur only in these acid streams.

Aridification along the Western Cape coast became more pronounced with the development of the Benguela Ocean current upwelling (Siesser, 1980) in the late Miocene. The xeric climatic conditions were more pronounced during the Pliocene/Pleistocene, favouring dry, fire-adapted vegetation; this potentially limited any dispersal among the allopatric populations confined to the relictual high lying forest patches. This was also the inception of the winter rainfall climate in the GCFR, the Pliocene uplift creating a rainfall gradient resulting in more summer arid in the west and wetter conditions in the east. This gradually led to slightly different rainfall zones within the GCFR, namely; winter-mesic, winter-arid, aseasonal-mesic and aseasonal-arid (Allsop *et al.*, 2014). This may have affected mayfly emergence times, and created an environmental barrier against dispersal by shifting reproduction cycles out of sync. The Breede River is covered by three of these zones (winter-mesic, aseasonal-arid and aseasonal-mesic) which roughly separates the eastern, southwestern parts of the catchment, where there appears to be little gene flow for various groups within the genera (Chapter 4 – Barrier analysis).

Lithogloea underwent a rapid radiation in the late Plio/Pleistocene, possibly in response to the cyclical climatic changes that are characteristic of this time. The clade spread mostly through the Breede catchment, followed by dispersal to the Palmiet in the late Pliocene. This dispersal to the Palmiet from the Breede system is also seen in the *N. crassi* complex (Figure 5.5; node 11) and the *Ephemerellina* E2 clade (Figure 5.3; node 10), suggesting that there was link between the Breede and Palmiet catchments during this period, possibly an off-shore link during a regression (arrowed, Figure 5.1).

Within the Pleistocene period, the Teloganodidae were generally limited to dispersal and vicariance of populations within catchments or between close neighbouring catchments, possibly due to the stabilisation of the river captures and courses from this time until present. Furthermore, many populations are restricted to fragmented forest palaeogenic zones, and so dispersal is limited.

5.5 Conclusions

Broad correlations between invertebrate diversity and the combination of landscape features and palaeoclimate shifts in the GCFR are not practical. The processes involved are far too complex and phylogeographic patterns are largely dependent on species life-history, adaptability and dispersal capabilities (Allsop *et al.,* 2014). Understanding these adaptive processes will lead to an understanding of the factors that have produced these patterns. Making broad generalisations of phylogeographic patterns across taxa is difficult, especially for invertebrates as there are often other factors not taken into account, such as associations with various vegetation types. General trends for invertebrates include a broad division between western and eastern regions, and often a Cape Peninsula clade (Allsop *et al.,* 2014). On a broad scale this can be seen within the southern African Teloganodidae.

From this study alone, the evolutionary drivers within a single family differ. Three important factors significantly affecting the phylogeographic patterns of Teloganodidae in the GCFR are seen, which align closely with those discussed by Cotterill and De Wit (2011): Firstly, the dispersal ability of each population or species, which is also linked to habitat speciality. *Lestagella* occurs under rocks in riffles and so dispersal in terms of available habitat suitability is less restricted than for other genera that are dependent on specific vegetation types. However *Lestagella* shows the most phylogeographic structure, indicating that it is possibly a poor long-distance disperser (i.e. able to disperse only in favourable conditions, possibly between rivers via protected, widespread forest habitats, and remaining sheltered in catchments in its alate stages, so little affected by passive wind migration). It is the smallest genus and possibly has the weakest flying ability (Chapter Six). Secondly, and closely linked with the first factor, is the distribution of the population or species at the ***time*** of tectonic or climatic events. For example, the non-fringed teloganodid lineages are not present in the Cape Peninsula possibly because they had not yet dispersed far enough south to use the land bridge that connected the Cape Peninsula with the mainland. Thirdly, the ability of the population to adapt and occupy available niches (displacement of other organisms as niches gradually become available during times of environmental change). There are of course numerous other factors at play that affect this process, especially pertaining to life history (emergence time and reproductive success).

Most of the lineage and species divergences occurring in the southern African Teloganodidae are due to allopatric speciation as a result of ancient population fragmentation occurring

largely in the mid to late Tertiary. With a few instances where some lineages have managed to re-enter certain areas, it is uncertain at this point whether the populations compete for resources or find available micro-niches to occupy (*Nadinetella* 'Robertson' and *N. brincki* complex Robertson populations are both found in the same habitat, associated with *Wardia hygrometica* moss).

The diversity of the Teloganodidae was greatly affected by shifts in ancient drainage patterns, climatic (including associated sea level changes) and physical events (uplift). As with any ecosystem, there is an intricate integration between its components. Vegetation has also played an important role in Teloganodidae diversification, in terms of generic habitat preference (*Wardia* or *Isolepis*) and protection from unfavourable climatic conditions (the ancient Afrotemperate forest association).

Similar patterns of dispersal and vicariance between lineages also provides information on possible events (e.g. corridors between catchments opening for dispersal) and timelines, which may be useful in a biogeographical context in other studies investigating phylogeography in the GCFR.

Ancestral reconstructions presented here indicate that Afrotropical Teloganodidae have a single origin. However these deeper nodes must be inferred with caution as it is not possible to determine the relationships with the Oriental lineages with certainty, and the use of more samples to provide better representation of the Oriental Teloganodidae is crucial to confidently inferring the origin of the family. Although this study favours the older divergence using a continental separation calibration, a later dispersal event to Madagascar cannot be dismissed completely. Further study incorporating a more extensive Oriental teloganodid range, possibly with more calibration points using other known continental fragmentation points (such as the Madagascar/India separation, or colonisation of certain South East Asian islands would add substantial value to the understanding of the global Teloganodidae.

Chapter Six

Geometric Morphometrics: Wing Shape Variation, Evolutionary Allometry and Integration

SUMMARY

This chapter explores the characteristics and quality of a morphometric dataset on Teloganodidae fore and hind wing shape-data at genus and species levels. Basic geometric morphometric analyses (PCA, CVA, BG-PCA) are presented to reveal any shape variation patterns between and within taxa. One group (*Lestagella*) displayed phenotypic variation related to catchment locality suggesting that there are geographic clines in the wing shape of this genus across its range. The morphometric data was found to contain phylogenetic signal at the species level analysis only. A phylogeny obtained using molecular methods was mapped into the wing shape-space to provide insight into the processes of shape evolution in teloganodid wings. Taxon means and independent contrasts were used to demonstrate the effects of evolutionary allometry and integration of the fore and hind wings. Variation was found to be concentrated in a single direction (i.e. most variation in a single principle component), indicating that shape changes show consistent evolutionary divergence, and is strongly associated with integration between fore and hind wings. While evolutionary allometry was a significant contributing factor to variation and integration, it did not account for all variation observed. Amplexiform wing-coupling in Teloganodidae is thought to be an important factor influencing shape change and integration, and there is some evidence of plausible phenotypic change between both wings to integrate wing coupling. Other factors (e.g. environmental, functional, and/or developmental) probably contribute to the shape changes and integration, and so this study inspires further investigation into the evolutionary divergence patterns of shape change in Teloganodidae wings.

6.1 Introduction

Bookstein (1982) developed the idea of using "landmark" data on objects that are quantifiable, that is, repeatable between similar landmarks on related objects. This chapter uses the geometric morphometrics (GMM) method (e.g. Bookstein, 1996) making use of landmark coordinates to determine shape and centroid size. Centroid size is the square root of the sum of squared distances of all landmarks from a "centre of gravity", and therefore measures the "spread" of the landmarks, which is then used comparatively.

The methods used here make use of a Procrustes superimposition, where only the shape information from landmark configurations is extracted by standardising all other components of variation (Gower, 1975; Dryden and Mardia, 1998). That is, standardised size, position and orientation so that landmark configurations are shifted to a common position. This Procrustes shape information is then referred to as the shape-space, shape-dimension or "morpho-space".

After this superimposition, the standardisation of variable components (size, position and orientation) results in a loss of shape variable information (i.e. dimensions of the data). Therefore the data has more landmark coordinates than the actual number of shape variables (dimensions). That is, four degrees of freedom are lost in the data after superimposition (one degree for standardisation of size, two degrees for fixing the configuration position because of the X and Y coordinates and one degree for orientation). For example, a 2-dimensional dataset with 10 landmark configurations effectively has 20 coordinates (each landmark has an X and Y coordinate) and 20 degrees of freedom, but after Procrustes superimposition, four degrees of freedom are removed (for size, position and orientation), leaving the dataset with 16 degrees of freedom. The dimensionality (number of shape variables) of the dataset being used needs to be considered in terms of sample size. If the sample size is small, and the number of dimensions is higher than the number of observations (sample size), this can lead to "over-dimensionality" (too many degrees of freedom) relative to sample size, rendering any statistical results unviable (low statistical power). This can lead to an over-estimation of results, showing false patterns and trends within the data (Drake and Klingenberg, 2008). Dimensionality has been taken into account in all analyses presented in this chapter, where landmarks configurations have been removed where necessary to prevent over-estimation of statistics used.

The Procrustes shape space is curved, as opposed to a flat Euclidean space (Viscosi and Cardini, 2011). This is problematic for standard statistical methods that require data to be in a flat Euclidean space. Therefore the Procrustes shape coordinates need to be projected into a Euclidean shape tangent (similar to the projection method where a three-dimensional cartographic world globe is used to create flat, two-dimensional world maps). The tangent between the Procrustes and Euclidean spaces represents the sample mean shape of the data, and this approximation of the tangent has been found to be a considerably effective representation of the mean in almost all biological datasets investigated (Adams *et al.,* 2004; Viscosi and Cardini, 2011).

Using independent phylogenetic data (e.g. from molecular methods) to determine historical shape change in morphometric data is a developing field (e.g. Klingenberg and Ekau, 1996; Klingenberg and Gidaszewski, 2010; Figueirido *et al.*, 2010; Klingenberg *et al.*, 2012). It allows testing of the phylogenetic signal of morphometric data, by incorporating phylogenetic structure into morphometric datasets (Klingenberg and Gidaszewski, 2010). This is done using Partial Least Squares (PLS: Rholf and Corti, 2000) and Independent Contrasts (IC: Felsenstein, 1985; Rohlf, 2001) methods. The PLS method is becoming popular for characterising patterns of morphological integration (e.g. Klingenberg and Zaklan, 2000; Klingenberg *et al.,* 2001, 2003; Monteiro *et al.,* 2005; Mitteroecker and Bookstein, 2008; Kulemeyer *et al.,* 2009; Klingenberg and Marugán-Lobón, 2013).

The aim of this chapter is to examine the geometric morphometrics of South African Teloganodidae wings in a phylogenetic context. The presence or absence of phylogenetic signal in teloganodid fore and hind wings is investigated. A multilevel approach is used to explore shape variation patterns of evolutionary integration and allometry in teloganodid wings, and assess evolutionary divergence between and within taxa. Differences and adaptations in wing shape between river catchment localities within *Lestagella* were also examined.

Due to limited samples available, variation between sexes or alate life stages (subimago and imago) could not be considered, and these are therefore not differentiated in the analyses. Statistical analyses were conducted on species averages, so differences between the sexes or alate stages were averaged out over the data, and each species (including this variation) is represented. As this may increase within-taxon variation, results should be interpreted with

caution and this chapter should be considered as an exploratory study to determine underlying trends in the data.

6.2 Methods

6.2.1 Specimen Preparation

Teloganodidae adults successfully reared through from nymph to subimago / imago stage were analysed for diagnostic characters as well as photographed for geometric morphometric (GMM) analyses. Both the fore and hind wings (left and right) were removed from 79 adults from across the genera (Appendix 6A) The genus *Lestagella* was best represented in the dataset, with a total of 35 individuals with fore wing data and 32 individuals with hind wing data (some hind wings were damaged; Table 6.1). The genera *Ephemerellina*, and two other taxa of uncertain placement in terms of taxonomic hierarchy, which will be referred to as Species B and Species C were poorly represented, each with only two individuals per taxon for the fore wing and hind wing datasets (see Table 6.1 and Appendix 6A for details on all individuals used in these analyses).

Table 6.1. Individuals included in geometric morphometric analyses, with number of representatives at each taxon level presented for right-side fore and hind wings.

Taxon		**Number of specimens**			
Genus	**Species**	**Fore wing**	**Total number in genus**	**Hind wing**	**Total number in genus**
Ephemerellina	*E. barnardi*	2	2	6	6
Lestagella	*L. penicillata*	5		5	
	L. sp.1	6		6	
	L. sp.2	5		5	
	L. sp.3	3		3	
	L. sp.4	4		4	
	L. sp.5	1		1	
	L. sp.6	9		5	
	L. sp.7	2	35	2	31
Lithogloea	*L. harrisoni*	6	6	5	5
Nadinetella	*N. brincki*	10		10	
	N. crassi	5	15	5	15
Species A	Species A	11	11	11	11
Species B	Species B	2	2	2	2
Species C	Species C	2	2	2	2
			73		**72**

The wings were temporarily slide mounted in an ethanol-based gel, and left and right wings photographed. Wings were then placed into individual vials with the rest of the specimen and are housed at the Albany Museum in Grahamstown (AMGS). Each photograph was named according to a string of classifiers to facilitate subdivision of the dataset for various analyses, which were used later in the GMM software, MorphoJ v1.06d (Klingenberg, 2011). The string consisted of: the first three letters of the genus name, followed by one random letter denoting the separate recognised species (if this was applicable), a three-letter code pertaining to each individual specimen, one letter for the sex of the specimen (m / f), four letters representing the river locality where each specimen was collected, four letters for the catchment, one letter signifying the side of the wings (left or right) and two letters denoting which wing (fore- or hind wing), i.e.:

GenusnameSpeciesIndividualSexRiverCatchmentSide(L/R)wing(FW/HW)

For example: ***nadd062mdassbreerfw*** (*Nadinetella crassi*, individual number 062, male, river Dassieshoek in the Breede catchment, right fore wing).

To avoid large amounts of text within diagrams, the *Lestagella* populations from each catchment were assigned species numbers (Table 6.1) which are associated with the catchment localities as outlined in previous chapters (see Table 6.2).

Table 6.2. River catchment localities associated with the species numbers assigned to the *Lestagella* genus, higher clade group indicated in brackets (corresponding to Chapter Four analyses).

Assigned species number	**Associated Catchment**
L. sp1	Breede (L2 group)
L. sp2	Eerste
L. sp3	Keiskamma
L. sp4	Olifants
L. sp5	Outeniqua (AFR)
L. sp6	Tsitsikamma (AFR)
L. sp7	Breede-East (L1 group)
L. penicillata	Cape Peninsula

6.2.2 Morphometric Data Features

Photographs were converted into TPS file types (format needed for digitisation of photographs) using TPSUtil v1.58 (Rohlf, 2015). Separate datasets were made for the fore wings and hind wings. The software TPSDig v2.17 (Rohlf, 2015) was used to digitize landmarks on each image for each specimen (7 genera and 15 species, see Table 6.1).

A set of 16 landmarks were placed on distinctive points along the main veins on the fore wings and 13 landmarks placed on the hind wings (Figure 6.1). Landmark points along main veins were chosen so that the configurations were repeatable and corresponding for each wing. The landmarks were chosen to cover the wing shape as completely as possible, while ensuring that the landmarks could be located unambiguously on all specimens throughout the taxa. The fore wing dataset had 28 dimensions (with 73 observations/samples) and the hind wings had 22 dimensions (with 72 observations/samples).

A warped outline drawing of a Teloganodidae wing was made in TPSDig v2.17 (Rohlf, 2015), which is an estimated mean shape or frame of the structure under investigation. This is purely for visualisation of the shape changes between samples in MorphoJ v1.06d, all wing shape changes are presented in this chapter as warped outline drawings.

Analyses were conducted at generic and species levels (Table 6.1). The genus *Lestagella* was the best represented in the datasets, so was chosen for a small pilot analysis investigating river population relation with catchment locality (Figure 6.2). For this, landmark configurations were reduced to nine for the fore and hind wings (Figure 6.1) to avoid over-generalising the results due to over-dimensionality.

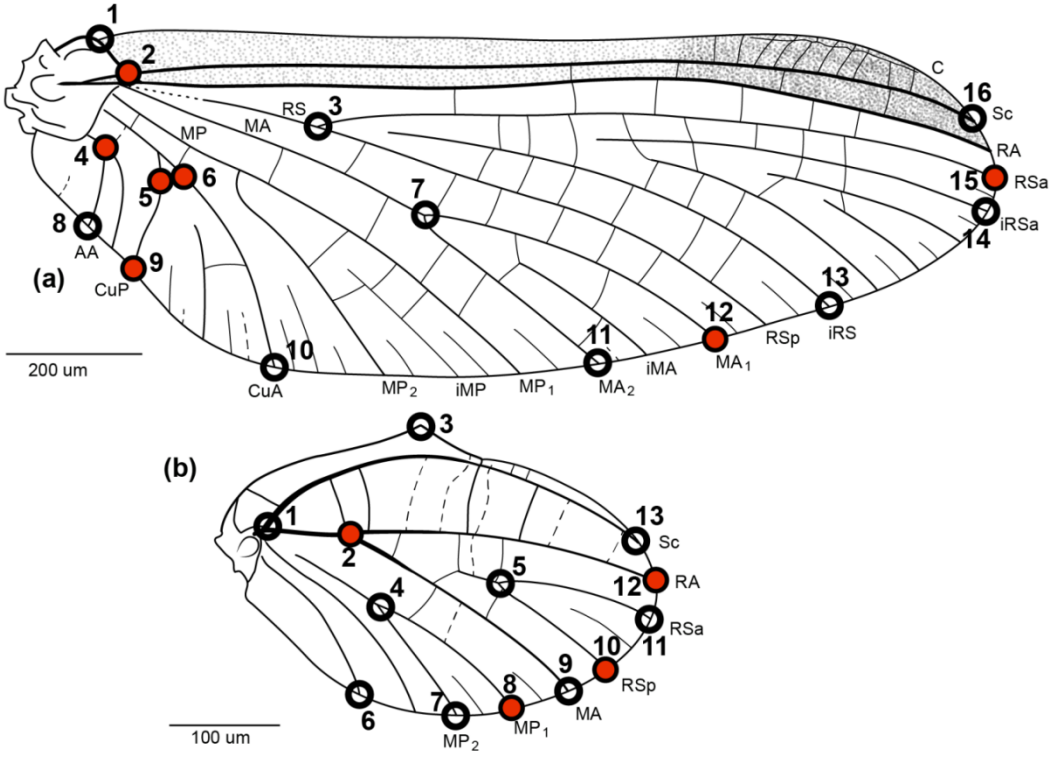


Figure 6.1. General Teloganodidae wings with venation labels for the (a) fore wing showing 16 landmark configurations chosen for this study, landmarks in red were removed for the *Lestagella* dataset analyses, (b) hind wing showing 13 landmark configurations, landmarks in red were removed for the *Lestagella* dataset analyses.

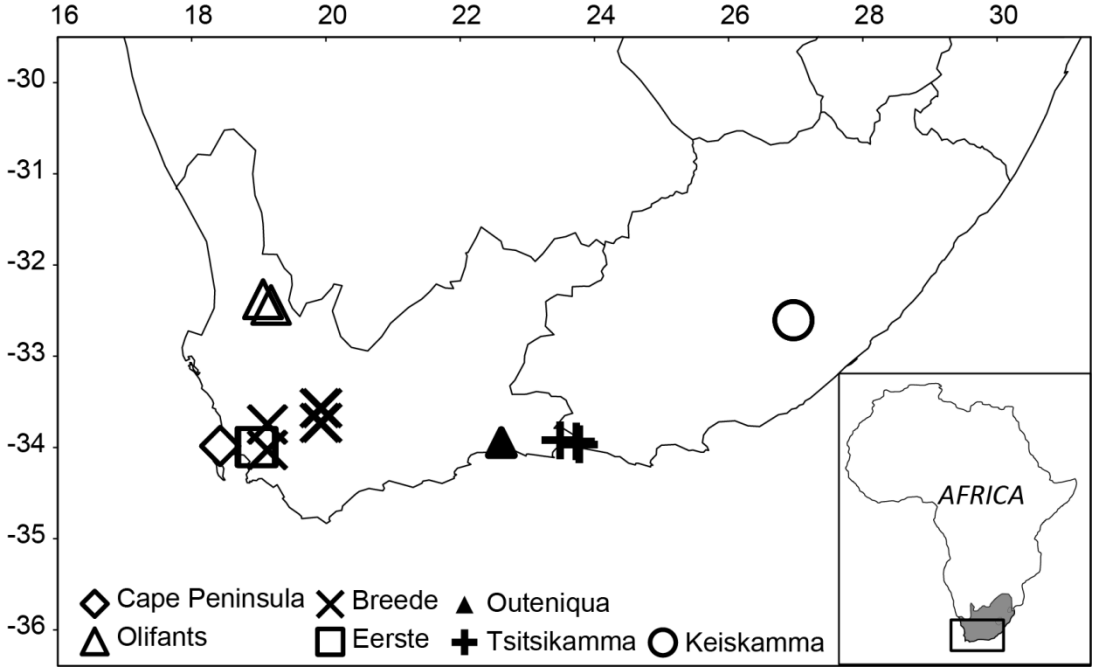


Figure 6.2. Distribution map showing the localities for each of the *Lestagella* specimens included in the GMM analyses, each symbol represents river localities within the labelled catchments.

The tangent space approximation was tested using TPSSmall (Rohlf, 2015), which uses Euclidean and Procrustes shape distances (from the .TPS file) and tests whether the variation in shape among the set of specimens in the dataset is too large, thus rendering statistical methods based on the tangent space approximation unviable. Both the slope and correlation in the regression should be close to the value one for the approximation to be considered acceptable.

All morphometric analyses from this point onwards were conducted using MorphoJ v1.06d (Klingenberg, 2011) unless otherwise stated. A Procrustes superimposition was used to extract the shape information from the landmark coordinates (Dryden and Mardia, 1998), followed by generation of a variance-covariance matrix. The raw data was checked for outliers using a plot of the cumulative distribution of the (Procrustes) distances from the average (this is a function for finding outliers in the MorphoJ software).

Before analyses were conducted on the full datasets, the quality of the data capture was tested, and characteristics of variation were explored. A subset of 25 fore wings and 25 hind wings (from across the genera) were used to examine the extent of digitising error; how much individuals differ between and within species in terms of shape and centroid size; whether these differences are explained by the measurement error, and if there is any asymmetry between the left and right wings. Each photo was digitized twice and the error assessed by Procrustes ANOVA. The Procrustes ANOVA performs MANOVA multivariate statistics on the shape variation (Pillay's trace and associated P-value), which gives an idea of whether there is any direction in the shape variation.

6.2.3 Exploring shape space with PCA, BG-PCA and CVA

A principle component analysis (PCA) examines original shape space (where spatial relationships between observations remain unaltered) and displays general patterns of variation according to the amount of variation for which they account, without considering any structure or specific hypotheses in the data.

Canonical coordinates for variables analyses (CVA) are mostly used in taxonomic or evolutionary studies (e.g. Duarte *et al.,* 2000; Klingenberg *et al.,* 2003; Elmer *et al.,* 2010; Florio *et al.,* 2012; Klingenberg *et al.,* 2012) as they are based on the assumption of group structure in the data. CVA is an ordination method for datasets that contain more than two

groups, whereas discriminant function analyses (DFA) are more appropriate for two group datasets. DFAs were not conducted in this study as the data presented represent more than two groups and there is a chance that the features that best summarize the overall differences among groups do not coincide with those that best distinguish any two groups.

CVA is an efficient method for identifying separation between taxa as it maximises the differences between taxa relative to the variation within taxa. This is done by transforming the data in such a way that the difference between means relative to the variation within groups is maximised; in multivariate analyses this measure is known as the Mahalanobis distance (Mardia *et al.*, 1979; Klingenberg and Monteiro, 2005: pg. 680, Figure 1). The statistical significance of pairwise differences in mean shapes was assessed with permutation tests (10 000 permutations) using Mahalanobis distance as the test statistic (Appendix 6B). CVA however assumes that all groups share the same covariance matrix, i.e., they need to be sufficiently similar so that the pooled estimate that is not too far from the within-group variation of any of the groups under study. If the covariance matrices are not similar however, the transformation of the space will be unable to produce equal or comparable amounts of within-group variation in all directions of multivariate space. CVA plots should show scatters of points for designated groups that should be similar in size – this is a visual way of determining that the covariance matrices within groups are sufficiently similar to meet the assumption of equal within-group dispersion for the CVA.

Small samples sizes can lead to inflated Mahalanobis distance estimates due to underestimated within-group variation. This would result in a poor reflection of the true variability in groups and can produce misleading results. If this is the case then a between-groups PCA (BG-PCA) can be used to provide useful ordination (Boulesteix, 2004). The BG-PCA is based on the covariance matrix among the group means (and not the covariance matrix among the individual observations as is in a standard PCA). BG-PCAs can reveal the main features of variation among species (Klingenberg and Spencer, 1993) and have been used to assist taxonomic studies based on morphometrics (Yazdi and Adriaens, 2013).

Shape variation between genera was visualised and compared using standard PCA and CVA. At the species level, BG-PCA and CVA were performed on the shape variables (both approaches were taken to examine the effects of CVA overestimation due to small sample sizes). For *Lestagella*, a BG-PCA and CVA was conducted on the shape variables grouped by catchment. The PCA, BG-PCA and CVA are presented by scatterplots showing scores that

account for maximum variation in the data, along with a warped outline drawing showing the shape changes in the right wings.

The mean shapes for each genus and species were computed (separately) by averaging the right wing shapes of all specimens within each genus or species (Table 6.1). These new datasets (fore wing and hind wing) of observations averaged by taxa were then used for further comparative analyses.

6.2.4 Phylogeny and comparative approach

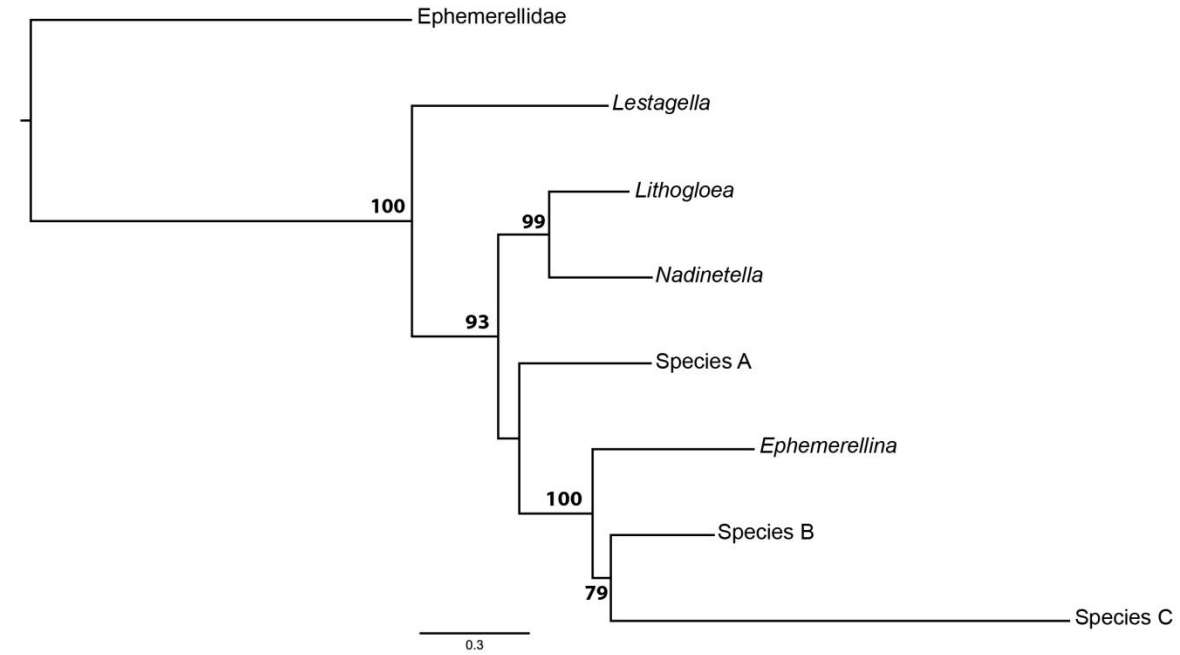
Morphometric data has continuous variation rather than discrete character steps. Squared-change parsimony (Maddison, 1991) is used to combine the continuous shape information with phylogenetic trees. Shape variables used to derive cladistics variables and shape space distances have been used for estimating phylogenetic trees, with recent approaches treating landmarks separately (Catalano *et al.*, 2010; Goloboff and Catalano, 2011; Catalano and Goloboff, 2012).

The comparative analyses are based on a phylogenetic tree created separately from a subset of data obtained in Chapter Four. Using a representative specimen from each genus and from each species group (see Table 6.1) for the molecular phylogeny, a RAxML maximum likelihood analysis using five genes (12S, 16S, 28S, H3 and COI – see Chapter Four for details on molecular methods), where models for partitions are selected by the RAxML software (Stamatakis, 2014) which was used via the CIPRES Science Gateway cluster (Miller *et al.*, 2010). The best supported tree for the genus and species level, with 500 bootstrap iterations, was used for further comparative analyses with the geometric data (Figure 6.3 - 6.4). Branch lengths were used to weight the analyses.

Phylogenetic signal can be defined as the association between phylogenetic relationship and phenotypic similarity; a strong phylogenetic signal exists if closely related taxa tend to be more similar to each other than remotely related taxa (Cole *et al.*, 2002; Cardini and Elton, 2008; Klingenberg and Gidaszewski, 2010). For data to have phylogenetic signal, the tree from the original data is shorter than the trees from data where shape values have been randomly exchanged among the terminal nodes of the tree. Ultimately, if there is signal, then the comparative analyses of the data need to take into account the phylogenetic structure.

To determine if the Teloganodidae wing datasets contained phylogenetic signal, a permutation approach was used (Klingenberg and Gidaszewski, 2010), with a null hypothesis of no phylogenetic signal in the data. The test used 10 000 random permutations and a significant test result indicates that the data has phylogenetic structure and therefore phylogeny needs to be taken into account for further comparative analyses. Where the permutation test provided evidence against the null hypothesis of no phylogenetic signal, the data from the landmark configurations were mapped onto the phylogeny to generate independent contrasts (IC) of shape variation in the sample (Felsenstein, 1985). The residuals of these IC (adjusted for phylogeny) take the relationship of the species (whereas original data treats each species independently with no relation to each other) into account. If phylogeny plays an important role on phenotypic changes in the wings, a different result from the analyses on the original dataset is expected.

(6.3)



(6.4)

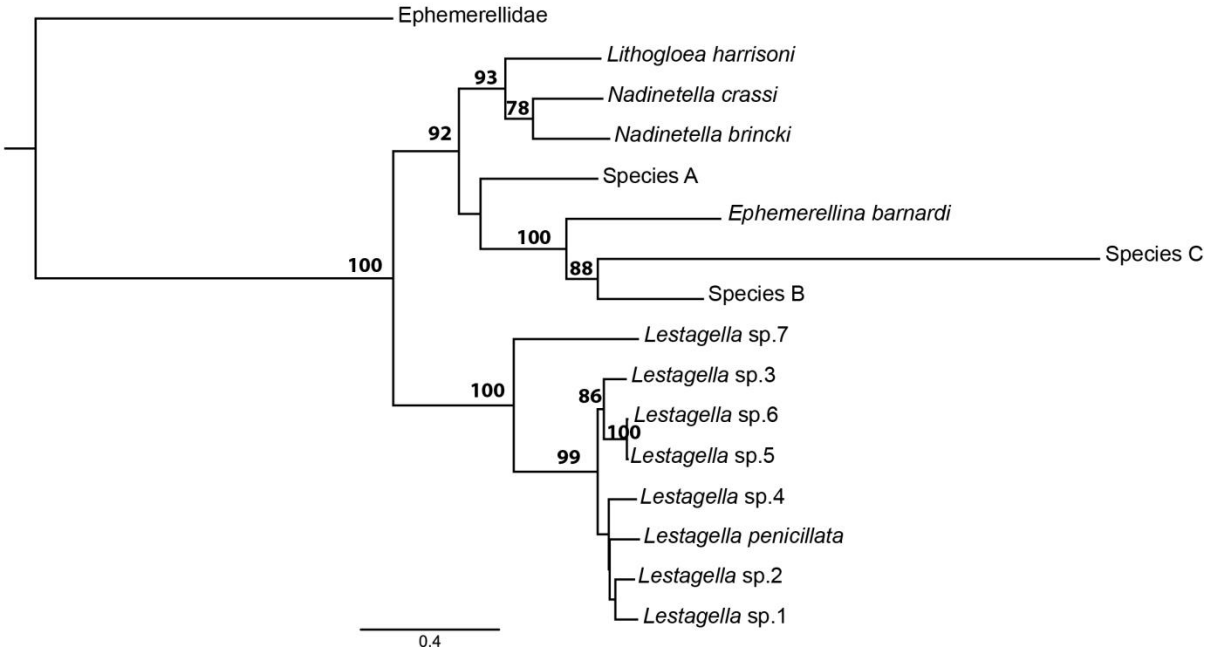


Figure 6.3 - 6.4. Maximum Likelihood phylogeny used to map morphometric data for (6.3) genus and (6.4) species. Bootstrap support values >70 are shown.

6.2.5 Patterns of Evolutionary Diversification in shape space

The phylogenetic history of shape change was reconstructed by projecting the phylogeny into the shape tangent space, where it is drawn onto plots of multivariate ordinations of the species means. This graphical approach provides an intuitive idea of how specific clades diversified and dispersed through morphometric variable space, at least as far as it is possible to infer from the shape information provided by the terminal taxa (e.g. Klingenberg and Ekau, 1996; Figueirido *et al.*, 2010, 2013; Klingenberg and Gidaszewski, 2010; Monteiro and Nogueira, 2011; Klingenberg *et al.*, 2012; Meloro and Jones, 2012).

The shapes corresponding to the internal nodes of the phylogeny in these plots are reconstructed by squared-change parsimony (Maddison, 1991; Rohlf, 2001), which is the most widely used standard for mapping these continuous characters (shape variables) onto phylogenies. The squared-change parsimony method is a maximum likelihood estimate of the ancestral state (Maddison, 1991), and searches for trait values of the internal nodes that minimize the differences of the trait over the whole tree. The analysis finds shapes for the internal nodes that minimize the length of the tree in Procrustes distance units. This approach is used to map the morphometric data onto the phylogenetic tree (Klingenberg and Ekau, 1996).

The phylogenetic trees are projected in principal component (PC) score scatterplots, whereby the PCA is computed from the covariance matrix among the average shapes of the terminal taxa (species). To examine whether these patterns of diversification may relate to patterns observed within taxa, a PCA of the pooled within-taxon covariance matrix was computed. This is a joint estimate of the covariance within-taxa, as opposed to the among-taxon covariance matrices used in the comparative analyses, which is calculated in these analyses by averaging species in the datasets for fore wing and hind wing. Matrix correlations between the pooled within- and among-taxon matrices were computed to test the similarity of divergence in these matrices.

In this analysis, specific lineages and clades are the main focus of the mapped phylogenetic displays and not the overall evolutionary process; therefore the analyses use the species means and not IC (which takes phylogeny into account) as the units of this analysis. These phylogenies mapped onto PCA plots were conducted for both the original mean shape space and the size-corrected data space to remove any effects of evolutionary allometry (see section

6.2.6). Any large differences between the two analyses would indicate that evolutionary allometry plays a role in wing shape in Teloganodidae.

6.2.6 Evolutionary Allometry and Size Correction

Allometry, the variation in shape that is associated with variation in size (Gould, 1966; Mosimann, 1970) is a factor that can contribute substantially to integration or morphological traits, and is investigated using a multivariate regression of shape onto size (Drake and Klingenberg, 2008). Evolutionary allometry is the primary interest in this section, it is the shape and size variation seen among different species which indicate evolutionary processes that have formed the observed shape (Drake and Klingenberg, 2008; Klingenberg and Zimmermann, 1992). In the context of diversification among taxa, the focus is on evolutionary allometry, the evolutionary change of shape associated with the evolutionary change of size (Cock, 1966; Klingenberg, 1996).

The effect of evolutionary allometry and its significance across the datasets was investigated using a multivariate regression of shape variable against the log transformed centroid size. This was conducted on the fore and hind wing IC, with the significance tested using 1000 permutations. The IC are used as the units of the analysis (Figueirido *et al.*, 2010; Perez *et al.*, 2011; Klingenberg *et al.*, 2012). The shape variable that has the most correspondence with size is extracted from this analysis. The evolutionary allometry concerns the covariation of shape change and size among phylogenetic branches that are derived from a common ancestor (Klingenberg, 1996). From this, the association of shape and size in the evolutionary process can be examined and an interpretation of how they are formed can be provided. The residuals from the regression can be used for further analyses involving integration and modularity (e.g. Klingenberg, 2009). To eliminate the effect of evolutionary allometry, the size correction based on the regression of the IC can also be applied to the species averages. The vector of regression coefficients from the IC were used to compute the residual component of variation in the species averages, which provided size-corrected shape scores free of evolutionary allometry, which were then used in a PCA so that the shape changes are visualised.

6.2.7 Patterns of variation in evolutionary shape changes

Morphological integration across species arises from associations among aspects of shape in the evolutionary changes along the branches in the phylogeny. This integration can be studied by examining patterns of variation in the covariance matrix of IC for wing shape. PCA is useful in this context, as the first few eigenvectors identify the dominant features of shape variation and the corresponding eigenvalues indicate the amount of variation associated with each PC (e.g. Pearson, 1901; Jolliffe, 2002).

A PCA is computed for the complete variation in the IC and then another PCA which takes the allometric effects into account. The eigenvectors and eigenvalues can be interpreted in the same way as for PCA in other contexts. This is similar to the phylogenetic PCA which was proposed by Revell (2009), but with a slightly different algorithm due to the use of the IC (Felsenstein, 1985; Rohlf, 2001).

6.2.8 Evolutionary integration between fore and hind wing

A Partial Least Squares (PLS) analysis creates a new variable axis that accounts for the largest amount of correlation between two datasets by singular value decomposition (Rholf and Corti, 2000). The scores were plotted onto the configuration defined by the first PLS axis of the fore wing against the hind wing to visualise the correlation between them. To analyse the covariation of evolutionary changes between the fore and hind wing, the PLS analysis uses independent contrasts (IC) of the shape variables as the units. Covariation between IC of the shape coordinates for fore and hind wing indicates evolutionary integration of shape between them.

PLS axes computed from IC therefore identify shape features with maximal evolutionary covariation (Rholf and Corti, 2000). To analyse the patterns of integration between both wings as a whole, a Procrustes superimposition was computed for each set of landmarks and the resulting separate blocks of shape coordinates were used in a PLS analysis between these partitions using IC as the data. PLS analysis uses a singular value decomposition of the uncentred matrix of covariances between two sets of variables to extract pairs of PLS axes, one for each set, that have maximum covariance with each other and provides a summary of the total covariation between sets in a minimum number of dimensions (Rohlf and Corti, 2000).

The interpretation of the results of the PLS is similar to other shape changes and amounts of covariation associated with the different PLS axes. However, due to the arbitrary ordering of sister nodes, the scatterplots can be ambiguous; therefore the strength of association is presented for the pairs of PLS axes using the RV coefficient of overall association (Escoufier, 1973; Klingenberg, 2009). The RV coefficient has been used to measure integration in various studies (e.g. Klingenberg, 2009; Gómez-Robles and Polly, 2012; Klingenberg and Marugán-Lobón, 2013).

To test the covariation between the fore and hind wing, a permutation test was used (Good, 2000), with a null hypothesis of total independence. Note here that the observations are IC of Procrustes coordinates, meaning that they are shape changes (rather than actual shapes) and have an average close to zero for every coordinate, and therefore cannot be used directly in the Procrustes superimposition. Therefore the mean shape needs to be added to the vectors of IC for the Procrustes fits as part of the permutation approach (Klingenberg and Marugán-Lobón, 2013).

6.2.9 Angular Comparisons of results from PCA, PLS and Regression

Shape changes visualised from PCA, PLS and regression can indicate that the vectors are similar or even identical. For example, if allometry accounts for most of the variation, then the allometric regression vector from a shape on size regression may be expected to coincide with the PC1. Angles between vectors is a way of quantitatively assessing the similarity of two vectors in shape tangent space (e.g. Klingenberg and Zimmermann, 1992; Klingenberg *et al.*, 1998; Klingenberg and Zaklan, 2000; Klingenberg *et al.*, 2001, 2003). The smaller the angle, the more similar the vectors; the P-values presented are tested against the null hypothesis that the vectors have random directions in shape tangent space (see Li (2011) for details on these methods). Klingenberg and Marugán-Lobón (2013) provide details of assessing these angles, with brief equations and comparison of this method with work done by other authors.

6.3 Results

6.3.1 Exploring patterns in original shape space

Tangent space and outliers

The tangent space approximation (tested in TPSSmall; Rohlf, 2015) was acceptable for both the fore- and hind wing data. Slope and correlation for the fore wing was 0.9888 and 0.9999 respectively and for the hind wing, 0.9478 and 0.9999 respectively. This means that the variation in shape among the set of specimens in the dataset is small, thus rendering statistical methods based on the tangent space approximation viable.

No serious outliers were found in the datasets, suggesting that there were no specimens with unusual shapes in the datasets.

Sources of variation

The Procrustes ANOVA on the subset sample of 25 specimens was computed with the following classifiers for effects: *Error 1*: the replicates for calculating measurement error in digitising, *Side*: the left and right wings, *Individual*: each specimen observation; *Species (Main Effect)*: individuals grouped into species (see Table 6.1). Although investigating asymmetry was not the primary aim in this study, this analysis of the data subset gave an impression of the effects of fluctuating and directional asymmetry (*Individual*-by-*Side* and *Side* interactions respectively).

In the hierarchical Procrustes ANOVA tables (Table 6.3 – 6.6), "*Species"* is placed at the top of each table and is the main effect or largest biological effect of interest. The smallest biological effect of interest in this analysis was the individual-by-side (*Ind*Side*) interaction. The most important information within the tables is the Mean Squares (MS), which are the estimates of the variances associated with an effect in the ANOVA, and the *F*-ratios (ratios of the MS values). The following results are considering the MS values and F-ratios within Tables 6.3 – 6.6:

The measurement error caused by digitizing for the centroid size and shape in the fore wings was 0.004 % and 0.1 % of the individual variation respectively (Table 6.3 and Table 6.4). For the hind wings, 0.03 % and 0.1 % of the individual variation for centroid size and shape respectively, can be attributed to measurement error (Table 6.5 and Table 6.6). In terms of individuals and species (the main focus of the study), this amount of error is negligible and is

at least one order of magnitude less than the effect of biological interest (individuals), therefore it is considered unnecessary to digitise all the specimens more than once.

In the fore wings, intraspecific variation in centroid size (*Individual*, Table 6.3) and shape (*Individual*, Table 6.4) was 31 % and 34 % of the species groups, respectively. In the hind wings, intraspecific variation in centroid size (*Individual*, Table 6.5) and shape (*Individual*, Table 6.6) accounted for 9 % and 44 % of the species groups. All of these were highly significant (95 % confidence interval). This is unsurprising as the high amount of intraspecific variation could be attributed to the distinct clades (and apparent new species) seen within these taxa (Chapter Four and Chapter Five).

If we look at the Mean Square (MS) values for individual-by-side interaction (fluctuating asymmetry) for size and shape of fore wings (Table 6.3 – 6.4) and hind wings (Table 6.5 – 6.6), they range from being ca. 7 to 93 times larger than the variation between digitising replicates, further confirming that measurement error is not a serious concern for these datasets. The high significance of these interactions indicates that the biological effect (fluctuating asymmetry) is evident through the "noise" of measurement error.

Looking at the main effect of size variation, there is no significant difference in size between the left and right wings for fore wings (Table 6.3), yet there is a slight significant difference between left and right in the hind wings (Table 6.5). There is also indication of significant variation in shape between the left and right side for both the fore- and hind wings (Table 6.4 and 6.6). However there are not enough samples to compute the MANOVA significance of directionality of this variation for the fore wings (Table 6.4), and the directionality appears to be insignificant for the hind wings (Table 6.6, $P = 0.1278$).

The right and left wing sides were not available for every specimen in the full dataset (Table 6.1) and the sample size of this particular exploratory study is too small to go into detailed analyses on fluctuating and directional asymmetry. The Procrustes ANOVA has shown potential for investigating these asymmetries, which can be explored from a developmental, ecological or evolutionary perspective (Klingenberg *et al.*, 1998). More comparative studies are needed to further investigate and understand the evolution of directional asymmetry. Judging by these preliminary findings from 25 samples, a future, detailed study of asymmetry in Teloganodidae wings could contribute to the understanding of these processes.

As asymmetry was found to be present in the data, only the right-side wings for the remainder of the study were used because there were more data available for this side. Two datasets were prepared for both fore and hind wings, one where observations were averaged by genus, and the other averaged by species.These datasets were used throughout the remainder of the study.

Table 6.3. Procrustes ANOVA for Centroid size variation, for the left and right Fore wings of 25 individuals.

Effect	SS	MS	Df	F	P
Species	44.71	6.387	7	3.21	0.0235
Individual	33.86	1.992	17	413.59	<.0001
Side	0.0254	0.02543	1	3.21	0.0857
Ind*Side	0.1899	0.007915	24	93.21	<.0001
Digitising Error	0.004246	0.000085	50		

Table 6.4. Procrustes ANOVA for Shape variation for the left and right Fore wings of 25 individuals.

Effect	SS	MS	Df	F	P	Pillai tr.	P
Species	0.0447	2.28×10^{-4}	196	2.92	<.0001		
Individual	0.0371	7.80×10^{-5}	476	12.06	<.0001		
Side	0.000394	1.42×10^{-5}	28	2.18	0.0005		
Ind*Side	0.00435	6.47×10^{-6}	672	8.18	<.0001	16.32	<.0001
Digitising Error	0.00111	7.91×10^{-7}	1400				

Table 6.5. Procrustes ANOVA for Centroid size variation for the left and right Hind wings of 25 individuals.

Effect	SS	MS	Df	F	P
Species	1.09×10^{7}	1.81×10^{6}	6	11.13	<.0001
Individual	2.93×10^{6}	1.63×10^{5}	18	446.24	<.0001
Side	2904.29	2904.29	1	7.96	0.0095
Ind*Side	8757.75	364.91	24	7.67	<.0001
Digitising Error	2377.85	47.56	50		

Table 6.6. Procrustes ANOVA for Shape variation for the left and right Hind wings of 25 individuals.

Effect	SS	MS	Df	F	P	Pillai tr.	P
Species	0.2014	1.53×10^{-3}	132	2.24	<.0001		
Individual	0.2696	6.81×10^{-4}	396	8.67	<.0001	16.31	<.0001
Side	0.0043	1.96×10^{-4}	22	2.5	0.0002	0.97	0.1278
Ind*Side	0.0415	7.85×10^{-5}	528	106.78	<.0001	17.03	<.0001
Digitising Error	0.00081	7.35×10^{-7}	1100				

6.3.2 Exploring patterns in shape space (PCA, CVA, BG-PCA)

Wings at genus level

The PCA and CVA of wing shape variation shows that a large proportion of the variation is contained in relatively few dimensions for all PCAs and CVAs. A standard PCA was used for fore wing and hind wing measurements at genus level (Figure 6.5 – 6.6), to examine any evidence of group separation in original shape space (before any generic level averaging).

Fore wing PCA and CVA (Figure 6.5a- d)

The first two PCs accounted for over half of the total variance in the sample for the fore wing at the genus level. There appears to be some separation between the genera in the original shape space, especially for Species A. The shape changes associated with the PCs (Figure 6.5b) show some of the main features of wing variation, PC1 is associated with a general lengthening and shape change of the wing from a shorter more rectangular shape, and the apical margin ranges from a blunt rounded shape to a slightly downward curved, more pointed apex. PC2 is associated with lengthening and broadening of the wing base and extended anal margin.

The CVA for fore wings show that taxa are distinct from each other at generic level (Figure 6.5 c – d). The Mahalanobis distances range from 4.39 (*Nadinetella* vs *Lithogloea*) and 14.74 (*Ephemerellina* vs Species B). All permutation tests indicated that mean shapes differ significantly among taxa, except for *Ephemerellina* vs Species C, in the pairwise permutation test between genera. The Procrustes distances ranged from 0.019 (*Nadintella* vs Species C) to 0.059 (*Ephemerellina* vs *Lestagella*), suggesting small to moderate shape differences between genera. The scatter plot of CV scores shows a gradient of a shorter, more rectangular wing to a longer and distally curved wing along the CV1 (Figure 6.5d), with a marked concentration of a shorter, more rectangular wing shape at the lower end of the CV1 scores. The CV2 shape follows a gradient from a more extended anal margin of the wing to a more slender and straighter anal margin across the CV2 scores (similar to the PCA).

Hind wing PCA and CVA (Figure 6.6 a – d)

Generic separation in the PCA for the hind wing was not as clear as observed in the fore wing. The first two PCs accounted for over half of the variation (Figure 6.6a). The shape changes associated with the PCs show some of the main features of wing variation. PC1 is

associated with a change from an oval-shaped hind wing to a shorter wing with an extended anal margin. PC2 shows a shape range from a narrowed to a more rounded apical margin, with a marked concentration of the genera clustering closer to the narrowed apex shape along the apical margin. (Figure 6.6a – b).

Separation of genera according to the hind wing was clear in the CVA (Figure 6.6b – d) with only slight overlap of *Lithogloea* and *Nadinetella*. The Mahalanobis distances ranged from 3.72 (*Nadinetella* vs *Lithogloea*) and 10.07 (*Ephemerellina* vs *Lestagella*). All permutation tests were significant, indicating that the mean shapes differed significantly between genera. Procrustes distances ranged from 0.029 (*Nadinetella* vs *Lithogloea*) and 0.116 (Species C vs Species A) suggesting comparatively large shape differences between genera. The CV1 scores show a shape change from an oval-shaped hind wing to a shorter wing with an extended hind margin (Figure 6.6d), with most genera clustering at the intermediate of these two extremes. The CV2 scores show a shape range from a narrowed to a more rounded apical margin, with a marked concentration of the genera closer to the narrowed apex shape along the apical margin, which is similar to the PCA.

Wings at species level

Due to smaller group sizes at species level, a between-group PCA (BG-PCA) was performed at species level for fore wing and hind wing (Figure 6.7 and 6.8). A CVA was included for comparison, and to examine effects on the larger group sizes.

Fore wings BG-PCA and CVA (Figure 6.7 a – d)

The BG-PCA does not separate the species as clearly as the CVA does (Figure 6.7a and c); possibly as a result of over-estimation of the CVA due to the small sample sizes for some of the groups. Species A separates out clearly in both BG-PCA and CVA. The Mahalanobis distances ranged from 3.54 (*Lestagella penicillata* vs *L. sp2*) to 18.56 (*L. sp3* vs *Ephemerellina barnardi*). Possibly due to the small sample sizes in some species represented here (Table 6.1), not all pairwise permutation tests indicated that mean shapes differed significantly among taxa (Appendix 6B). Procrustes distances ranged from 0.012 (*Lestagella penicillata* vs *L. sp2*) to 0.073 (*L. sp3* vs *Ephemerellina barnardi*). This suggests small to moderate shape differences between species. The CV1 and CV2 scores scatter plot show similar trends as the CV1 and CV2 trends seen for the genera (see above).

Hind wing species level CVA and BG-PCA (Figure 6.8 a – d)

As seen in the fore wings, the BG-PCA does not show such clear, distinct groups as the CVA. The possible reasons for this are outlined in the fore wing section above. The shape change for BG-PCA is similar as in the CVA (Figure 6.8b and d). When considering the hind wing at species level, there is a degree of separation of taxa (Figure 6.8a), however some groups show overlap. The Mahalabobis distances range from 3.31 (*L. sp3* vs *L. sp1*) to 12.74 (*L. sp5* vs *Ephemerellina barnardi*), not all pairwise permutation tests were significant (Appendix 6B). Procrustes distances had a range from 0.023 (*Nadinetella brincki* vs *Lithogloea harrisoni*) to 0.137 (*L. sp5* vs *Ephemerellina barnardi*), indicating that most are large shape differences. The CV1 and CV2 scores scatter plot show similar trends as the CV1 and CV2 trends seen for the genera (see above).

Lestagella fore and hind wing shape variation between catchments (Figure 6.9 – 6.10)

As an exploratory part of this study, the separation of groups due to catchment was investigated (see map in Figure 6.2). *Lestagella* was used as a model dataset as this genus had the largest sample size. The landmark number was reduced to 9 landmarks for the fore and hind wings (Figure 6.1, red-marked landmarks removed).

Fore wings CVA and BG-PCA (Figure 6.9a – d)

The BG-PCA does not separate groups as clearly as the CVA; however both show the same shape changes as the CVA (Figure 6.9b, d). Looking at the various catchment localities for the BG-PCA (Figure 6.9a), Keiskamma and Tsitsikamma (AFR) are distinct groups. The CVA for *Lestagella* fore wing shows overlap in wing shape between some of the catchments (Figure 6.9c), particularly for the Olifants, Breede and Eerste catchments, while Keiskamma and the Cape Peninsula (Liesbeek) catchments appear well separated, Outeniqua (AFR) is only represented by one datapoint, but this was not excluded from the analysis as the basic shape was still of interest, even though the grouping may not be statistically viable. According to shape, the Outeniqua (AFR) is more similar to the Tsitsikamma (AFR) group for the hind wing than the fore wing (Figure 6.9a, c).

The Mahalanobis distances ranged from 1.72 (Breede vs Eerste) to 7.30 (Cape Peninsula vs Outeniqua[AFR]), and pairwise permutation tests were significant for most groups except

those seen in Appendix 6B. The Procrustes distances ranged from 0.013 (Olifants vs Eerste) to 0.038 (Tsitsikamma [AFR] vs Cape Peninusla), indicating small to moderate shape differences between catchments. The CV1 scores scatter plot shows a gradient from a narrow apical margin with a more extended anal margin to a more rounded apical margin with a less prominent anal margin (Figure 6.9d). CV2 scatter plot of scores show a shape range from shorter wing with a broader wing base to a slightly longer wing with a narrower wing base.

Hind wings CVA and BG-PCA (Figure 6.10a –d)

The BG-PCA shows some separation at species level, with some overlap. Clear distinctions can be seen between some of the catchments. Shape changes are similar between the two analyses. The CVA for hind wing shows distinct group separation according to catchment (Figure 6.10c).

The Mahalanobis distances ranged from 3.95 (Breede vs Keiskamma) to 9.72 (Keiskamma vs Outeniqua [AFR]). Pairwise permutation tests indicated that most mean shapes differed significantly between catchments (Appendix 6B), with the exception of the Outeniqua catchment which paired with the Eerste, Keiskamma and Olifants catchments (this could be as a result of the small sample size representing Outeniqua, see fore wing section above). The Procrustes distances ranged from 0.021 (Breede vs Keiskamma) to 0.093 (Cape Peninsula vs Outeniqua[AFR]), suggesting moderate shape differences between catchments. The CV1 scatterplot of CV1 scores shows a gradient change from a more pointed apical margin to a rounded hind wing, CV2 from a shorter, more rounded hind wing to a longer and more oval shape.

The arrangement of groups in the plots relate to the geographic locations of the river catchments, suggesting that there are geographic clines in the wing shape of this genus across its range.

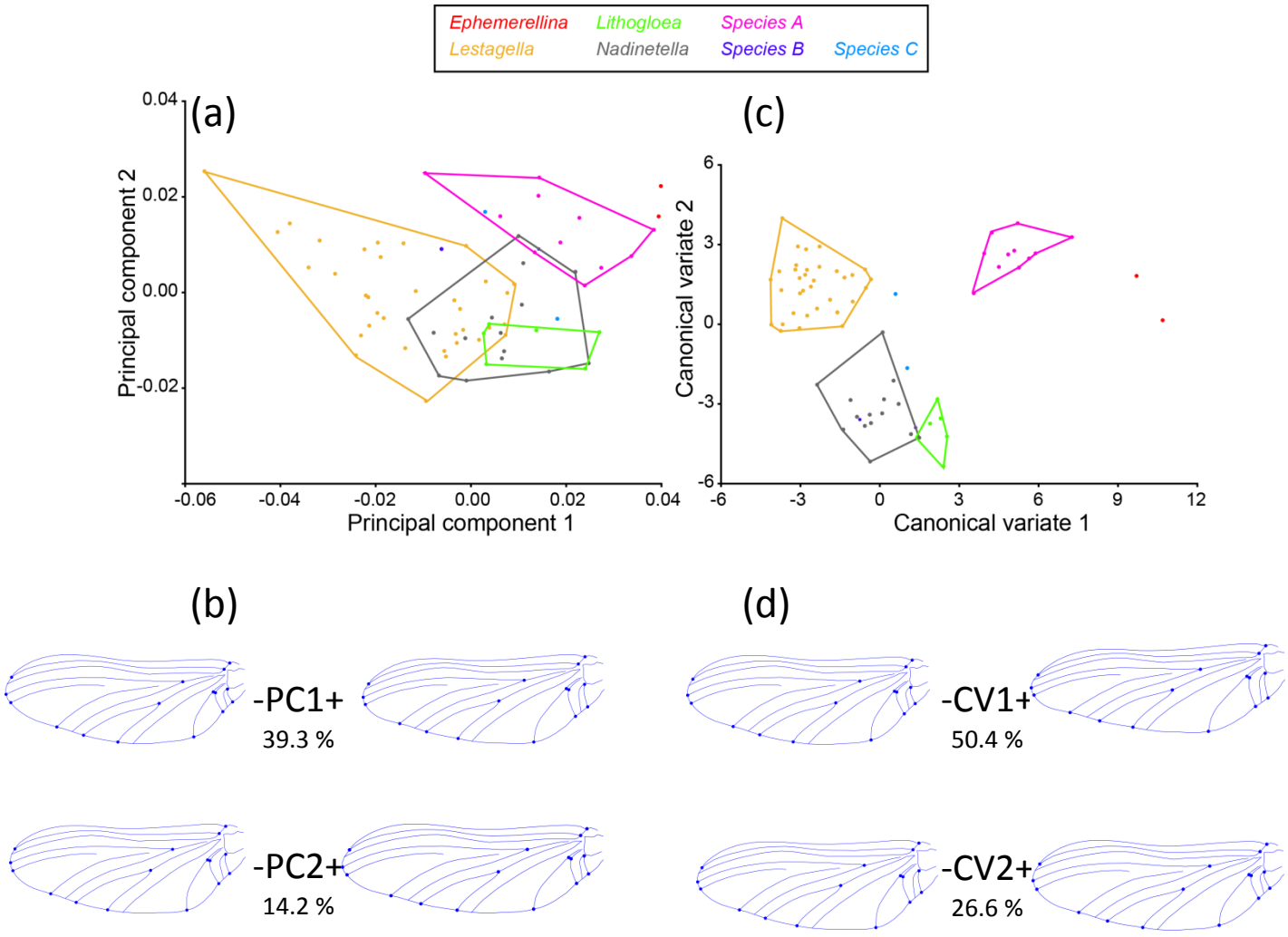


Figure 6.5. Generic level fore wing shape variation of (a) standard PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate.

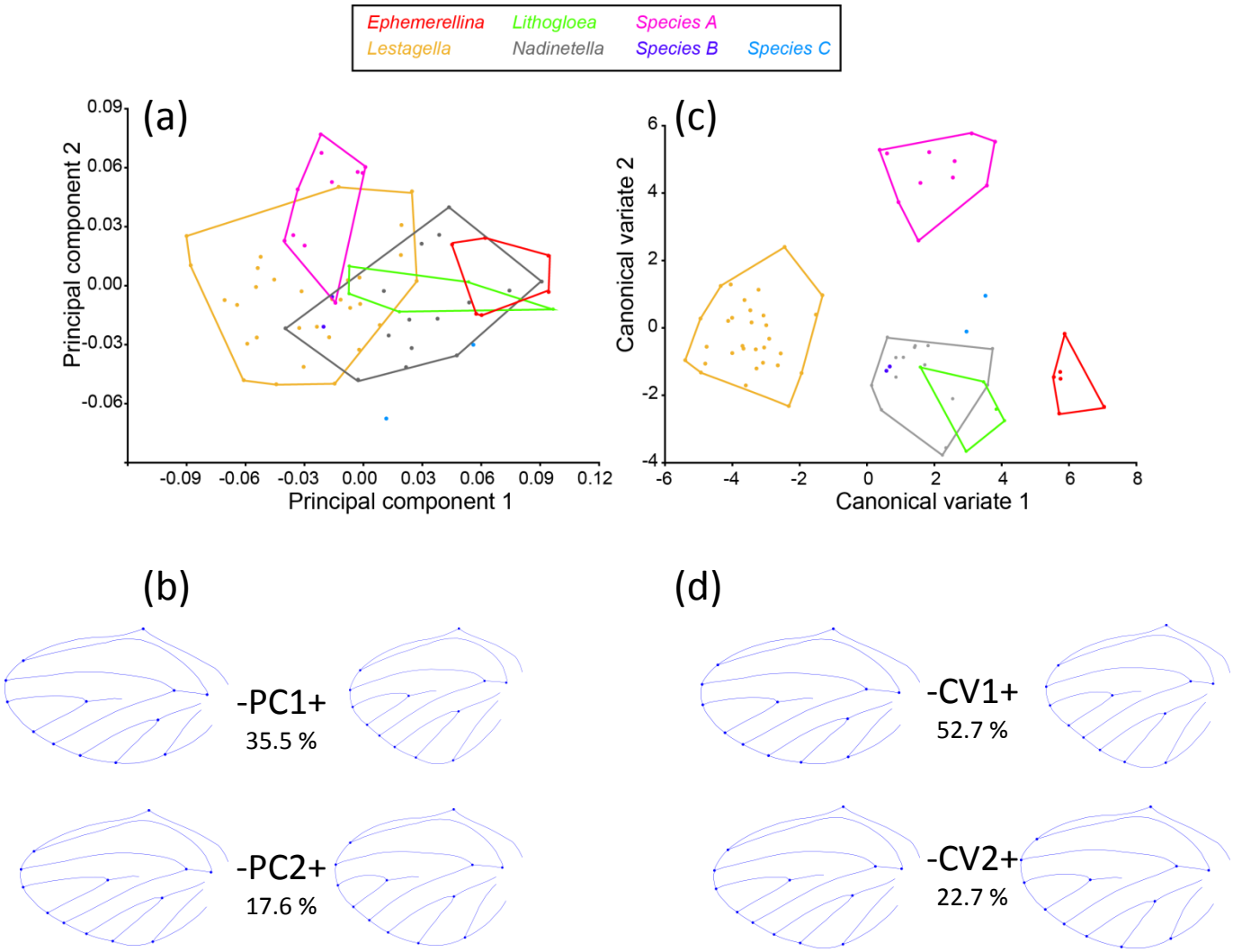


Figure 6.6. Generic level hind wing shape variation of (a) standard PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate.

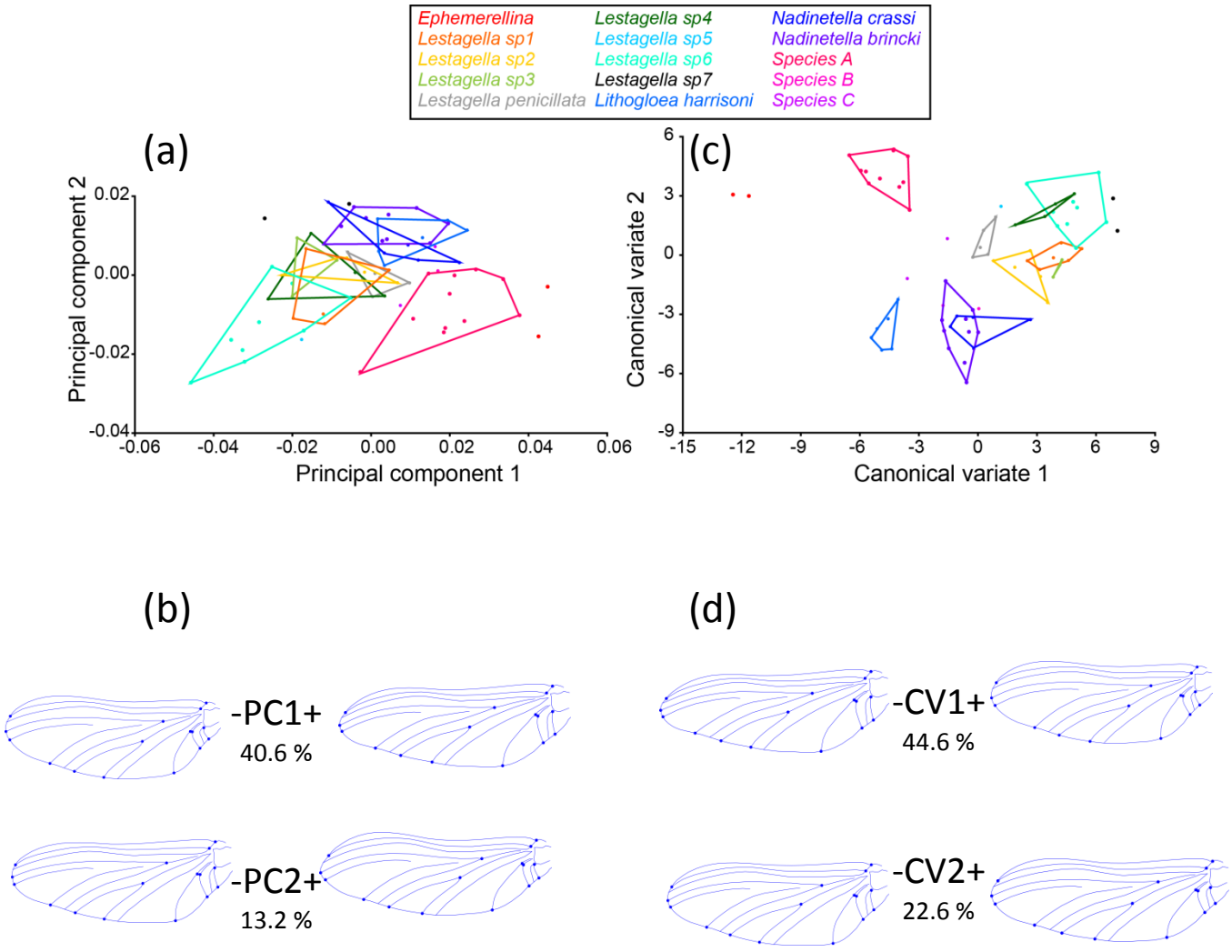


Figure 6.7. Species level fore wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate.

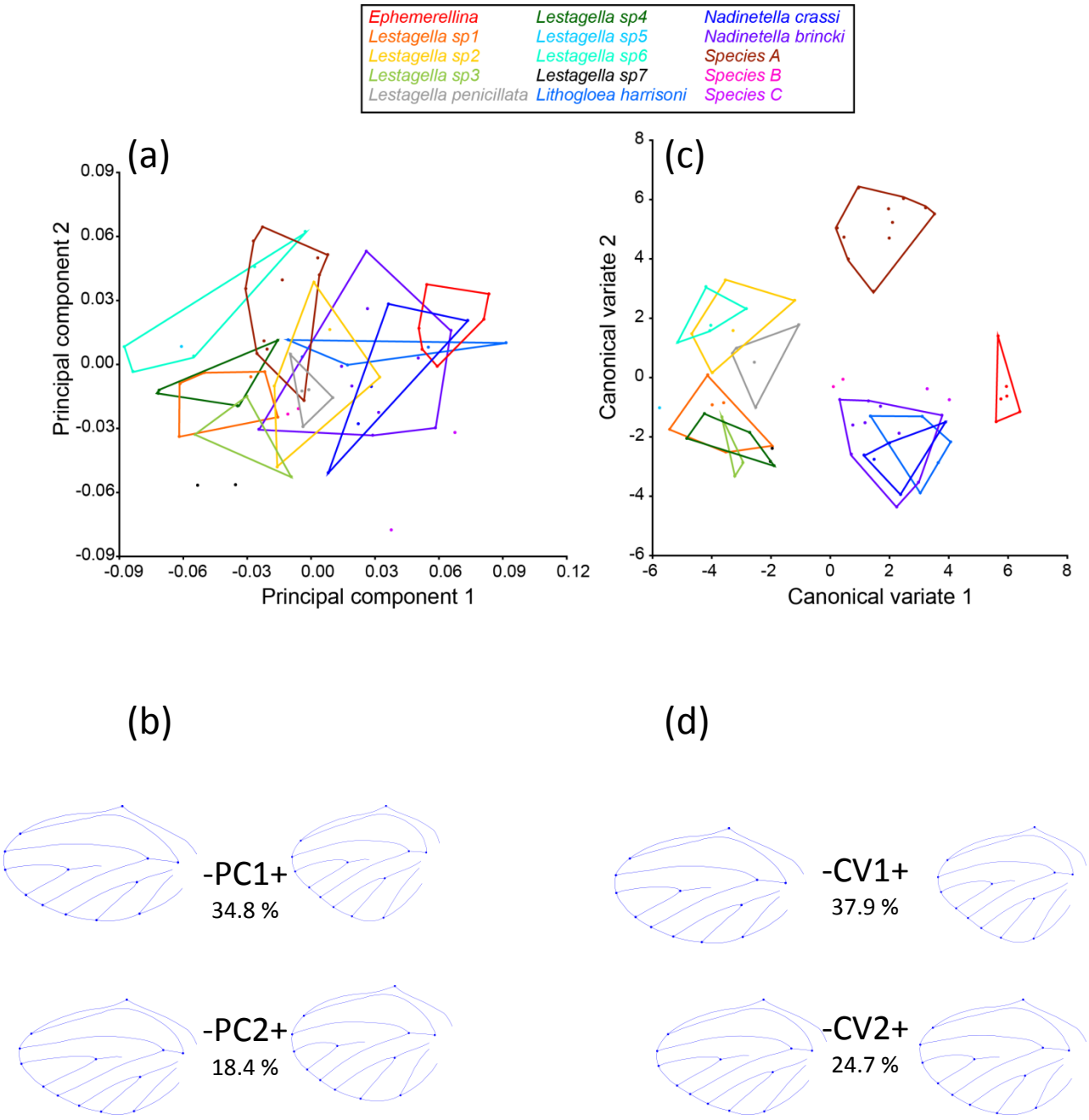


Figure 6.8. Species level hind wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate.

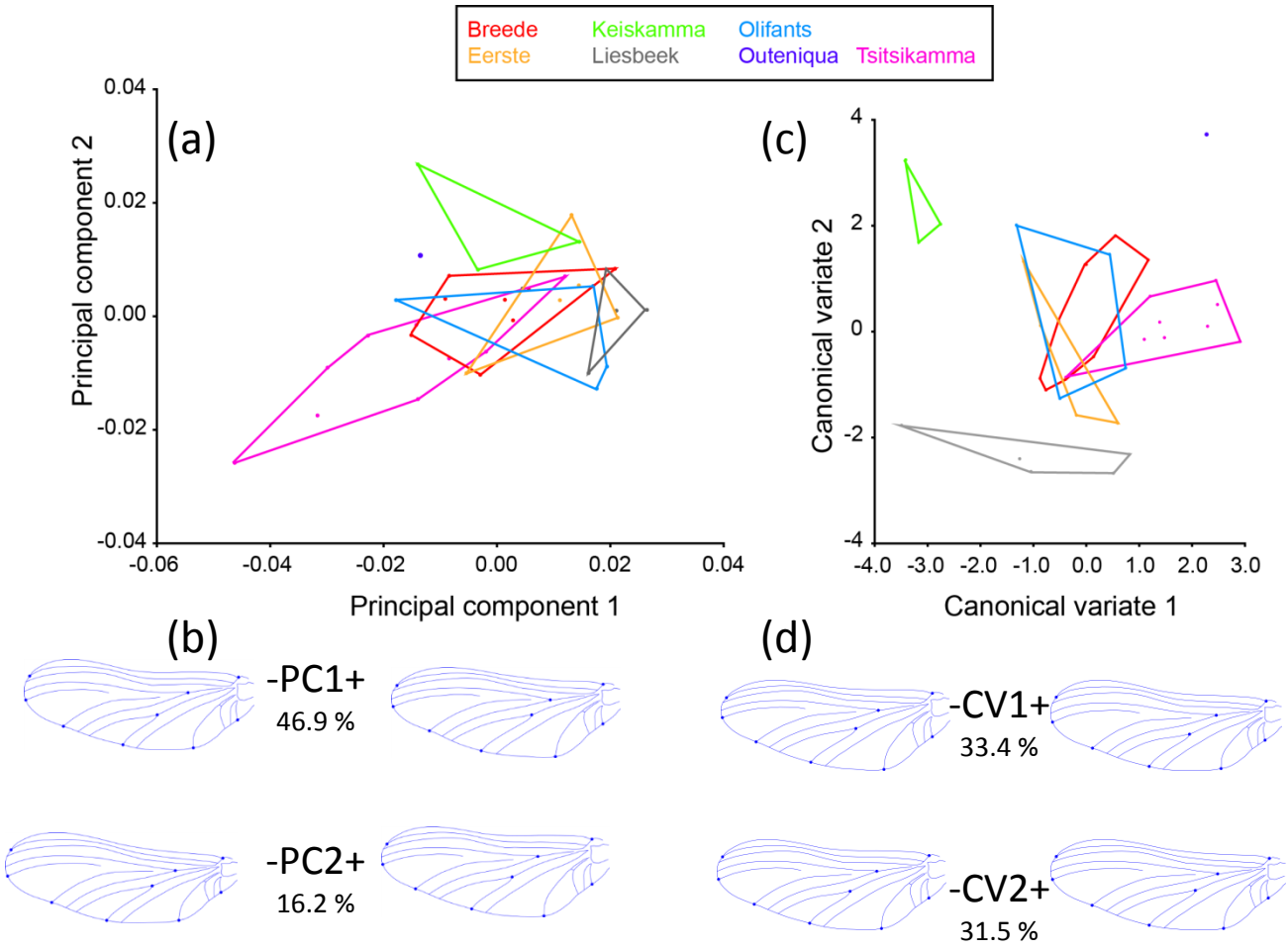


Figure 6.9. *Lestagella* populations separated by river catchment locality, fore wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate. Data analyses conducted with nine landmark configurations.

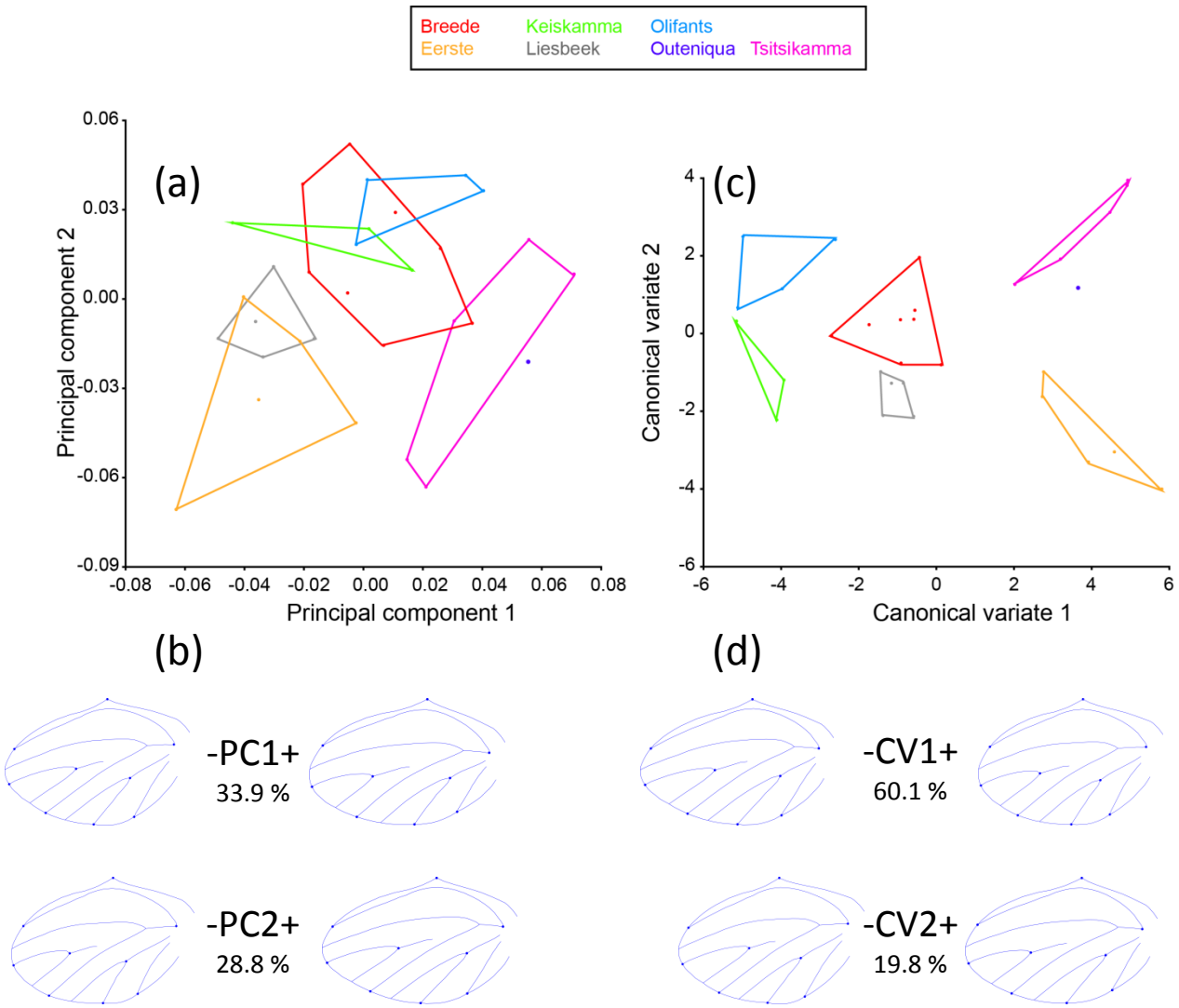


Figure 6.10. *Lestagella* populations separated by river catchment locality, hind wing shape variation of (a) BG-PCA scatter plot with convex hulls to show trends among groups and (b) shape changes shown for the first two principle components (where majority of shape variation was found). (c) CVA scatter plot with convex hulls depicting trends among groups and (d) shape changes shown for the first two canonical variates (where majority of shape variation was found). Percentage values indicate amount of variation accounted for within corresponding component or variate. Data analyses conducted with nine landmark configurations.

6.3.3 Phylogeny and Patterns of Evolutionary Diversification

PCA mapped phylogenies

The projection of the phylogenetic tree into the PC plots by squared change parsimony for the fore wing at species level shows a small amount of branch crossing (visual indication of homoplasy). The data clearly has phylogenetic structure, reflected in the shape space (Figure 6.11a – b).

Mapping the fore and hind wing shapes onto the phylogeny (Figure 6.11) using squared-change parsimony returns the tree length (Table 6.7) in squared Procrustes distance units. The permutation tests for phylogenetic signal in the shape data for both fore and hind wing at genus level were not significant. For species, both fore and hind wing shape data were significant (Table 6.7). Therefore, comparative analyses were only performed on the species level for both fore and hind wing.

Table 6.7. Tree lengths and phylogenetic signal permutation test results for the generic and specific taxon levels for the fore and hind wings.

	Fore wing		**Hind wing**	
	Genus	**Species**	**Genus**	**Species**
Tree length	0.00226	0.00367	0.0158	0.0243
Phylogenetic signal (P-value)	0.109	0.0001*	0.5883	0.0108*

* Significant result, and rejection of the null hypothesis of no phylogenetic signal

The PCA of the variation of mean wing shape (wings are averaged by taxon) shows that most of the variation is contained in relatively few dimensions, with the first three PCs accounting for approximately 80 % and more of the total variance in the sample for genus (fore wing: Table 6.8, hind wing: Table 6.9). The shape changes associated with the PCs show some of the main features of wing variation (Figure 6.11a – b).

Fore wing species level mapped PCA of taxon means (Figure 6.11a)

There is phylogenetic structure in the fore wing data, some branch crossing within the Species A, Species B and Species C indicates homoplasy in these groups. The *Lestagella*

groups are well separated, the L1 group (*L.* sp7) and the Outeniqua/Tsitsikamma (AFR: *L.* sp5 and *L.* sp6) have a distinct shape, while the remainder of the group have fairly similar shapes. *Nadinetella* and *Lithogloea* exhibit morphometric similarities, which supports the phylogenetic relationship.

PC1 is associated with a lengthening of the fore wing as a whole, going from a rounded, blunter apex to a more downwardly pointed curve of the apical margin. PC2 represents an axis of variation between the relative length and prominent medio-anal margin extension of the wing and PC3 features variation between wings with a relatively broad wing base, more slender wing to a more extended, margin and narrower wing base and longer **MA_1** and **MA_2** veins.

Hind wing species level mapped PCA of taxon means (Figure 6.11b)

The three PCs in the hind wing data show that Species A, B and C are better separated, however *Lestagella*, *Nadinetella* and *Lithogloea* indicate moderate homoplasy. PC1 is associated with a costal-anal margin broadening, while PC2 ranges from a more oblong and narrow hind wing to a more rounded shape with a shortening of the **RSa** and **RSp** veins. PC3 features variation between hind wings where the anal margin becomes more extended, while the apex of the wings stays relatively constant in shape.

There is a trend emerging, with a consistent direction of shape variation within the first three PCs for both the fore and hind wings, from one extreme side of the plot (*Lestagella*) to the other extreme side (*Ephemerellina* and Species A, B and C).

Pooled within- and among-taxa variation (Figure 6.12)

The first two PCs of the PCA of pooled within-taxon covariance matrix (Figure 6.12) account for almost half of the variation in both the fore and hind wing at species level. The fore wing PC1 is associated with a shortening of the wing combined with a slight extension of the anal margin while PC2 is associated with a rounding of the wing apex, resulting in a blunter apical margin. The hind wing PC1 shows a shape change with a broadening of the costal-anal margin combined with a rounding of the entire wing shape and a shortening of the **RSa** and **RSp** veins. PC2 features variation between hind wings where the anal margin becomes more extended or rounded, while the apex of the wings stays relatively constant in shape (also with a shortening of the **RSa** and **RSp** veins).

A matrix correlation between the pooled within-taxa covariance matrix and the covariance matrices among-taxon means was computed as a measure of the overall similarity of the covariance structures, and then the correlation between pooled within-taxa matrices and the among-taxa IC of wing shapes for species means was calculated. Fore wing and hind wing species pooled within-taxa and among-taxa matrix correlations had fairly strong matrix correlations at 0.64 and 0.72 respectively and both highly significant ($P < 0.0001$). The pooled within-taxon and IC of wing shapes for fore wing and hind wing species had fairly strong matrix correlations at 0.76 and 0.71 respectively, both highly significant ($P < 0.0001$). This indicates that both the fore and hind wings show similar patterns of divergence in the within- and among-taxa matrices, therefore there are no serious anomalies in the datasets.

Table 6.8. Fore wing PCA of variation among the shapes of generic means, both for original and size-corrected shape data.

	Species means			
	Uncorrected		**Size-corrected**	
	Eigenvalues	**% Total variance**	**Eigenvalues**	**% Total variance**
PC1	0.000341	60.86	0.000181	51.57
PC2	0.000111	19.82	0.000101	28.72
PC3	0.0000600	10.72	0.0000378	10.77
PC4	0.0000263	4.70	0.0000227	6.47
PC5	0.0000142	2.54	0.00000867	2.47
PC6	0.00000768	1.37		

Table 6.9. Hind wing PCA of variation among the shapes of generic means, both for original and size-corrected shape data.

	Species means			
	Uncorrected		**Size-corrected**	
	Eigenvalues	**% Total variance**	**Eigenvalues**	**% Total variance**
PC1	0.00149	44.49	0.00123	53.44
PC2	0.00104	31.04	0.000508	22.12
PC3	0.000413	12.35	0.000338	14.73
PC4	0.000237	7.08	0.000201	8.73
PC5	0.000148	4.42	0.0000226	0.98
PC6	0.0000211	0.63	0.00123	53.44

Table 6.10. Fore wing PCA of variation among the shapes of species means and independent contrasts, both for original and size-corrected shape data.

	Species means				**Contrasts**			
	Uncorrected		**Size-corrected**		**Uncorrected**		**Size-corrected**	
	Eigenvalues	% Total variance	Eigenvalues	% Total variance	Eigenvalues	% Total variance	Eigenvalues	% Total variance
PC1	0.000328	55.03	0.000203	45.89	0.000782	41.74	0.000432	36.20
PC2	0.0000761	12.77	0.0000686	15.55	0.000392	20.96	0.000242	20.26
PC3	0.0000683	11.46	0.0000580	13.13	0.000252	13.46	0.000186	15.59
PC4	0.0000497	8.35	0.0000433	9.80	0.000164	8.74	0.000148	12.45
PC5	0.0000269	4.52	0.0000287	6.51	0.000102	5.45	0.0000613	5.14
PC6	0.0000178	2.99	0.0000146	3.31	0.0000578	3.09	0.0000525	4.41
PC7	0.0000122	2.05	0.00000908	2.06	0.0000487	2.60	0.0000385	3.23
PC8	0.00000734	1.23	0.00000860	1.95	0.0000404	2.16	0.0000160	1.34
PC9	0.00000510	0.86	0.00000503	1.14	0.0000156	0.84	0.0000101	0.85

Table 6.11. Hind wings PCA of variation among the shapes of species means and independent contrasts, both for original and size-corrected shape data.

	Species means				**Contrasts**			
	Uncorrected		**Size-corrected**		**Uncorrected**		**Size-corrected**	
	Eigenvalues	% Total variance	Eigenvalues	% Total variance	Eigenvalues	% Total variance	Eigenvalues	% Total variance
PC1	0.00152	47.16	0.00126	43.31	0.00473	40.78	0.00383	45.02
PC2	0.000691	21.43	0.000814	28.00	0.00363	31.25	0.00250	29.45
PC3	0.000414	12.83	0.000341	11.75	0.00130	11.19	0.000696	8.19
PC4	0.000198	6.15	0.000150	5.15	0.000582	5.02	0.000419	4.93
PC5	0.000117	3.64	0.000110	3.77	0.000404	3.48	0.000390	4.59
PC6	0.0000916	2.84	0.0000882	3.03	0.000326	2.81	0.000267	3.15
PC7	0.0000846	2.62	0.0000614	2.11	0.000203	1.75	0.000155	1.83
PC8	0.0000401	1.25	0.0000393	1.35	0.000160	1.38	0.0000986	1.16
PC9	0.0000364	1.13	0.0000252	0.87	0.000117	1.01	0.0000782	0.92

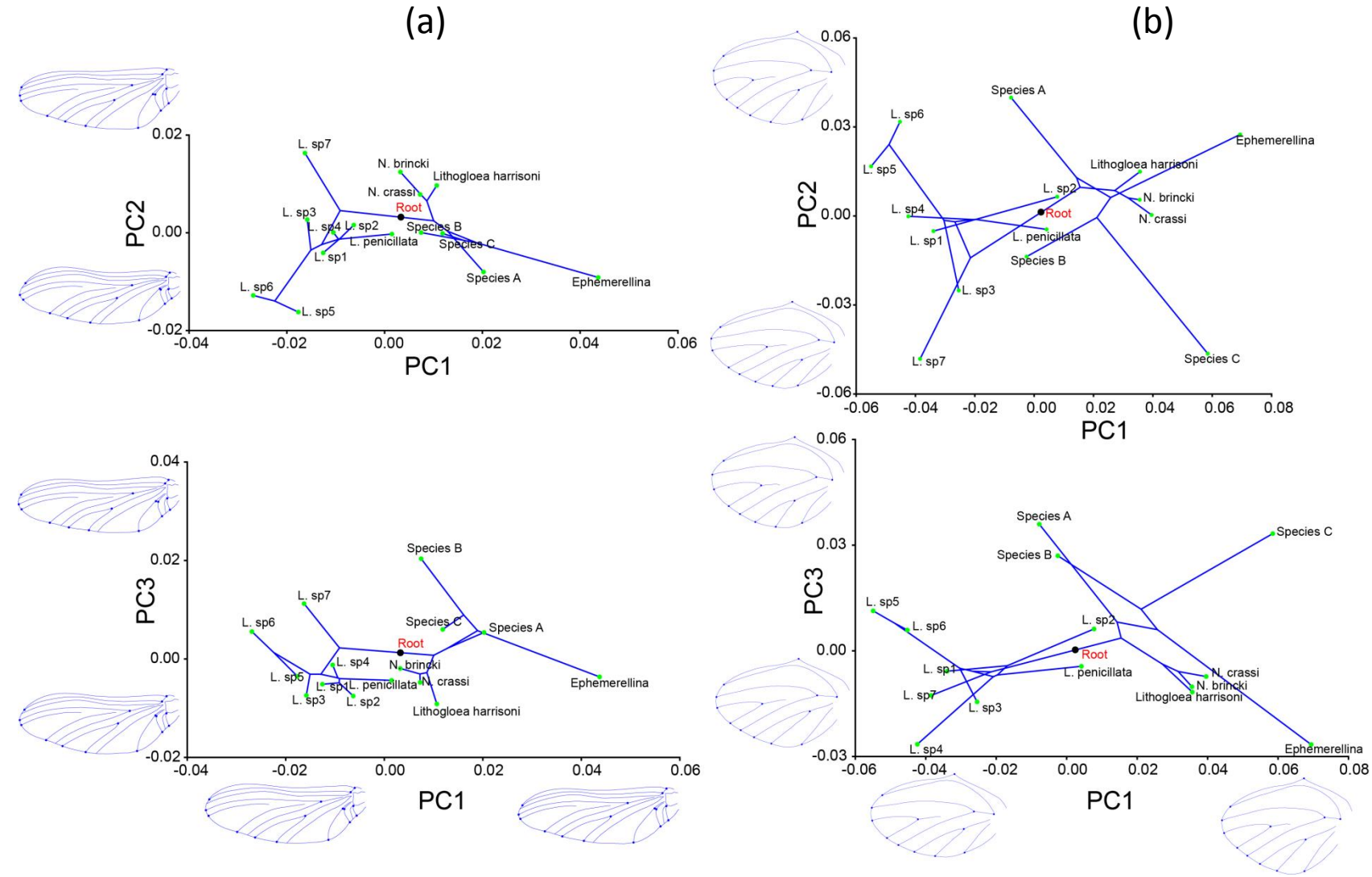


Figure 6.11. PCA-mapped phylogenies showing phylogenetic structure for uncorrected shape variation and changes for, (a) fore wing at species level, showing shape changes for PC1 – 3, and (b) hind wing at species level, showing shape changes for PC1 -3.

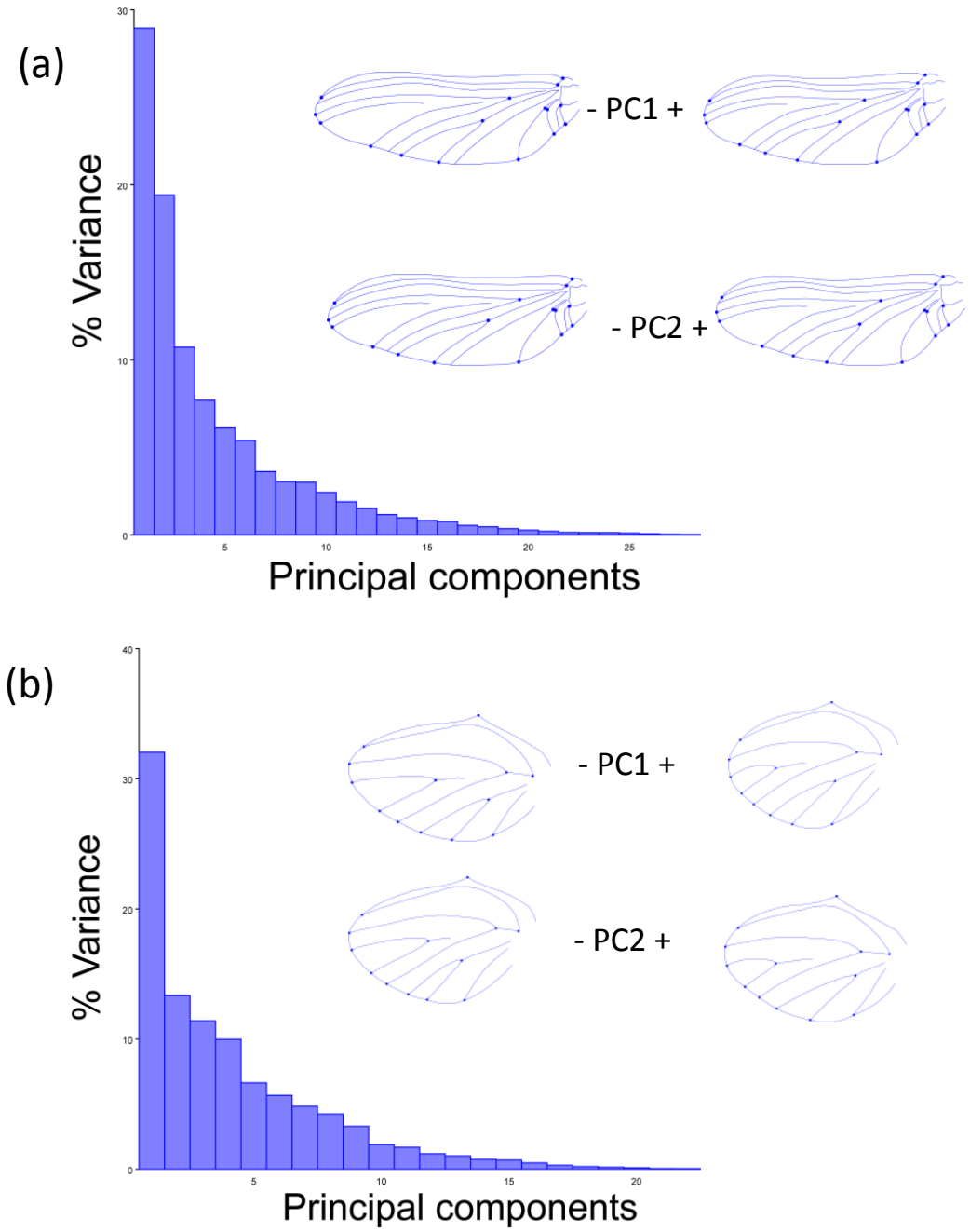


Figure 6.12. PCA histogram plots of Pooled within-taxa covariance matrix of wing shape, showing the first two PCs for (a) Fore wings at species level (showing scale factors of shape change from -0.04 to +0.04), and (b) hind wings at species level (-0.09 to +0.09).

6.3.4 Evolutionary Allometry (Figure 6.13)

The *Lestagella* sample from the Outeniqua (*L.* sp5) was found to be a considerable outlier that affected the regression analyses and skewed the results in the data for analyses on size; this sample was not found to be an outlier for shape. On checking the source files and the scale settings, the source image proved to be correctly captured and the specimen is unusually small. This could be explained by varying environmental factors between localities; sizes of individuals (particularly aquatic invertebrates) may vary slightly depending on factors such as temperature or altitude (this pattern has been seen in Simuliidae, de Moor 1982, 1989). Seasonality and temperature were not factors as the individual was collected in the summer season (as were the other samples) and temperatures were similar. The specimen was collected at a lightly lower altitude (150 m, while other Tsitsikamma sites were higher than 240 m), this is unlikely to have had an effect. Another difference observed between sites was an unusually high value for the Total Dissolved Solids (TDS) and Electrical Conductivity (EC), almost double that of the other sites. It is not possible to determine the exact cause at this stage, possibly attributed to the geology or perhaps fertilizer from possible farming upstream. Unfortunately there is only one specimen from this locality, so it is not possible to establish if there is an unusual trend within this group. Until there is a larger sample size for this locality, the observation was removed from any analyses that took size into account.

As the dataset covers a range of taxa within the family, allometry may be an important potential factor. To examine whether allometry influences patterns of variation, the IC of shape were regressed onto the IC of log transformed centroid size. The multivariate regression shows a clear relationship that accounts for 30.96 % and 17.82 % of the shape variation for fore wing and hind wing species respectively, with high significance for the fore wings (P = 0.0005) and just significant for hind wings (P = 0.0450). It thus indicates strong evolutionary allometry in the fore wings and moderate evolutionary allometry for the hind wings.

Size-corrected fore wing species level mapped PCA of taxon means (Figure 6.13a)

Incorporating the effects of allometry has resulted in good separation between Species A, B and C in the first two PCs, however some homoplasy is evident in the third PC for Species B and Species C, and between *Nadinetella* and *Lithogloea*. Within *Lestagella*, *L.* sp7 has

become more distinct, *L.* sp3 (Keiskamma catchment) has separated from the general cluster and *L*. sp6 has clustered with the other *Lestagella* populations with some branch crossing.

The shape change (Figure 6.13a) associated with evolutionary allometry in the fore wing is a relative lengthening of the fore wing combined with a narrowing of the apical or disto-costal wing margin and extension of the proximo-anal margin with increasing size. This shape change may be an important factor is aiding the larger mayflies (e.g. *Ephemerellina*) in flight.

Size corrected hind wing species level mapped PCA of taxon means (Figure 6.14b)

After the effects of allometry were removed, homoplasy within the hind wing increased considerably, particularly within *Lestagella*. Species A, C, *Nadinetella, Lithogloea* and *Ephemerellina* are well separated.

The shape change associated with evolutionary allometry of hind wing is relative narrowing of apical margin and broadening of the costal-anal margin shape of the wing near the base, along with the lengthening of the **RSa** and **RSp** veins with increasing size (Figure 6.13b).

Even after the effects of allometry were removed, the trend of a consistent direction of shape variation within the first three PCs for both the fore and hind wings, from one extreme side of the plot (*Lestagella*) to the other extreme side (*Ephemerellina* and Species A, B and C) is still apparent. Size-corrected shape changes seen for each genus, compared with the average wing shape, can be seen in Appendix 6C, to further illustrate wing shape variation.

Size-corrected pooled within- and among-taxa variation (Figure 6.13)

The pooled within-taxon regression accounts for 5.64 % and 3.55 % of the fore wing and hind wing shape variation within the species respectively and are both statistically significant ($P = 0.0005$ and $P = 0.0214$ for fore wing and hind wing respectively). The within-taxon allometry is associated with the fore wing shape that is similar to the among-taxon allometry result, except not as pronounced (Figure 6.13c). The result is similar for the hind wing where the allometry is also not as extreme as shown in the among-taxon, evolutionary allometry assessment.

The within-taxon allometry was used to correct for the effects of size in the data by computing residuals, the results of the PCAs for all these size corrected data are similar to the analyses presented above (Figure 6.12), (size-corrected PCA results not shown), it is

important to note that the dominance of the PC1 in the size-corrected analyses (for both fore wing and hind wing) are more pronounced than in the original shape data. Also, the high and statistically significant matrix correlations between the different levels (see previous section) of variation remain the same.

Applying the regression vector retrieved from the regression with IC and computing the residual component of variation in taxon averages provides shape scores that are free of the effects of evolutionary allometry (Figure 6.14). PC1 of this analysis now accounts for less than in the analysis of size-uncorrected shape, in terms of both the absolute amount (eigenvalue) and the proportion of the total variance (Table 6.10 and 6.11). The shape features associated with the first three PCs are similar to those of the PCA of total shape variation; however the size-corrected shapes are not as prominent (Figure 6.14, Appendix 6C).

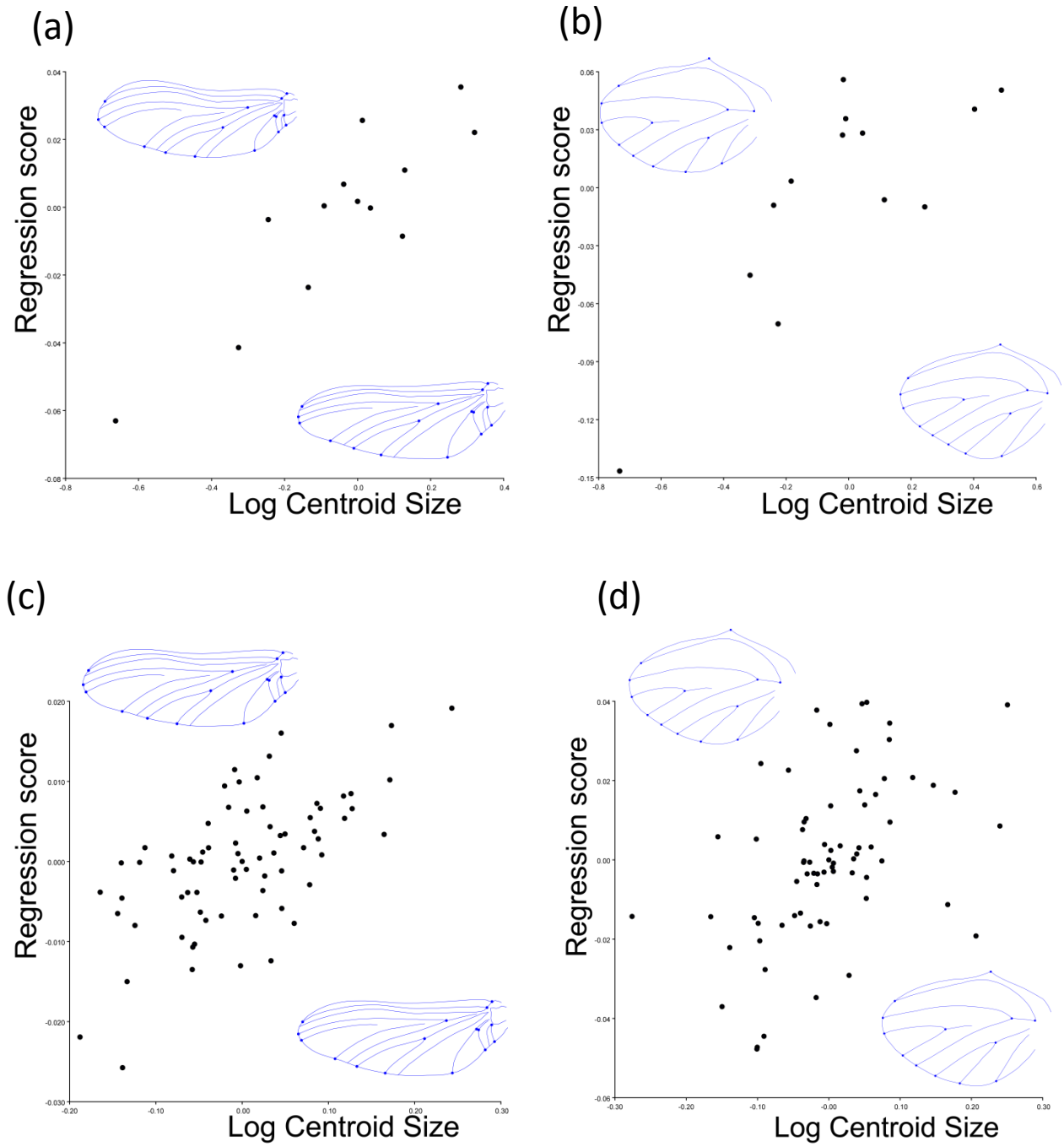


Figure 6.13. (a) evolutionary allometry between species in fore wing, (b) Evolutionary allometry between species in the hind wing (c) allometry within-taxa in the fore wing, (d) hind wing pooled within-taxa, (c and d) characterised by pooled within-group regression of shape of log-transformed centroid size. The scatter shows the individual deviations from the taxon means of shape and log transformed centroid size.

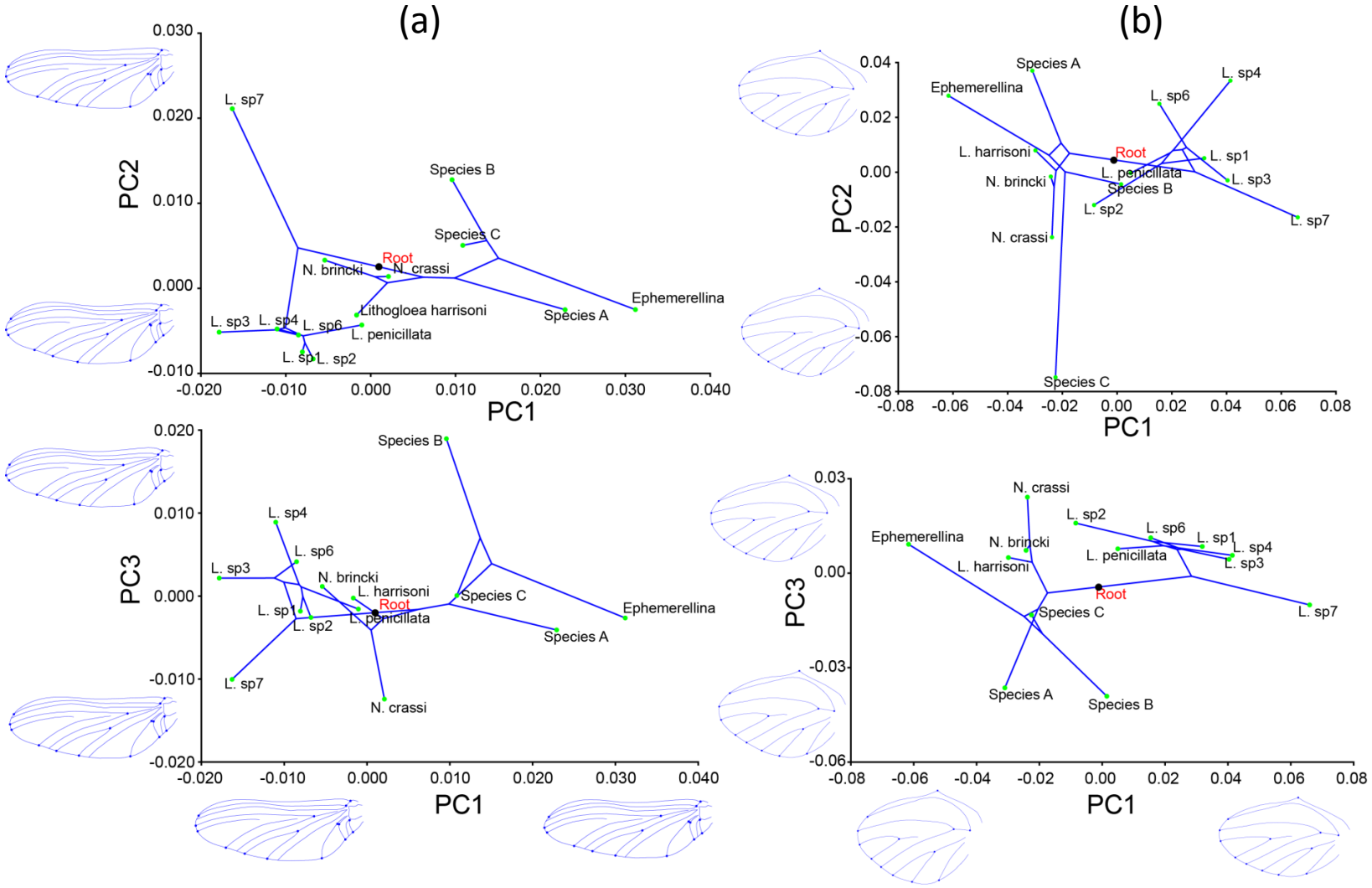


Figure 6.14. PCA-mapped phylogenies showing phylogenetic structure for size-corrected shape variation for, (a) fore wing at species level, showing changes for PC1 – 3, and (b) hind wing at species level, showing shape changes for PC1 -3. Shapes representing each genus after size correction can be seen in Appendix 6C.

6.3.5 Patterns of variation in evolutionary changes

Fore wing

To examine the patterns of evolutionary variation in the wing shape, the covariance matrix of IC was examined with a PCA (Fig 6.15 and Table 6.10). The shape changes associated with the first two PCs (Fig 6.15a) clearly resembles the corresponding PCs in the analysis of species means (Table 6.12 Angular comparison). PC3 in the analysis of species means is more similar to PC4 in the IC (Table 6.12). The eigenvalues of the IC and the corresponding eigenvalues in the PCA for species-means both show the majority of variation accounted for in the first three PCs (Table 6.10).

The shape change for the PC1 of IC (Figure 6.15a) is similar to the shape change associated with the allometric regression vector (Figure 6.13a; angle 17.0°, $P < 0.00001$). As the PC1 takes up 41.7 % of the total variation of IC of shape (Table 6.10) and the allometric regression accounts for 31.0 %, it appears that the evolutionary allometry is an important factor in fore wing shape diversification, although other processes probably contribute to the same features of evolutionary shape variation (such as some of the other PCs in the analysis, but in smaller degrees).

To characterize the patterns of evolutionary change without allometric effects, a PCA of the residuals from the regression of IC of shape on the IC of log-transformed centroid size was used (Figure 6.15b). There is a clear resemblance between the shape change for PC1 for size corrected contrasts and PC2 for uncorrected contrasts, and PC2 of size-corrected contrasts with PC3 of uncorrected contrasts, both PC1s (for size-corrected and uncorrected) also show some significant resemblance (Table 6.13).

Hind wing

The shape changes associated with the first three PCs (Fig 6.16a) clearly resembles the corresponding PCs in the analysis of species means (Table 6.12. Angular comparison). The eigenvalues of the IC and the corresponding eigenvalues in the PCA for species means both show that the majority of variation is accounted for in the first three PCs (Table 6.11).

The shape change for the PC2 of IC (Figure 6.16a) is similar to the shape change associated with the allometric regression vector (Figure 6.13b; angle 38.4°, $P < 0.00001$). As the PC2 takes up 31.2 % of the total variation of IC of shape and the allometric regression accounts

for 17.8 %, it appears that the evolutionary allometry is a factor in the hind wing shape diversification, although other processes probably contribute to the same and other features of evolutionary shape variation.

To characterize the patterns of evolutionary change without allometric effects, a PCA of the residuals from the regression of IC of shape on the IC of log-transformed centroid size was used (Figure 6.16b). There is a striking resemblance between the shape changes for both PC1s (Table 6.13), and a clear resemblance of the PC2s. The PC3 of the size-corrected IC shows some resemblance to the uncorrected contrasts PC3 and PC4, therefore leaving the correspondence ambiguous (Table 6.13).

Table 6.12. Angular comparisons between PCA for species means for complete shape variation and PCA for independent contrasts for complete shape variation, to examine patterns of evolutionary variation in wing shape. Permutation test against the null hypothesis that the vectors have random directions.

Species means	Independent Contrasts	Angle	*P*-value
Fore wing:			
PC1	PC1	34.2°	<.00001
PC2	PC2	46.8°	0.00004
PC3	PC4	39.4°	<.00001
Hind wing:			
PC1	PC1	27.2°	<.00001
PC2	PC2	29.7°	<.00001
PC3	PC3	40.4°	0.00002

Table 6.13. Angular comparisons between PCA for independent contrasts for complete shape variation and size corrected PCA for independent contrasts, to characterise for patterns of evolution without allometric effects. Permutation test against the null hypothesis that the vectors have random directions.

Size corrected	Uncorrected	Angle	*P*-value
Fore wing:			
PC1	PC1	54.5°	0.00096
PC1	PC2	37.9°	<.00001
PC2	PC3	35.5°	<.00001
Hind wing:			
PC1	PC1	8.2°	<.00001
PC2	PC2	27.3°	<.00001
PC3	PC3	53.9°	0.00307
PC3	PC4	40.9°	0.00003

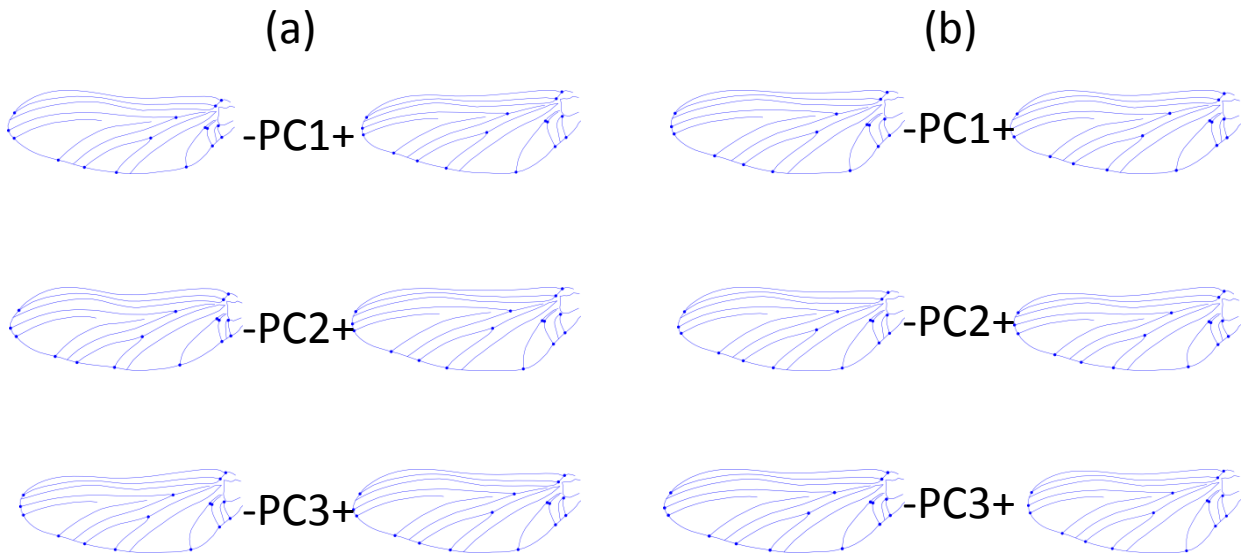


Figure 6.15. Patterns of evolutionary diversification in fore wing shape: a PCA for independent contrasts for complete variation in (a) the species level and, (b) PCA for the residuals from the regression of IC of shape on the IC of log transformed centroid size species.

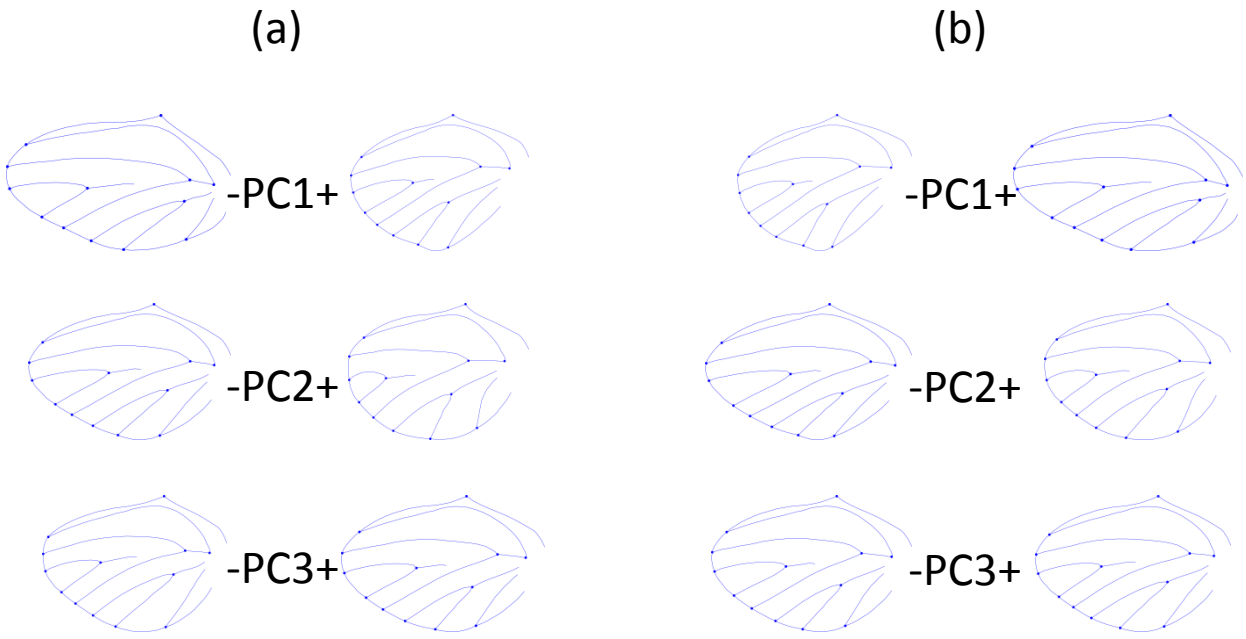


Figure 6.16. Patterns of evolutionary diversification in hind wing shape: PCA for independent contrasts for complete variation a) at species level and (b) PCA for the residuals from the regression of IC of shape on the IC of log transformed centroid size species.

6.3.6 Evolutionary integration

The analysis of independent contrasts (IC) indicates that there is clear evolutionary integration between the fore and hind wing, where the RV coefficient is 0.60, indicating a covariation of fairly high strength, with a significant permutation test (P = 0.0024). For the PLS analysis of IC without size correction, the first three pairs of PLS axes account for 67.0 %, 24.7 % and 3.5 % of the total squared covariance between fore and hind wing, respectively.

The patterns of covariation show agreement with the PCA (of IC) in the fore wings where the PLS1 and PC1 are strikingly similar, while PC2 and PLS2 as well as PC3 and PLS3 show a clear resemblance (Table 6.14).

In the hind wings, the PLS1 shows some resemblance to both PC1 and PC2, making the correspondence somewhat ambiguous, while there was a clear resemblance found between PLS2 – PC2 and PLS3 – PC3 (Table 6.14).

Because allometry is a potential integrating factor, the role in the fore-hind wing integration is assessed by examining the evolutionary covariation between fore and hind wing after eliminating allometric effects from IC of shape. The RV coefficient for size-corrected contrasts is 0.58, only slightly lower than for the analysis without size correction, and is still significant (P = 0.0016). The first three pairs of PLS axes account for 56.5 %, 32.3 % and 5.8 % of the total squared covariance between fore and hind wing, respectively.

For the fore wing, the shape changes of the first two pairs of PLS axes between the uncorrected and size-corrected contrasts do correspond (Table 6.15), however PLS 2 and PLS3 of the uncorrected PLS analysis do also have a significant resemblance to the PLS1 and PLS 2 (respectively) of the size-corrected analysis, therefore creating some ambiguity in this correspondence.

For the hind wing, the size-corrected PLS1 closely resembled the uncorrected PLS2, and size-corrected PLS 2 had a resemblance to the uncorrected PLS1, while the size-corrected PLS 3 most clearly resembled the PLS4 form the uncorrected analysis (Table 6.15).

These patterns of evolutionary can be visualised in Figure 6.17, where size-corrected and uncorrected variation are remarkably similar. Although evolutionary allometry is a contributing to the shape variation, the corrected patterns of shape variation are slightly less

prominent. The basic patterns of integration found between the wings (Figure 6.17) is an associated broadening of the costal-anal margin at the hind wing base with the relative lengthening and extension of the proximo-anal margin of the fore wing (Figure 6.17a, PLS1 and 6.17b; PLS2). A broadening of the apical or disto-costal wing margin of the fore wing is associated with a rounding of the entire hind wing shape (Figure 6.17a, PLS2 and 6.17b, PLS1 and PLS2). In the uncorrected PLS2 and corrected PLS1/PLS2 integration shapes, there is a distinct pattern where a narrowing of the hind wing is associated with an extension of the proximo-anal margin of the fore wing.

Table 6.14. Angular comparisons between PCA for independent contrasts for complete shape variation and Partial Least Squares (PLS) analysis of integration between the fore wing and hind wing. Permutation test against the null hypothesis that the vectors have random directions.

	Angle	***P*-value**
Fore wing:		
PC1 – PLS1	17.3°	<.00001
PC2 – PLS2	30.2°	<.00001
PC3 – PLS3	45.4°	0.00002
Hind wing:		
PC1 – PLS1	35.8°	<.00001
PC2 – PLS1	56.7°	0.00669
PC2 – PLS2	34.2°	<.00001
PC3 – PLS3	48.9°	0.00065

Table 6.15. Angular comparisons between uncorrected Partial Least Squares (PLS) analysis of integration between the fore wing and hind wing and size corrected PLS analysis. Permutation test against the null hypothesis that the vectors have random directions.

		Angle	***P*-value**
Uncorrected	**Size-Corrected**		
Fore wing:			
PLS1	PLS1	45.0°	0.00002
PLS2	PLS1	48.7°	0.00010
PLS2	PLS2	53.0°	0.00055
PLS3	PLS2	54.2°	0.00086
Hind wing:			
PLS1	PLS2	29.5°	<.00001
PLS2	PLS1	41.5°	0.00004
PLS3	PLS4	29.8°	<.00001

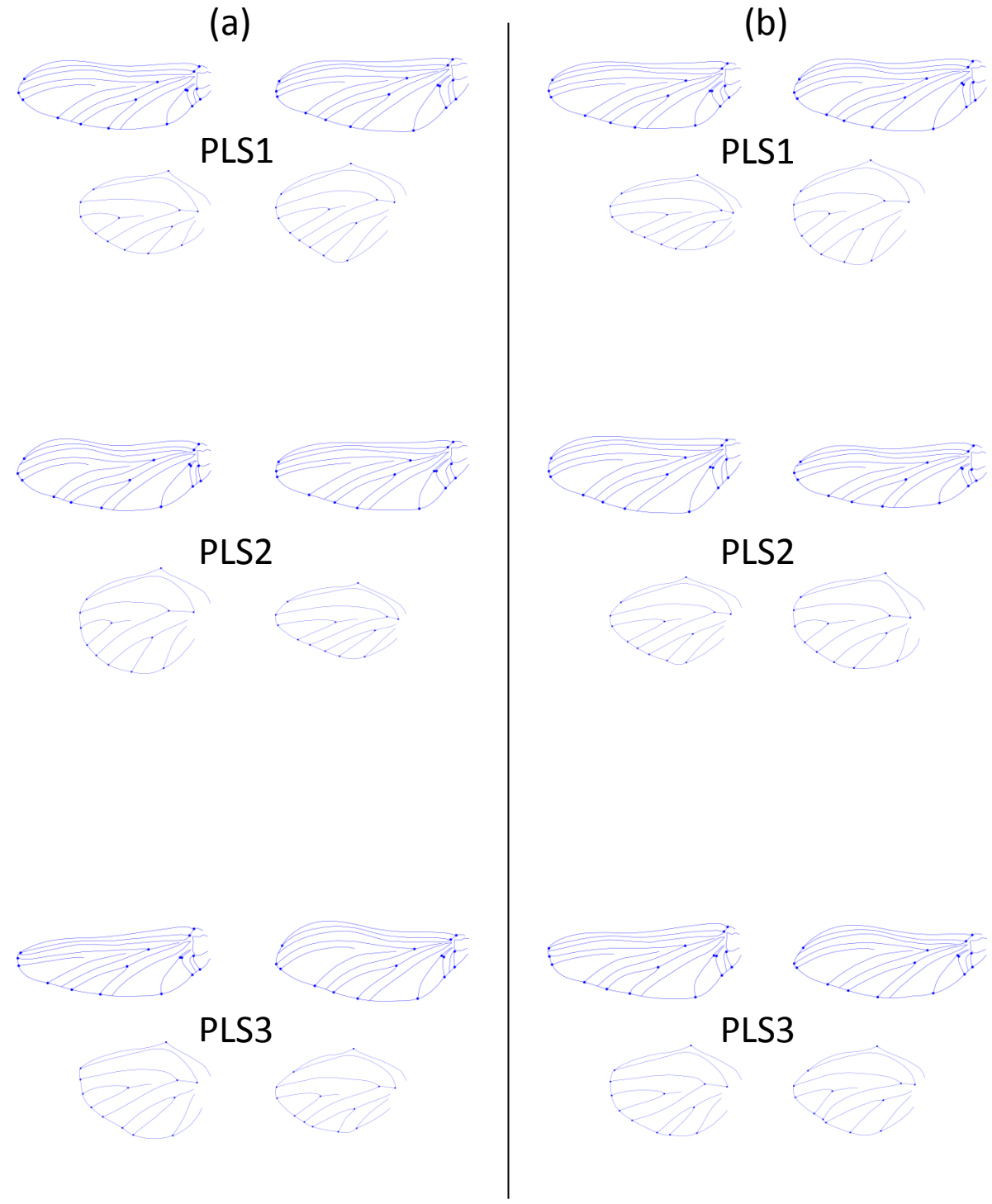


Figure 6.17. Patterns of evolutionary integration between fore wing and hind wing shape: column a) PLS for independent contrasts for complete variation in in the species, column b) PLS (size-corrected) for the residuals from the regression of IC of shape on IC of log-transformed centroid size among species.

6.4 Discussion

This chapter used a multilevel approach in geometric morphometrics that investigates the variation of Teloganodidae wings shapes and their evolution in a comparative context. It explores the distribution of wing shapes in Procrustes shape space and investigates evolutionary allometry and evolutionary integration between the fore and hind wings. The results at generic level did not reveal any phylogenetic signal, and so comparative analyses were based on predetermined species or populations. The analyses showed that there is strong integration of evolutionary changes throughout the wings. The fore and hind wings appear to be integrated strongly and seem to evolve as a single integrated unit, the wings are presumably under the same evolutionary pressures as they are both used in flight. Allometry is a contributing factor to this integration, but it does not account for all the integration in the two wings.

Taxa Variation

From the Procrustes ANOVA determining measurement error on the subset of 25 individuals, there appears to be evidence of directional (one side is systematically larger than the other) and fluctuating asymmetry in the fore wings, which may relate to some developmental and environmental interactions. These interactions on a greater scale can cause phenotypic change, eventually leading to evolution and speciation. The cause of this asymmetry would make an interesting future study (including more samples).

The CVA of wing shape at genus level clearly separates each genus from the other in both fore and hind wings, with some overlap between *Lithogloea* and *Nadinetella* based on the hind wing CV1 – CV2. At the species level, there is clear separation at species level according to the fore wing and hind wing, with *Lithogloea* and *Nadinetella* groups overlapping in the hind wing. Despite considerable differences in morphology between nymphs of *Lithogloea* and *Nadinetella*, molecular evidence (see Chapter Four) and morphometric data show a close phylogenetic relationship.

The BG-PCAs and CVA analyses show that *Lestagella* can be separated according to catchment (Figure 6.10), especially in the hind wing. However the mapped PCA shows branch crossing for some of the populations, indicating a level of homoplasy, especially in the hind wing. However, the results hold true that *Lestagella* can be separated according to catchment. There is also molecular evidence (Chapter Four) for groups (especially

Lestagella) being isolated by catchment, and preliminary phenotypic evidence appears to support the molecular data.

Comparative studies

The shape variation in both for and hind wings at species level contains a clear phylogenetic signal, indicating the importance of phylogenetic methods for investigating the patterns of variation among taxa. There are, however, a few instances of evolutionary divergence among closely related taxa (evidence of crossed branches in the mapped PCAs), and evidence for some convergent evolution or homoplasy in the wing shape (Figure 6.11 and 6.14). There is some divergence among closely related taxa that possibly reflect the plasticity of the wings to evolve, for instance, in response to selection on functional morphology.

Many large shape differences between taxa underscore that there is a substantial evolutionary potential in the wing shapes. Given this potential for evolutionary change, it is not surprising that the species level analysis found highly significant phylogenetic signal. However at genus level, there was no clear phylogenetic signal. It is possible that this result is due to insufficient sample size (averaged data only returned 7 genera), or weakly supported nodes in the molecular phylogeny. Furthermore, a phylogenetic tree cannot accurately represent an evolutionary history that includes hybridisation events (which may cause abrupt changes in morphological traits and therefore affect the evolution of wing shape), therefore producing a non-significant phylogenetic signal. Unfortunately, it is not possible to determine conclusively with the current evidence why no significant phylogenetic signal for genus level was found.

The shape changes associated with the PCs of species means (Figure 6.11 and 6.14) and the PCs of independent contrasts (Figure 6.15 and 6.16) showed clear correspondence. This is frequently the case in comparative studies (Klingenberg and Gidaszewski, 2010), but cannot always be expected. Analyses of variation across the tips of a phylogeny provide estimates of evolutionary patterns that are unbiased, but have higher variance than estimates from comparative methods such as IC (Rohlf, 2006). For all PCAs of the fore and hind wing, PC1 takes up almost half of the variation, far more than any other PC (Table 6.10 and 6.11; Figure 6.14). This indicates that the shape variation in each data set is highly concentrated in a single direction. Moreover, the shape changes associated with the PC1s are remarkably consistent across the levels of analysis: evolutionary divergence among taxa (analyses with taxon means

or IC) and pooled within-taxon variation. This correspondence is also apparent from the high matrix correlations between the covariance matrices for within-taxon variation and evolutionary divergence.

Because allometry produces shape variation that is concentrated in a single dimension of shape space, it is a factor that can contribute substantially to integration throughout an entire structure or structures, and it is therefore important to consider allometry in studies of morphological integration (Klingenberg *et al.*, 2001; Rosas and Bastir, 2004; Klingenberg, 2009; 2013). Evolutionary allometry accounts for 31.0 % of the shape variation in IC in the fore wings and 17.8 % in the hind wings. This is relatively high for the fore wings, but in the hind wings it is comparable to estimates in other studies of the proportion of shape variation for which evolutionary allometry accounts (Figueirido *et al.*, 2010, Klingenberg *et al.*, 2012). Removing the effects of allometry by using residuals from the regression of shape on size, clearly affects the patterns of overall variation of wing shapes (Figure 6.13 – 6.16) and patterns of integration between fore and hind wing (Figure 6.17) Although these effects of allometry are noticeable and removing them reduces the RV coefficient between fore and hind wing only slightly from 0.60 to 0.58, the residuals from the allometric regression still shows strong integration of both wings together.

Shape changes associated with the within- and among- taxon allometries are similar. Such agreements between levels of allometry have been reported before (Klingenberg and Zimmerman, 1992; Klingenberg *et al.,* 2012). The allometric shape changes coincide with the PC1 patterns of shape variation at all levels analysed in the study (Figure 6.11 – 6.17). It is therefore plausible that the process responsible for the common pattern of shape variation is related to size variation and growth. However, analyses correcting for allometry by regression do not eliminate the coherent patterns of shape variation, so that allometry is clearly not the only source of integration (seen in other studies such as Klingenberg *et al.*, 2012; Klingenberg and Marugán-Lobón, 2013). There are other factors that contribute to this integration, such as developmental, environmental, behavioural or functional interactions.

PLS analysis has been used to analyse covariation among parts within a configuration in several earlier studies, but mostly at intraspecific level (Klingenberg and Zaklan, 2000; Klingenberg *et al.,* 2003; Mitteroecker and Bookstein, 2008; Kulemeyer *et al.,* 2009). In this chapter, PLS analysis is applied in a phylogenetic context to investigate evolutionary

integration (e.g. Klingenberg and Marugán-Lobón, 2013) between fore and hind wings in an interspecific context.

The shape variation, integrated throughout the wings is further highlighted by the correspondence between the PCs of overall fore wing and hind wing variation and PLS axes that characterise covariation between fore and hind wing. Although the shape changes associated with the PCs and PLS axes are not identical, there is a clear correspondence between the PCs and PLS axes (Table 6.14). Because the PLS axes are computed exclusively from information about covariation between the fore and hind wing, the correspondence between PCs and PLS axes implies that features of integrated evolution in fore and hind wing are among the dominant features of wing shape variation. Accordingly this correspondence of PCs and PLS axes is evidence of overall integration of the two structures as a whole (Klingenberg and Zaklan, 2000). Similar correspondence of PCs and PLS axes has been found in other studies of various organisms (Klingenberg and Zaklan, 2000; Klingenberg *et al.*, 2001; Monteiro *et al.*, 2005; Kulemeyer *et al.*, 2009; Klingenberg and Marugán-Lobón, 2013).

Coupling of fore and hind wings often result in high integration between wings (e.g. Breuker *et al.*, 2007). This has been reported for some Ephemeroptera (Edmunds and Traver, 1954; Peters and Campbell, 1991), where a hind wing costal projection couples with the fore wing. Ephemeroptera are considered to have amplexiform wings, where the wings overlap and operate in one phase, but with no specific mechanical connection (Dudley, 2002).

A costal projection is found in the Teloganodidae hind wing (Figure 6.1), and this would explain the strong integration between fore and hind wings. Rare footage of sub-imaginal Teloganodidae in flight gives some visual support to this family for wing-coupling (Figure 6.18). The distinct integrative pattern seen in the PLS2 for uncorrected and size-corrected shapes could be related to this coupling mechanism, whereby a narrower hind wing corresponds to an extended anal margin, so that wings will be in contact during flight. While this does not account for all integration, it is certainly a contributing factor.



Figure 6.18. Teloganodidae in flight, demonstrating the amplexiform wing coupling, where the costal projection of the hind wing assists in coupling (taken by Geoff McIlleron, NIKON D200, 17/12/2008, at 20h51, permission granted: 12/01/2016).

6.5 Conclusions

There is strong phylogenetic signal at the species level within Teloganodidae, indicating that the morphometric data of wing shape can infer phylogeny for this group. The arrangement of groups in the plots relate to the geographic locations of the river catchments, suggesting that there are geographic clines in the wing shape of this genus across its range.

The strong dominance of a single PC and the relative consistency of patterns across the data suggest that a common process may be channelling the phenotypic variation in a single direction. Because the wing shape variation within-taxa is concentrated considerably in a

single dimension of the shape spaces, evolutionary changes are also most likely in that direction. It is thus possible that the PC1s act as "lines of least resistance", where this direction of variation controls or limits evolutionary change (Schluter, 1996; Arthur, 2001; Hunt, 2007; Klingenberg, 2010). It is also important to remember that no single explanation can account for the variation across any taxa (Efroni *et al.*, 2010). Multilevel analyses may improve our understanding of the interface between micro- and macroevolution and will allow inferences on the evolutionary processes involved in the diversification of major clades (Monteiro *et al.*, 2005; Hunt, 2007; Klingenberg, 2010).

The strong integration and common pattern of variation at multiple levels suggest a shared mechanism underlying this variation, which is not completely explained for by allometry. A possible contributing factor is the necessity for the fore and hind wings to couple during flight, therefore directing morphological change in the wings to ensure that wings are able to couple. The ability of the wings to couple and the shape of the wings may contribute to relative strength of flight, therefore affecting dispersal abilities. As demonstrated by *Lestagella*, geographic catchment locality can affect phenotypic variation; this may influence dispersal abilities (and possible vicariance) in relation to the physical and environment characteristics of each catchment.

The factors addressed here however, still do not account for all the variation in the wings. This raises an interesting question of the possible factors contributing to the evolution and integration of wings in the Teloganodidae, and is at least a step towards understanding their evolution.

Chapter Seven

General Discussion and Conclusions

This final chapter outlines the findings from the previous chapters and contextualises the main conclusions. To date, the research presented here provides the most comprehensive analysis of phylogenetic relationships within southern African Teloganodidae. It provides considerable insight into the historic factors which have been the driving forces shaping the generic and species distribution of the teloganodid lineages seen today.

7.1 A Multi-faceted Approach

The multi-faceted approach taken in this thesis is inspired by the unified species concept of de Queiroz (2007), where numerous species-delimiting properties provide support or opposition for the hypothesis that metapopulation lineages are evolving separately.

The properties explored throughout the chapters have included biological isolation (allopatric speciation), phylogenetic monophyly, genealogy, historical phylogeography and phylogenetic geometric morphometrics. These properties have provided insight into various drivers and mechanisms for diversification within the clade.

From a phylogenetic monophyly and genealogical perspective, southern African Teloganodidae across the GCFR can be divided into 27 species and possibly six genera, from a starting point of four genera and five species previously recorded (McCafferty and Wang, 1997). Clades within the genera show trends where distinct lineages are closely linked with catchment locality and their associated watersheds, which has been reported for other organisms (Australian Atyidae shrimps: Hughes *et al.* 1996), in southern Africa (Diptera: Wishart and Hughes, 2001; Cicadidae: Price *et al.* 2007, Price *et al.* 2010; Cyprinidae, *Pseudobarbus*: Swartz *et al.* 2008). This association with catchments was not found to be a consequence of isolation by distance, and the isolation by barrier tests sometimes revealed stronger barriers between close catchments and weaker barriers between more distant catchments, indicating that the reason for these differences are complex. Strong phylogeographic structure was found within most genera, especially *Lestagella*.

This led to investigation into the mechanisms and drivers of diversification by considering the phylogeography and ancestral reconstruction of the genera. Each clade within the genera presents different patterns when examined at the finer scale, however a few common mechanisms for diversification were observed. One of the largest mechanisms contributing to the diversification of Teloganodidae was probably the fragmentation of Afrotemperate forests from the Miocene, primarily as a result of sea level changes and tectonic uplift. This created palaeogenic (refuge) zones that would have acted as refugia against unfavourable conditions for the ancestral lineages. This would have led to allopatric speciation of some lineages. Some lineages originally isolated in one catchment in the Micoene, were able to disperse and "re-enter" other catchments in the Plio-Pleistocene, possibly due to the climatic changes associated with the glacial and interglacial cycles, and possibly river piracies and captures. Similar patterns have been reported in numerous other GCFR taxa, such as Plecoptera: Notonemouridae (Stevens, 2008); Anabantidae and Cyprinidae fish (Skelton, 1994); Onychophora (velvet worms: Daniels *et al.*, 2009; McDonald and Daniels, 2012); southwestern Cape moss, *Wardia hygrometrica* (Wardiaceae, Mwafongo, 2003), Diptera: Blephariceridae (Wishart and Hughes, 2001, 2003); Megaloptera: *Platychauliodes* (Liu *et al.*, 2011); Megaloptera: *Taeniochauliodes* (Liu *et al.*, 2013) and Decapoda: Potamonautidae (Phiri, 2014) to name a few.

Phylogeographic patterns for Teloganodidae are dependent on three key points: 1) dispersal ability, which is affected by environmental boundaries such as vegetation habitat associations, 2) distribution of lineages at the time of climatic or geological changes that would have either inhibited or promoted dispersal, allowing some lineages and not others to disperse to new localities. This can be seen as an instance of "right place, right time, right habitat preference", allowing *Lestagella* to disperse to the Cape Peninsula, while the land bridge was above sea level, and 3) the ability of the population to adapt and occupy available niches. This process would be simpler for more generalist organisms (*Lestagella* found under stones in current) not reliant on a certain specialised biotope such as a type of vegetation, compared to those with specialist requirement (*Nadinetella* with its dependency on *Wardia* moss).

The adult stage of teloganodid mayflies is short-lived and relatively morphologically conserved especially compared to the distinctive nymphal stage. The geometric morphometrics method can examine minute changes in shape structure, and was able to

determine strong phylogenetic signal within the wing shapes of the species clades. The lineage found to exhibit the greatest phylogeographic structure, *Lestagella*, showed geographic clines in the wing shape of this genus across its range. This implies possible evolutionary adaptations to differing landscape or environmental parameters within specific catchments, and understanding these adaptive processes will lead to an understanding of the factors that have produced these patterns.

Higher Taxon Inference

There is a closer relationship between taxa from Madagascar and Africa than between Madagascar and India, a trend seen in other Ephemeroptera (McCafferty, 1999; Sartori *et al.* 2000; Monaghan *et al.* 2005), however for this study this may be a result of poor sampling of the Oriental fauna. Ephemerythidae and Melanemerellidae share a link that needs to be further investigated. The relationship of the Asian Teloganodidae lineage to the Afrotropical Teloganodidae (African and Madagascan) and the Melanemerellidae/Ephemerythidae clade is uncertain; more samples are needed to make any conclusions with confidence. Insufficient sample sizes, limited accessibility of certain material, fresh material for molecular work, and issues where collecting and export permits are difficult to acquire were limiting factors for the higher taxon inferences in this study.

Importance of Species Level Taxonomy and its Implications

This study has shown a considerable increase in biodiversity of Teloganodidae in the southwestern Cape, with many previously unknown species. The next, critical step is to morphologically characterize and describe these species. This is not only important for the knowledge of biodiversity of the area, but will provide the foundation for future research and policies for managers of conservancies, water affairs, and environmental consultants. Detailed species level knowledge also has direct applied implications for water quality management and conservation as Ephemeroptera are well known as valuable indicator species in aquatic biomonitoring (e.g. Brittain, 1982; Bauernfeind and Moog, 2000; Beketov, 2004). Too often biomonitoring focuses only on family level (Barber-James and Pereira-da-Conceicoa, 2016), and this work shows the importance of species level studies to determine the true diversity of an ecosystem,

7.2 Future Directions

It was realised that a revision of the southern African Teloganodidae was necessary when some specimens examined were remarkably different from described species. Due to the variability seen between the diagnostic characters between the genera and with added problems of species discrimination in some cases (e.g. *N. crassi* and *N. brincki*), it is important to clearly define genera and species, and publish the descriptions of the newly discovered taxa. It is also important to keep in mind the exceptional amount of work that this study represents, including different skills and expertise needed in terms of bioinformatics and laboratory protocols. While this study emphasizes the need to examine biodiversity in more detail, especially in the GCFR, it is unrealistic to expect in-depth studies such as this, for all species from different taxon groups. There is considerable time pressure to recognise the earth's biodiversity before it disappears, especially in Africa. A possible solution to this challenge involves the recent advances in DNA technologies, which have now made it possible to sequence mixed, bulk samples of organisms in one run using NGS (Next Generation Sequencing). This ultimately reduces the amount of expertise needed, is becoming more cost-effective and greatly reduces the amount of processing time for the amount of information retrieved. Techniques using environmental DNA (eDNA) are also being developed (Herder *et al.*, 2014; Moyer *et al.*, 2014; Deiner *et al.*, 2015a; Deiner *et al.*, 2015b, Goldberg *et al.*, 2015), where trace DNA left in sediment or water columns can be detected, allowing all species occurring in that area/river section to be identified.

This is the first comprehensive biodiversity study of southern African Teloganodidae, and the first detailed estimate of their origin and the historic evolutionary pathways taken resulting in the species we have today. This new knowledge on the diversity of these mayflies needs to be shared through published species descriptions and production of keys to assist easy end user identification. This knowledge needs to be disseminated to scientists, managers and the public by means of identification keys and other educational projects, to promote understanding of why it is important to preserve biodiversity and maintain healthy, functioning ecosystems. Cooperation and understanding from a well-educated populace can add much value to biodiversity knowledge, as has been seen in the United Kingdom with the Riverfly, BugLife initiative (www.buglife.org.uk). Citizen Science is becoming increasingly popular, bringing hope to the future of taxonomy as a science (Pocock *et al.*, 2014).

The higher taxon relationships require a formal assessment with more samples, to elucidate the relationships between the closely related families investigated in this thesis. Particularly for the Oriental Teloganodidae, additional molecular analyses including a more extensive range of taxa from India, Sri Lanka and South East Asia would clarify the deeper relationships within the global Teloganodidae. Mountain range orientation, rainfall zone and vegetation structure are a few factors that can have a considerable effect on catchment landscapes, which may in turn mould its faunal component. Looking at comparative phylogeography in terms of landscape structure can provide insight to common mechanisms for diversification. The high degree of phylogeographic structure found within *Lestagella*, in combination with the geographic clines established from the geometric morphometric data, make this group an excellent candidate for examining how landscape structure may genetically and morphologically mould its fauna.

Further studies investigating the extent at which community overlap and phylogeography of fauna and flora that are found in Afrotemperate mountain forest fragments throughout Africa would provide valuable insight and knowledge into areas of endemism and origins of biodiversity in these regions. This would have direct implications for conservation of these areas, allowing predictions of impacts from factors such as deforestation and climate change on the biodiversity. Climate change has already shown effects on the emergence and reproductive patterns of dragonflies (Doi, 2008) and other aquatic insects (Jonsson *et al.*, 2015) over the last few decades. Even during the fieldwork conducted to collect the teloganodids for the research presented here, a change in the emergence time for some species was found, comparing records by Barnard (1932), with a delay of nearly two months for some species due to a delayed winter. This aspect was not a focus of this research, but it warrants further study. Understanding these processes and how they affect different faunal groups may help with future predictions and management of systems to avoid loss of biodiversity.

7.3 Concluding Remarks

The biodiversity of the Teloganodidae is much richer than portrayed from previous literature. As the Cape Floristic Region is a recognised biodiversity hotspot (Linder, 2003), the trends shown in the western and southern Cape Teloganodidae are probably not unique, suggesting much undiscovered biodiversity in this area. Aquatic macroinvertebrates are in general, understudied and underestimated in South Africa. This study has shown that a detailed analysis of biodiversity can have many valuable contributions, from directing future research focus and fine-tuning previous phylogeographical and evolutionary hypotheses, to improving management and conservation decisions in order to preserve these endemic biodiversity hotspots.

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Appendix

Appendix headings contain a number which is associated with the corresponding chapter number. Multiple appendices for a particular chapter are labelled with letters of the alphabet and in order (e.g. A, B, C …). There are no appendices for Chapters One, Two or Seven.

Appendix 3A. Character list generated in DELTA from Lestagella penicillata redescription (Pereira-da-Conceicoa and Barber-James, 2013). To be used in descriptive texts and analyses for future species descriptions of *Lestagella* and other Teloganodidae

Character List

#1. <♂A> <head> antennae <length>/
    1. half width of head capsule/
    2. equal to width of head capsule/
    3. twice width of headcapsule/
#2. <♂A> <head> dorsal portion of compound eye <colour>/
    1. reddish-brown/
    2. red/
    3. black/
#3. <♂A> <head> dorsal portion of compound eye <shape>/
    1. spherical/
    2. oblong/
#4. <♂A> <head> dorsal portion of compound eye <separation>/
    1. slightly separated dorsally/
    2. well separated dorsally/
    3. not separated dorsally/
#5. <♂A> <thorax> forewing <shape>/
    1. narrow/
    2. broad/
#6. <♂A> <thorax> <forewing> pterostigma/
    1. present/
    2. absent/
#7. <♂A> <thorax> <forewing> <pterostigma> with/
    1. 8–10 crossveins/
    2. no crossveins/
#8. <♂A> <thorax> Wing venation follows notation of
/
#9. <♂A> <thorax> <forewing> **RS** <vein>/
    1. basally faint/
    2. basally distinct/
#10. <♂A> <thorax> <forewing> marginal intercalaries/
    1. present/
    2. absent/
#11. <♂A> <thorax> <forewing> marginal intercalaries
/
    1. sometimes paired/
    2. single/
#12. <♂A> <thorax> <forewing> <marginal intercalaries>/
    1. absent between **RA** and **RSa**/
    2. present between **RA** and **RSa**/
#13. <♂A> <thorax> hind wings/
    1. ovoid/
    2. round/
#14. <♂A> <thorax> <hind wings> with costal elevation
/
#15. <♂A> <thorax> ventral margins of fore and hind wings
/
#16. <♂A> <thorax> prothoracic legs <length>/
    1. with tibia just over twice the length of femora/
    2. with tibia subequal to length of femora/
#17. <♂A> <thorax> <prothoracic legs> tarsal segment I <length>/
    1. subequal to segment II/
    2. twice length of segment II/
#18. <♂A> <thorax> <prothoracic legs> segment III <length>/

1. half-length of segment II/
2. subequal to segment II/

#19. <♂A> <thorax> <prothoracic legs> segment IV <length>/
1. is ca. one third length of segment III/
2. is ca. half length of segment III/

#20. <♂A> <thorax> <prothoracic legs> femora <surface>/
1. with subtriangular, scale-like projections and few scattered setae/
2. without subtriangular, scale-like projections and few scattered setae/

#21. <♂A> <thorax> <prothoracic legs> tibia and tarsi <surface>/
1. with smaller scale-like projections and scattered seate/
2. without smaller scale-like projections and scattered seate
/

#22. <♂A> <thorax> <prothoracic legs> foreleg claws <pads>/
1. modified into two pad-like lobes/
2. modified differently/

#23. <♂A> <thorax> <prothoracic legs> foreleg claws <dorsal>/
1. with flat scale-like dorsal surface/
2. without flat scale-like dorsal surface/

#24. <♂A> <thorax> <prothoracic legs> foreleg claws <ventral>/
1. and smooth slightly grooved ventral surface/
2. and smooth ventral surface without groove/

#25. <♂A> <thorax> mid and hind legs with tibiae ca./
times femora length/

#26. <♂A> <thorax> mid and hind legs tibiae/
1. few setae scattered, becoming more frequent nearer the tarsi/
2. few setae scattered, not becoming more frequent nearer the tarsi
/

#27. <♂A> <thorax> <mid and hind legs> tarsi/
1. one thrid the length of tibiae/
2. not one third the length of tibiae/

#28. <♂A> <thorax> <mid and hind legs> tarsal segments I - III
/
1. subequal in length/
2. not subequal in length/

#29. <♂A> <thorax> <mid and hind legs> tarsal segments I - III/
1. with many short, simple setae/
2. without many short, simple setae/

#30. <♂A> <thorax> <mid and hind legs> tarsi/
1. segment IV twice the length of segment III/
2. segment IV not twice the length of segment III/

#31. <♂A> <thorax> <mid and hind legs> Distal end of tibiae and tarsal segments I - III/
1. with distinct spike on ventral surface/
2. without distinct spike on ventral surface/

#32. <♂A> <thorax> <mid and hind legs> claws <pads>/
1. with one pad-like lobe and one hooked/
2. not as above/

#33. <♂A> <thorax> <mid and hind legs> dorsal surface <claw>/
1. with flat scales as in foreleg/
2. without flat scales/

#34. <♂A> <abdomen> gill socket vestiges/
1. present/
2. absent/

#35. <♂A> <abdomen> male abdomen/
1. ca. two thirds thickness of female abdomen/
2. not as above/

#36. <♂A> <abdomen> genital forceps/
1. three segmented/
2. not three segmented/

#37. <♂A> <abdomen> genital forceps/
1. segment I more broad than other segments/

2. segment I not more broad than other segments/
#38. <♂A> <abdomen> genital forceps/
1. ca. two thirds the length of segment II/
2. not two thirds the length of segment II
/
#39. <♂A> <abdomen> <genital forceps> segment III/
1. reduced/
2. not reduced/
#40. <♂A> <abdomen> <genital forceps> segment III/
1. bending inwardly, nearly forming a right angle/
2. not as above/
#41. <♂A> <abdomen> <genital forceps> Inner lateral margins of genital forceps/
1. with ovoid scale-like processes on all three segments/
2. with ovoid scale-like processes not on all three segments/
#42. <♂A> <abdomen> <genital forceps> ventral, dorsal and outer margin surfaces of segment I and II/
1. covered in few simple setae scattered and small subtriangular scale-like processes/
2. not as above/
#43. <♂A> <abdomen> <genital forceps> ventral, dorsal and outer margin surfaces of segment I and II
/
1. while thrid segment has many, larger, more elongate subtriangular scale-like processes/
2. not as above/
#44. <♂A> <abdomen> penis lobes
/
1. fused with small medial notch or indentation/
2. not fused/
#45. <♂A> <abdomen> penis lobes <distal end>/
1. slightly broadened distally/
2. tapering distally/
#46. <♂A> <abdomen> penis lobes/
1. longitudinal groove vestigial/
2. not vestigial/
#47. <♂A> <abdomen> penis lobes/
1. elongated but shorter than genital forceps/
2. longer than genital forceps/
#48. <♂A> <abdomen> styliger plate/
1. distinctly convex/
2. flattened/
#49. <♂A> <abdomen> three caudal filaments/
1. ca. subequal in length/
2. not subequal/
#50. <♂A> <abdomen> three caudal filaments/
1. one third longer than body length/
2. not as above/
#51. <♂A> <abdomen> three caudal filaments/
1. few setae scattered all over surface/
2. not as above/
#52. <♀A> <head> antennae <length>/
#53. <♀A> <head> compound eyes/
#54. <♀A> <head> compound eyes/
#55. <♀A> <thorax> forewings/
#56. <♀A> <thorax> fore mid and hind legs/
#57. <♀A> <abdomen> gill socket vestiges/
1. present/
2. absent/
#58. <♀A> <abdomen> three caudal filaments/
#59. <N> colouration of immature nymphs/
1. pale, straw coloured/
#60. <N> mature nymphs/
1. darker with more prominent abdominal markings/
#61. <N> Body/

1. dorso-ventrally flattened/

#62. <N> <head>/
1. with well-developed marginal fringe of setae extending to lateral margin of head with setae longer anteriorly and shorter laterally/

#63. <N> <head> male nymphs/
1. show developing dorsal compound eyes/

#64. <N> <head> *labrum*/
1. width three times length/

#65. <N> <head> <labrum> lateral margins/
1. rounded/

#66. <N> <head> <labrum> lateral margins/
1. anterior with slight emargination/

#67. <N> <head> <labrum> basal margin/
1. with elongated, squared notch medially/

#68. <N> <head> <labrum> apico-dorsal surface/
1. up to one third covered with densely clustered, feathered setae/

#69. <N> <head> <labrum> apico-dorsal surface/
1. few feathered setae extend up to two thirds along lateral margin/

#70. <N> <head> <labrum> dorso-medial, transverse row of setae/
1. 34times larger than apico-dorsal setae/

#71. <N> <head> <labrum> dorso-medial, transverse row of setae/
1. not feathery and possible remnant setal bases situated towards dorso-basal margin/

#72. <N> <head> <labrum> dorso-lateral margin/
1. with single cluster of setae/

#73. <N> <head> <labrum> dorso-lateral margin <with single cluster of setae>/
times length of dorso-medial transverse setae/

#74. <N> <head> <hypopharynx> lingua/
1. similar in size to superligua/

#75. <N> <head> <hypopharynx> lingua and superlingua/
1. both rounded apically/

#76. <N> <head> <hypopharynx> lingua and superlingua/
1. narrowing posteriorly/

#77. <N> <head> <hypopharynx> lingua/
1. with variable distomedial notch, ranging from deep indentation to none/

#78. <N> <head> <hypopharynx> dorsal surface/
1. with tuft of fine setae antero-laterally/

#79. <N> <head> <hypopharynx> dorsal surface/
1. very short brush setae covering dorsal to ventral surface anteriorly/

#80. <N> <head> <hypopharynx> dorsal surface/
1. small cluster of larger setae postero-laterally/

#81. <N> <head> <hypopharynx> superlingua/
1. with long, feathery setae on outer antero-lateral margin/

#82. <N> <head> <hypopharynx> superlingua/
1. becomin shorter and non-feathery on outer lateral margin closest to base/

#83. <N> <head> <hypopharynx> superlingua/
1. small, short setae sparsely covering antero-lateral surface extending from antero-dorsal to inner latero-ventral surface/

#84. <N> <head> <hypopharynx> superlingua setae/
1. covering two thirds toward base/

#85. <N> <head> *labium*/
1. with partly divided gloassae and paraglossae/

#86. <N> <head> <labium> paraglossae/
1. slightly falciform/

#87. <N> <head> <labium> paraglossae/
1. larger than glossae/

#88. <N> <head> <labium> paraglossae/
1. rounded apically/

#89. <N> <head> <labium> glossae and paraglossae/
1. apical surfaces with short-feathered seate/

#90. <N> <head> <labium> paraglossae/

1. lateral outer surface with long, thin feathered setae 2 times the length of apical setae/
#91. <N> <head> <labium> paraglossae and glossae/
1. dorsal surface withshort scattered setae/
#92. <N> <head> <labium> paraglossae and glossae/
1. two rows of longer setae situated obliquely from paraglossal base towards base of glossae/
#93. <N> <head> <labium> labial palps/
1. three-segmented/
#94. <N> <head> <labium> labial palps/
1. segment I as long as segment II and III combined/
#95. <N> <head> <labium> labial palps/
1. articulation between segment II and III distinct/
#96. <N> <head> <labium> labial palps/
1. segment II narrows slightly distally/
#97. <N> <head> <labium> labial palps/
1. segement III small with slight medial constriction and tapered distally/
#98. <N> <head> <labium> labial palps/
1. lateral surfaces of segment II and III with scattered setae/
#99. <N> <head> <labium> prementum and postmentum/
1. as seen in figure/
#100. <N> <head> <labium> postmentum/
1. posmentum covered with sparsely scattered simple setae and disc-shaped sensory pits or setal bases/
#101. <N> <head> *mandibles*/
1. elongate/
#102. <N> <head> mandibles/
1. length almost 4 times width/
#103. <N> <head> <mandibles> outer lateral margin/
1. with few long socketed-setae (<10) from middle towards base/
#104. <N> <head> <mandibles> incisors/
1. single/
#105. <N> <head> <mandibles> incisors/
1. prominent/
#106. <N> <head> <mandibles> incisors/
1. with small tuft of very fine setae on outer edge of incisor near distal end/
#107. <N> <head> <mandibles> ventral surface of mandibles/
1. with many disc-shaped sensory pits or setal bases/
#108. <N> <head> <mandibles> ventral surface of mandibles/
1. medio-transverse groove with small triangular scales or scleritized projections/
#109. <N> <head> <mandibles> left mandible/
1. with prostheca close to incisor/
#110. <N> <head> <mandibles> left mandible <proscthecа>/
1. protheca well-developed into long sclerotized setae/
#111. <N> <head> <mandibles> left mandible <proscthecа>/
1. longest seta feathered or brush-like/
#112. <N> <head> <mandibles> molar region/
1. prominent/
#113. <N> <head> <mandibles> molar region/
1. with 2–3 long, thin setae below mola/
#114. <N> <head> <mandibles> right mandible/
1. with prostheca well-developed/
#115. <N> <head> <mandibles> right mandible/
1. with branched scerotized setae/
#116. <N> <head> <mandibles> right mandible/
1. single feathery or brush-like seta protruding out towards mola/
#117. <N> <head> <mandibles> molar region/
1. with 3 sclerortized setalike projections distally/
#118. <N> <head> <mandibles> molar region/
1. with elongated, thumb-like proximal ridge/
#119. <N> <head> <mandibles> molar region/
1. row of (<10) setae below mola/
#120. <N> <head> *maxillae*/

1. uniform with maxillary palp absent/
#121. <N> <head> <maxillae> canines/
1. fused into single elongate canine/
#122. <N> <head> <maxillae> canines/
1. with depression along inner margin containing a sigle seta/
#123. <N> <head> <maxillae> outer face of canine base/
1. with cluster of long, thin setae/
#124. <N> <head> <maxillae> galea-lacinia/
1. with two dentisetae/
#125. <N> <head> <maxillae> galea-lacinia/
1. 6–7 long socketed setae/
#126. <N> <head> <maxillae> proximal dentiseta/
1. slightly serrated on the inner margin/
#127. <N> <head> <maxillae> dorso-lateral surface below galea-lacinia apex/
1. with one large seta present/
#128. <N> <head> <maxillae> inner lateral surface below single large seta/
1. with five simple setae as seen in FIGURE/
#129. <N> <head> <maxillae> lower, outer lateral margin of maxillae/
1. with few large, thin simple setae present/
#130. <N> <thorax> Outer edges of prothorax/
1. lined with spatulate setae/
#131. <N> <thorax> femur/
1. subequal to tibia in length/
#132. <N> <thorax> femur/
1. medially broadened, narrowing toward distal and proximal ends/
#133. <N> <thorax> <legs> dorsal surface of fore femur/
1. with medio-transverse spatualte setae present along transverse ridge/
#134. <N> <thorax> <legs> dorsal surface of fore femur/
1. size of medial gap between transverse ridges of spatulate setae variable/
#135. <N> <thorax> <legs> transverse ridge/
1. extends distally along postero-lateral margin where setae gradually become longer (ca. three times length of spatulate setae)/
#136. <N> <thorax> <legs> antero-lateral margin of femur/
1. with short spatulate and lanceolate setae present/
#137. <N> <thorax> <legs> antero-lateral margin of femur/
1. with few long setae scattered near proximal base/
#138. <N> <thorax> <legs> dorsal surface of tibia/
1. with single row of long, perpendicular setae/
#139. <N> <thorax> <legs> dorsal surface of tibia/
1. lateral margins interspersed with small simple setae/
#140. <N> <thorax> <legs> Tarsus/
1. ca. half length of tibia/
#141. <N> <thorax> <legs> Tarsus/
1. entire surface interspersed with small, simple setae/
#142. <N> <thorax> <legs> tarsal claw/
1. elongate/
#143. <N> <thorax> <legs> tarsal claw/
1. with single row of 4–6 variably sized smaller denticles followed by one large apical tooth/
#144. <N> <thorax> <legs> tarsal claw/
1. a single row of 4 small, subapical setae present apicolaterally on either side of claw/
#145. <N> <thorax> <legs> tarsal claw/
1. ventral base of claw with small sparsely scattered setae/
#146. <N> <thorax> <legs> mid and hind legs/
1. as seen in FIGURE/
#147. <N> <thorax> <legs> mid and hind legs/
1. femora slightly more elongate and without transverse ridge of spatulate setae/
#148. <N> <thorax> <legs> mid and hind legs/
1. antero-lateral margins with long setae/
#149. <N> <thorax> <legs> mid and hind legs/
1. poterolateral margins with spatulate setae/

#150. <N> <thorax> <legs> tibiae, tarsi and claws/
1. similar to foreleg/
#151. <N> <abdomen> abdominal segments/
1. with series of dark spot dorsolaterally, largest occuring on segment II/
#152. <N> <abdomen> postero-lateral processes/
1. subtriangular/
#153. <N> <abdomen> postero-lateral processes/
1. moderately developed and slightly separated from the base of following segment/
#154. <N> <abdomen> postero-lateral processes/
1. processes with single row of long setae/
#155. <N> <abdomen> filamentous gill I/
1. present/
#156. <N> <abdomen> filamentous gill I/
1. three-segmented/
#157. <N> <abdomen> filamentous gill I/
1. with long, thin, fine setae scattered around segment III/
#158. <N> <abdomen> <gills> lamellate gills/
1. on segments II to IV/
#159. <N> <abdomen> <gills> gill II/
1. fully operculate/
#160. <N> <abdomen> <gills> gill II/
1. upper lamellae ovoid with thin setae present, starting ca. two thirds from base/
#161. <N> <abdomen> <gills> gill II/
1. setae longer aneriorly and shorter laterally/
#162. <N> <abdomen> <gills> gill II/
1. lower lamellae bifid and highly lobed/
#163. <N> <abdomen> <gills> gill III/
1. upper lamellae more circular in shape with thin setae present starting ca. three quarters from base/
#164. <N> <abdomen> <gills> gill III/
1. setae longer aneriorly and shorter laterally
/
#165. <N> <abdomen> <gills> gill III/
1. lower lamellae singular and highly lobed/
#166. <N> <abdomen> <gills> gill IV/
1. almost semi-circular/
#167. <N> <abdomen> <gills> gill IV/
1. lower lamellae absent/
#168. <N> <abdomen> <gills> gill IV/
1. thin setae present along lateral margin to apex/
#169. <N> <abdomen> <gills> gill IV/
1. seate longer anteriorly and shorter laterally/
#170. <N> <abdomen> three caudal filaments/
1. banded at base/
#171. <N> <abdomen> three caudal filaments/
1. sparsely setose with setae becoming lightly longer distally/
#172. <N> <abdomen> three caudal filaments/
1. medial caudal filment well-developed/
#173. <N> <abdomen> three caudal filaments/
1. filaments ca. two thirds longer than body length/

Appendix 4A. Details for specimens usedin molecular analyses. "Afr/SAm", Afr/Mad", "Ephem", "Lest", "Nad/Lith" and "Mito" correspond to datasets in Table 4.2., and shows which specimens were used in each dataset.

									Sequenced					Datasets					
CODE	**CAT NO**	**GENUS**	**RIVER**	**CATCHMENT**	**COUNTRY**	**GENBANK ACCESSION**	**SOUTH**	**EAST**	**COI**	**16S**	**12S**	**H3**	**28S**	**Afr/SAm**	**Afr/Mad**	**Ephem**	**Lest**	**Nad/Lith**	**Mito**
TEL 001	HMJ 50	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√	√	√	√			X		X		X
TEL 002	HMJ 50	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√										
TEL 003	HMJ 50	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√										
TEL 004	HMJ 50	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√										
TEL 005	HMJ 50	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√	√		√							
TEL 006	HMJ 37	Lestagella	Groot R	AFR	South Africa	-	-33.9662	23.559	√										
TEL 007	HMJ 52	Nadinetella	Salt R	AFR	South Africa	-	-33.9225	23.4898	√	√		√						X	X
TEL 008	ZAMBIAN	Ephemerythidae	Like Like R		Zambia	-			√				√	X	X				
TEL 009	ZAMBIAN	Tricorythidae	Like Like R		Zambia	-			√	√	√	√							
TEL 010	CED 102B	Ephemerellina		Olifants	South Africa	-	-32.4555	19.1706	√		√	√							
TEL 011	HMJ 39	Nadinetella	Bloukrans	AFR	South Africa	-	-33.9558	23.6384	√										
TEL 012	HMJ 41	Nadinetella	Bloukrans	AFR	South Africa	-	-33.9558	23.6384	√	√	√	√						X	X
TEL 013	HMJ 27	Lestagella	Hogsback	Keiskamma	South Africa	-	-32.6052	26.966	√	√		√							
TEL 014	HMJ 46	Nadinetella	Salt R	AFR	South Africa	-	-33.9225	23.4898	√										
TEL 015	**HMJ 47**	**Species A**	**Salt R**	**AFR**	South Africa	-	-33.9225	23.4898	√	√	√	√			X	X	X	X	X
TEL 016	HMJ 49	Nadinetella	Salt R	AFR	South Africa	-	-33.9211	23.491	√										
TEL 017	HMJ 49	Lestagella	Salt R	AFR	South Africa	-	-33.9211	23.491	√										
TEL 018	HMJ 1	Species A	Groot R	AFR	South Africa	-	-33.9662	23.559	√										
TEL 019	HMJ 4	Species A	Elandsbos	AFR	South Africa	-	-33.9668	23.775	√										
TEL 020	HMJ 27	Lestagella	Hogsback	Keiskamma	South Africa	-	-32.6052	26.966	√	√	√	√			X		X		X
TEL 021	HMJ 5	Lestagella	Elandsbos	AFR	South Africa	-	-33.9668	23.775	√										
TEL 022	HMJ 5	Lestagella	Elandsbos	AFR	South Africa	-	-33.9668	23.775	√										
TEL 023	HMJ 11	Lestagella	Storms	AFR	South Africa	-	-33.9887	23.9191	√										
TEL 024	HMJ 11	Lestagella	Storms	AFR	South Africa	-	-33.9887	23.9191	√	√	√	√							
TEL 025	HMJ 27	Lestagella	Hogsback	Keiskamma	South Africa	-	-32.6052	26.966	√										
TEL 026	HMJ 17	Lestagella	Lottering	AFR	South Africa	-	-33.933	23.7294	√										
TEL 027	HMJ 25	Species A	Lottering	AFR	South Africa	-	-33.9558	23.6384	√			√							
TEL 028	HMJ 19	Lestagella	Lottering	AFR	South Africa	-	-33.9109	23.7421	√										
TEL 029	HMJ 19	Lestagella	Lottering	AFR	South Africa	-	-33.9109	23.7421	√										
TEL 030	HMJ 19	Lestagella	Lottering	AFR	South Africa	-	-33.9109	23.7421		√	√	√			X		X		X
TEL 031	HMJ 26	Species A	Lottering	AFR	South Africa	-	-33.9558	23.6384		√									
TEL 032	HMJ 58	Lestagella	Koornlands	Breede	South Africa	-	-33.9986	20.4568	√										
TEL 033	HMJ 58	Lestagella	Koornlands	Breede	South Africa	-	-33.9986	20.4568	√	√	√	√			X		X		X
TEL 034	HMJ 59	Ephemerellina	Koornlands	Breede	South Africa	-	-33.9986	20.4568	√	√	√	√			X	X			X
TEL 035	HMJ 60	Lestagella	Liesbeek	Table Mountain	South Africa	-	-33.983	18.4306	√	√	√	√			X		X		X
TEL 036	HMJ 60	Lestagella	Liesbeek	Table Mountain	South Africa	-	-33.983	18.4306	√										

TEL 037	HMJ 66	Nadinetella	Robertson	Breede	South Africa	-	-33.7414	19.8913	√	√	√	√						X	X
TEL 038	HMJ 66	Nadinetella	Robertson	Breede	South Africa	-	-33.7414	19.8913	√	√	√	√						X	X
TEL 039	HMJ 66	Ephemerellina	Robertson	Breede	South Africa	-	-33.7414	19.8913	√										
TEL 040	HMJ 66	Ephemerellina	Robertson	Breede	South Africa	-	-33.7414	19.8913	√										
TEL 041	**HMJ 66**	**Ephemerellina**	**Robertson**	**Breede**	South Africa	-	-33.7414	19.8913	√	√	√	√			X	X			X
TEL 042	HMJ 68	Ephemerellina	Koornlands	Breede	South Africa	-	-33.9991	20.4562	√										
TEL 043	HMJ 69	Ephemerellina	Rondegat?	Olifants	South Africa	-	-32.3982	19.0904	√	√	√	√			X	X			X
TEL 044	TSR 262 T	Ephemerellina	Upper Bobbejaans	AFR	South Africa	-	-33.8963	23.5554	√			√							
TEL 045	TSR 405E	Ephemerellina	Upper Bobbejaans	AFR	South Africa	-	-33.8963	23.5554	√	√	√	√			X	X			X
TEL 046	CED 121B	Lithogloea	Rondegat R site C2-C	Olifants	South Africa	-	-32.2853	18.9909	√	√		√							
TEL 047	CED 93E	Lithogloea	Rondegat R	Olifants	South Africa	-	-32.3995	19.0906	√		√	√							
TEL 048	CED 102D	Species B	Driehoeks R	Olifants	South Africa	-	-32.4555	19.1706	√										
TEL 049	CED 96A	Lestagella	Rondegat R	Olifants	South Africa	-	-32.3744	19.0612	√	√	√	√					X		X
TEL 050	CED 96A	Lestagella	Rondegat R	Olifants	South Africa	-	-32.3744	19.0612	√	√	√	√					X		X
TEL 051	CED 136K	Nadinetella		Olifants	South Africa	-	-32.2766	18.9746	√										
TEL 052	CED 102C	Nadinetella	Driehoeks R	Olifants	South Africa	-	-32.4555	19.1706	√	√	√	√						X	X
TEL 053	CED 100A	Nadinetella	Rondegat R	Olifants	South Africa	-	0		√		√	√							
TEL 054	HMJ 77	Ephemerellidae	Japan		Japan	-													
TEL 055	HMJ 77	Ephemerellidae	Japan		Japan	-			√	√	√	√	√	X					X
TEL 056	HMJ 77	Ephemerellidae	Japan		Japan	-													
TEL 057	HMJ 77	Ephemerellidae	Japan		Japan	-			√	√	√	√							
TEL 058	HMJ 77	Ephemerellidae	Japan		Japan	-													
TEL 059	HMJ 78	Dudgeodes ulmeri	Indonesia		Indonesia	-			√	√	√	√							
TEL 060	HMJ 78	Dudgeodes ulmeri	Indonesia		Indonesia	-			√	√		√	√	X					
TEL 061	HMJ 79	Dudgeodes celebensis	Indonesia		Indonesia	-				√		√	√						
TEL 062	HMJ 80	Lestagella	Rondegat	Olifants	South Africa	-	-32.3742	19.0615	√	√	√	√							
TEL 063	HMJ 80	Lestagella	Rondegat	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 064	HMJ 80	Lithogloea	Rondegat	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 065	HMJ 87	Lithogloea	Rondegat	Olifants	South Africa	-	-32.3742	19.0615	√	√	√	√						X	X
TEL 066	HMJ 82	Ephemerellina	Rondegat	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 067	HMJ 88	Ephemerellina	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√	√	√	√							X
TEL 068	HMJ 82	Nadinetella	RONDEGAT	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 069	HMJ 102	Nadinetella	RONDEGAT	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 070	HMJ 103	Lithogloea	RONDEGAT	Olifants	South Africa	-	-32.3742	19.0615	√										
TEL 071	HMJ 89	Lestagella	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√										
TEL 072	HMJ 89	Lestagella	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√										
TEL 073	HMJ 89	Lithogloea	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√										
TEL 074	HMJ 88	Nadinetella	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√										
TEL 075	HMJ 88	Ephemerellina	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√	√	√	√			X	X			X
TEL 076	HMJ 88	Species B	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946			√	√							
TEL 077	HMJ 94	Lestagella	Dwarsrivier (Sandrif)	Olifants	South Africa	-	-32.4884	19.2675	√										
TEL 078	HMJ 92	Nadinetella	Jan Dissels	Olifants	South Africa	-	-32.2468	19.0163	√	√	√	√						X	X

TEL 079	HMJ 94	Lithogloea	Dwarsrivier (Sandrif)	Olifants	South Africa	-	-32.4884	19.2675	√	√	√	√					X	X
TEL 080	HMJ 96	Ephemerellina	Driehoeks	Olifants	South Africa	-	-32.4553	19.1701	√		√	√						
TEL 081	HMJ 97	Lithogloea	Driehoeks	Olifants	South Africa	-	-32.4553	19.1701	√	√	√	√					X	X
TEL 082	HMJ 97	Lestagella	Driehoeks	Olifants	South Africa	-	-32.4553	19.1701	√	√	√	√	X		X			X
TEL 083	HMJ 98	Lestagella	Driehoeks	Olifants	South Africa	-	-32.4363	19.1819	√									
TEL 084	HMJ 99	Species A	Driehoeks	Olifants	South Africa	-	-32.4363	19.1819	√	√	√	√	X	X				X
TEL 085	HMJ 106	Lestagella	Jonkershoek	Eerste	South Africa	-	-33.9945	18.953	√									
TEL 086	HMJ 107	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9945	18.953	√	√	√	√					X	X
TEL 087	HMJ 108	Lestagella	Jonkershoek	Eerste	South Africa	-	-33.9899	18.9569	√	√	√	√	X		X			X
TEL 088	HMJ 110	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9939	18.975	√									
TEL 089	HMJ 111	Lithogloea	Jonkershoek	Eerste	South Africa	-	-33.9939	18.975	√									
TEL 090	HMJ 114	Ephemerellina	Molenaars	Breede	South Africa	-	-33.7308	19.118	√	√	√	√	X	X				X
TEL 091	HMJ 113	Lithogloea	Molenaars	Breede	South Africa	-	-33.7308	19.118	√			√						
TEL 092	HMJ 113	Lestagella	Molenaars	Breede	South Africa	-	-33.7308	19.118	√									
TEL 093	HMJ 113	Lithogloea	Molenaars	Breede	South Africa	-	-33.7308	19.118	√	√	√	√						
TEL 094	HMJ 114	Nadinetella	Molenaars	Breede	South Africa	-	-33.7308	19.118	√	√	√	√					X	X
TEL 095	HMJ 114	Ephemerellina	Molenaars	Breede	South Africa	-	-33.7308	19.118	√									
TEL 096	HMJ 114	Nadinetella	Molenaars	Breede	South Africa	-	-33.7308	19.118	√									
TEL 097	HMJ 116	Lestagella	Witte	Breede	South Africa	-	-33.5691	19.1387	√									
TEL 098	HMJ 116	Lestagella	Witte	Breede	South Africa	-	-33.5691	19.1387	√	√	√	√	X		X			X
TEL 099	HMJ 116	Lithogloea	Witte	Breede	South Africa	-	-33.5691	19.1387	√	√	√	√		X	X	X		X
TEL 100	HMJ 117	Nadinetella	Witte	Breede	South Africa	-	-33.5691	19.1387	√	√	√	√		X	X	X		X
TEL 101	HMJ 117	Nadinetella	Witte	Breede	South Africa	-	-33.5691	19.1387	√									
TEL 102	HMJ 118	Lestagella	Skeleton Gorge	Table Mountain	South Africa	-	-33.9831	18.4246	√									
TEL 103	HMJ 118	Lestagella	Skeleton Gorge	Table Mountain	South Africa	-	-33.9831	18.4246	√	√	√	√	X		X			X
TEL 104	HMJ 118	Lestagella	Skeleton Gorge	Table Mountain	South Africa	-	-33.9831	18.4246	√									
TEL 105	HMJ 121	Ephemerellina	Oudebos	Palmiet	South Africa	-	-34.3302	18.9567	√	√	√	√	X	X				X
TEL 106	HMJ 121	Nadinetella	Oudebos	Palmiet	South Africa	-	-34.3302	18.9567	√	√	√	√					X	X
TEL 107	HMJ 121	Species B	Oudebos	Palmiet	South Africa	-	-34.3302	18.9567										
TEL 108	HMJ 124	Lithogloea	Palmiet	Palmiet	South Africa	-	-34.3188	18.9638	√									
TEL 109	HMJ 126	Lithogloea	Palmiet	Palmiet	South Africa	-	-34.3188	18.9638	√	√	√	√					X	X
TEL 110	HMJ129	Nadinetella	Palmiet	Palmiet	South Africa	-	-34.3105	18.9495	√	√	√	√					X	X
TEL 111	HMJ 130	Nadinetella	Dwars	Palmiet	South Africa	-	-34.2879	18.9358	√									
TEL 112	HMJ 130	Nadinetella	Dwars	Palmiet	South Africa	-	-34.2879	18.9358	√	√	√	√					X	X
TEL 113	HMJ 131	Lithogloea	Dwars	Palmiet	South Africa	-	-34.2879	18.9358	√									
TEL 114	HMJ 130	Species B	Dwars	Palmiet	South Africa	-	-34.2879	18.9358	√	√	√	√	X	X				X
TEL 115	HMJ 130	Ephemerellina	Dwars	Palmiet	South Africa	-	-34.2879	18.9358	√									
TEL 116	HMJ 133	Lithogloea	Gobos	Breede	South Africa	-	-34.0386	19.6169	√	√	√	√					X	X
TEL 117	HMJ 133	Lithogloea	Gobos	Breede	South Africa	-	-34.0386	19.6169	√									
TEL 118	HMJ 134	Ephemerellina	Gobos	Breede	South Africa	-	-34.0386	19.6169	√	√	√	√	X	X				X
TEL 119	HMJ 134	Nadinetella	Gobos	Breede	South Africa	-	-34.0386	19.6169	√	√	√	√					X	X
TEL 120	HMJ 133	Lestagella	Gobos	Breede	South Africa	-	-34.0386	19.6169	√	√	√	√	X		X			X

TEL 121	HMJ 136	Lestagella	Jubilee Creek	AFR	South Africa	-	-33.8896	22.9776	√													
TEL 122	HMJ 136	Lestagella	Jubilee Creek	AFR	South Africa	-	-33.8896	22.9776	√													
TEL 123	HMJ 138	Lithogloea	Jubilee Creek	AFR	South Africa	-	-33.8896	22.9776	√	√	√											
TEL 124	HMJ 138	Nadinetella	Jubilee Creek	AFR	South Africa	-	-33.8896	22.9776	√													
TEL 125	HMJ 138	Nadinetella	Jubilee Creek	AFR	South Africa	-	-33.8896	22.9776	√													
TEL 126	HMJ 142		Jubilee Creek	AFR	South Africa	-																
TEL 127	HMJ 144		Touws	AFR	South Africa	-																
TEL 128	HMJ 145	Lestagella	Touws	AFR	South Africa	-	-33.9468	22.6131	√													
TEL 129	HMJ 146	Lestagella	Swart	AFR	South Africa	-	-33.9415	22.5095	√													
TEL 130	HMJ 149	Species A	Doring	AFR	South Africa	-	-33.8718	22.4451	√	√	√	√				X		X				X
TEL 131	HMJ 150	Nadinetella	Doring	AFR	South Africa	-	-33.8718	22.4451	√													
TEL 132	HMJ 151	Lestagella	Doring	AFR	South Africa	-	-33.8718	22.4451	√													
TEL 133	HMJ 152	Lestagella	Diepwalle	AFR	South Africa	-	-33.9474	23.141	√													
TEL 134	HMJ 154	Lestagella	Rooiels	AFR	South Africa	-	-33.9448	23.0373	√													
TEL 135	HMJ 155	Species A	Rooiels	AFR	South Africa	-	-33.9448	23.0373	√													
TEL 136	HMJ 156	Nadinetella	Rooiels	AFR	South Africa	-	-33.9448	23.0373	√	√	√	√									X	X
TEL 137	HMJ 158	Species A	Gouna	AFR	South Africa	-	-33.9906	23.0404	√													
TEL 138	HMJ 161	Lestagella	Prince Alfred Pass	AFR	South Africa	-	-33.869	23.1672	√	√	√	√				X			X			X
TEL 139	HMJ 161	Species A	Prince Alfred Pass	AFR	South Africa	-	-33.869	23.1672														
TEL 140	HMJ 162	Lestagella	Prince Alfred Pass	AFR	South Africa	-	-33.869	23.1672	√													
TEL 141	HMJ 170		Brazil		Brazil	-																
TEL 142	HMJ 170		Brazil		Brazil	-			√		√		√	X								
TEL 143	HMJ 170		Brazil		Brazil	-			√		√	√										
TEL 144	HMJ 171	Lestagella	Amatole	Keiskamma	South Africa	-	-32.5881	27.0498	√		√	√	√	X		X			X			X
TEL 145	HMJ 171	Lestagella	Amatole	Keiskamma	South Africa	-	-32.5881	27.0498	√		√	√										
TEL 146		Lestagella	Liesbeek	Table Mountain	South Africa	-					√	√										
TEL 147	HMJ 121	Species B	Oudebos	Palmiet	South Africa	-	-34.3302	18.9567														
TEL 148	HMJ 88	Ephemerellina	Jan Dissels	Olifants	South Africa	-	-32.2197	18.9946	√		√	√										
TEL 149	HMJ 64	Lestagella	Jan DuToits	Breede	South Africa	-	-33.5627	19.3404	√		√	√										
TEL 150	HMJ 161	Species A	Prince Alfred Pass	AFR	South Africa	-	-33.869	23.1672	√		√	√										
TEL 151	HMJ 182	Lestagella	Buffalo R	Keiskamma	South Africa	-																
TEL 152	HMJ 182	Lestagella	Buffalo R	Keiskamma	South Africa	-																
TEL 153	HMJ 204	Nadinetella	Tradouw	Breede	South Africa	-	-33.965	20.7048	√	√	√	√									X	X
TEL 154	HMJ 208	Lithogloea	Witte River	Breede	South Africa	-	-33.6315	19.1066	√	√	√	√									X	X
TEL 155	HMJ 208	Lithogloea	Witte River	Breede	South Africa	-	-33.6315	19.1066	√	√	√										X	X
TEL 156	HMJ 202	Species C	Huis R	Breede	South Africa	-	-33.965	20.7048	√	√	√	√	√	X		X		X				X
TEL 157	HMJ 202	Species C	Huis R	Breede	South Africa	-	-33.965	20.7048	√	√	√	√	√	X				X				X
TEL 158	HMJ 206	Ephemerellina	Witte River	Breede	South Africa	-	-33.6315	19.1066	√	√		√										
TEL 159	HMJ 206	Ephemerellina	Witte River	Breede	South Africa	-	-33.6315	19.1066	√	√		√										
TEL 160	HMJ 223	Ephemerellina	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√	√						X				X
TEL 161	HMJ 223	Ephemerellina	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√	√				X		X				X
TEL 162	HMJ 225	Nadinetella	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√	√									X	X

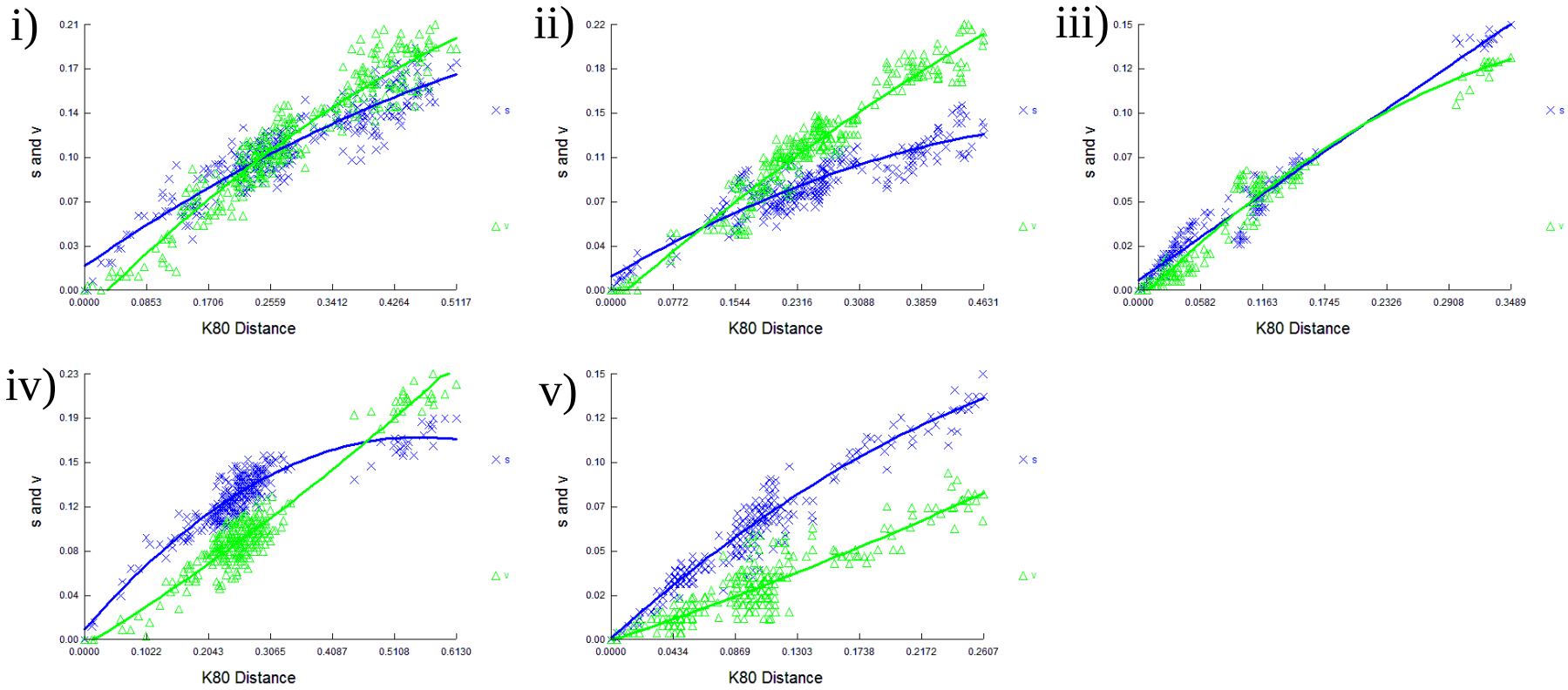
TEL 163	HMJ 225	Nadinetella	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√	√						X	X
TEL 164	HMJ 224	Lithogloea	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√								X
TEL 165	HMJ 224	Lithogloea	Huis R	Breede	South Africa	-	-33.9311	20.777	√	√	√	√						X	X
TEL 166	HMJ 213	Nadinetella	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√	√	√						X	X
TEL 167	HMJ 213	Nadinetella	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√	√	√						X	X
TEL 168	HMJ 213	Species A	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√	√	√			X	X			X
TEL 169	HMJ 214	Species A	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√	√	√				X			X
TEL 170	HMJ 216	Ephemerellina	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√	√	√			X	X			X
TEL 171	HMJ 216	Ephemerellina	Gourits	Gourits	South Africa	-	-33.9215	21.5114	√	√		√							
TEL 172	HMJ 231	Ephemerellina	JanJoubertsGat	Breede	South Africa	-	-33.9384	19.1612	√	√									
TEL 173	HMJ 231	Ephemerellina	JanJoubertsGat	Breede	South Africa	-	-33.9384	19.1612	√	√	√	√			X	X			X
TEL 174	HMJ 232	Lithogloea	JanJoubertsGat	Breede	South Africa	-	-33.9384	19.1612	√	√	√	√						X	X
TEL 175	HMJ 235	Species B	Swart R Caledon	Palmiet	South Africa	-	-34.2842	19.2541	√	√	√	√			X	X			X
TEL 176	HMJ 237	Nadinetella	Duiwelsbos	Breede	South Africa	-	-33.9936	20.4608	√	√	√	√						X	X
TEL 177	HMJ 240	Nadinetella	Koloniesbos	Breede	South Africa	-	-33.9955	20.4522	√	√	√	√						X	X
TEL 178	HMJ 240	Lestagella	Koloniesbos	Breede	South Africa	-	-33.9955	20.4522	√	√	√	√	√	X	X		X		X
TEL 179	MR 15/03/12	Leptohyphes				-				√									
TEL 180	P2150	Manohyphella	Madagascar		Madagascar	-				√		√							
TEL 181	P2150	Manohyphella	Madagascar		Madagascar	-						√			X				
TEL 182	P2189	Manohyphella	Madagascar		Madagascar	-				√									
TEL 183	P2189	Manohyphella	Madagascar		Madagascar	-			√	√					X				
TEL 184	HMJ 249	Lestagella	Witte R Gamtoos	Gamtoos	South Africa	-	-33.6527	24.5154	√	√		√	√	X	X		X		X
TEL 185	HMJ 249	Lestagella	Witte R Gamtoos	Gamtoos	South Africa	-	-33.6527	24.5154	√	√	√	√	√	X			X		X
TEL 186	HMJ 249	Lestagella	Witte R Gamtoos	Gamtoos	South Africa	-	-33.6527	24.5154	√		√								
TEL 187	HMJ 249	Lestagella	Witte R Gamtoos	Gamtoos	South Africa	-	-33.6527	24.5154	√	√	√								X
TEL 188	HMJ 253	Lestagella	Seven Weeks Poort	Gourits	South Africa	-	-33.4411	21.4094	√	√	√								X
TEL 189	HMJ 253	Lestagella	Seven Weeks Poort	Gourits	South Africa	-	-33.4411	21.4094	√	√	√	√	√	X	X		X		X
TEL 190	HMJ 253	Lestagella	Seven Weeks Poort	Gourits	South Africa	-	-33.4411	21.4094	√										
TEL 191	HMJ 256	Lestagella	N12 Herrie drift	Gourits	South Africa	-	-33.4255	22.5494	√	√	√	√			X		X		X
TEL 192	HMJ 256	Lestagella	N12 Herrie drift	Gourits	South Africa	-	-33.4255	22.5494	√										
TEL 193	HMJ 256	Lestagella	N12 Herrie drift	Gourits	South Africa	-	-33.4255	22.5494		√	√								
TEL 194	H8_3A	Species B	Steenbras		South Africa	-	-34.1807	18.9066		√									
TEL 195	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 196	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 197	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 198	H8_3A	Lithogloea	Steenbras		South Africa	-	-34.1807	18.9066		√									
TEL 199	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 200	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 201	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 202	H8_1		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 203	H8_1		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 204	H8_1	Lithogloea	Steenbras		South Africa	-	-34.1807	18.9066		√	√								

TEL 205	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 206	H8_3A		Steenbras		South Africa	-	-34.1807	18.9066											
TEL 207	V3_2	Nadinetella	Voevlei		South Africa	-	-33.3375	19.0336		√									
TEL 208	V3_2		Voevlei		South Africa	-	-33.3375	19.0336											
TEL 209	V3_1	Lithogloea	Voevlei		South Africa	-	-33.3375	19.0336		√									
TEL 210	V3a	Nadinetella	Voevlei		South Africa	-	-33.3375	19.0336		√									
TEL 211	V3_2		Voevlei		South Africa	-	-33.3375	19.0336											
TEL 212	HMJ 259	Ephemerellina	Gatbos (Tradouw)	Breede	South Africa	-	-33.9848	20.7195	√	√	√	√	√	**X**			**X**	**X**	**X**	**X**
TEL 213	HMJ 259	Ephemerellina	Gatbos (Tradouw)	Breede	South Africa	-	-33.9848	20.7195	√											
TEL 214	HMJ 259	Nadinetella	Gatbos (Tradouw)	Breede	South Africa	-	-33.9848	20.7195	√	√	√	√	√	**X**					**X**	**X**
TEL 215	HMJ 259	Nadinetella	Gatbos (Tradouw)	Breede	South Africa	-	-33.9848	20.7195	√											
TEL 216	HMJ 263	Species C	Huis R	Breede	South Africa	-	-33.9221	20.7565		√	√	√	√							
TEL 217	HMJ 265	Lestagella	Huis R	Breede	South Africa	-	-33.9221	20.7565	√	√	√	√	√	**X**	**X**	**X**	**X**	**X**	**X**	**X**
TEL 218	HMJ 265	Lithogloea	Huis R	Breede	South Africa	-	-33.9221	20.7565	√	√	√									**X**
TEL 219	HMJ 264	Species A	Huis R	Breede	South Africa	-	-33.9221	20.7565		√										
TEL 220	HMJ 264	Species A	Huis R	Breede	South Africa	-	-33.9221	20.7565	√	√	√	√	√	**X**	**X**	**X**				**X**
TEL 221	HMJ 263	Lestagella	Huis R	Breede	South Africa	-	-33.9221	20.7565	√											
TEL 222	HMJ 266	Lithogloea	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477		√	√	√	√	**X**						
TEL 223	HMJ 266	Lestagella	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477		√	√									**X**
TEL 224	HMJ 266	Lithogloea	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477	√											
TEL 225	HMJ 266	Lithogloea	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477	√					**X**					**X**	**X**
TEL 226	HMJ 266	Lestagella	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477	√											
TEL 227	HMJ 266	Lestagella	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477												
TEL 228	HMJ 266	Nadinetella	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477	√	√	√	√							**X**	**X**
TEL 229	HMJ 268	Nadinetella	Robertson DeHoop	Breede	South Africa	-	-33.7469	19.9477	√											
TEL 230	HMJ 269	Lestagella	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913		√	√									**X**
TEL 231	HMJ 269	Lithogloea	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913	√	√	√									**X**
TEL 232	HMJ 270	Nadinetella	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913	√	√	√	√	√	**X**					**X**	**X**
TEL 233	HMJ 270	Nadinetella	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913	√	√										
TEL 234	HMJ 270	Nadinetella	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913	√	√	√									**X**
TEL 235	HMJ 270	Nadinetella	Dassieshoek	Breede	South Africa	-	-33.7414	19.8913	√	√	√									**X**
TEL 236	HMJ 273	Ephemerellina	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√	√									**X**
TEL 237	HMJ 273	Ephemerellina	McGregor	Breede	South Africa	-	-34.028	19.9048	√											
TEL 238	HMJ 273	Species C	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√	√									**X**
TEL 239	HMJ 273	Species C	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√	√	√	√	**X**	**X**	**X**				**X**
TEL 240	HMJ 271	Species C	McGregor	Breede	South Africa	-	-34.028	19.9048												
TEL 241	HMJ 273	Nadinetella	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√	√									**X**
TEL 242	HMJ 273	Nadinetella	McGregor	Breede	South Africa	-	-34.028	19.9048	√											
TEL 243	HMJ 271	Lithogloea	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√	√									**X**
TEL 244	HMJ 271	Lithogloea	McGregor	Breede	South Africa	-	-34.028	19.9048	√											
TEL 245	HMJ 272	Species A	McGregor	Breede	South Africa	-	-34.028	19.9048	√											
TEL 246	HMJ 271	Lestagella	McGregor	Breede	South Africa	-	-34.028	19.9048	√	√										

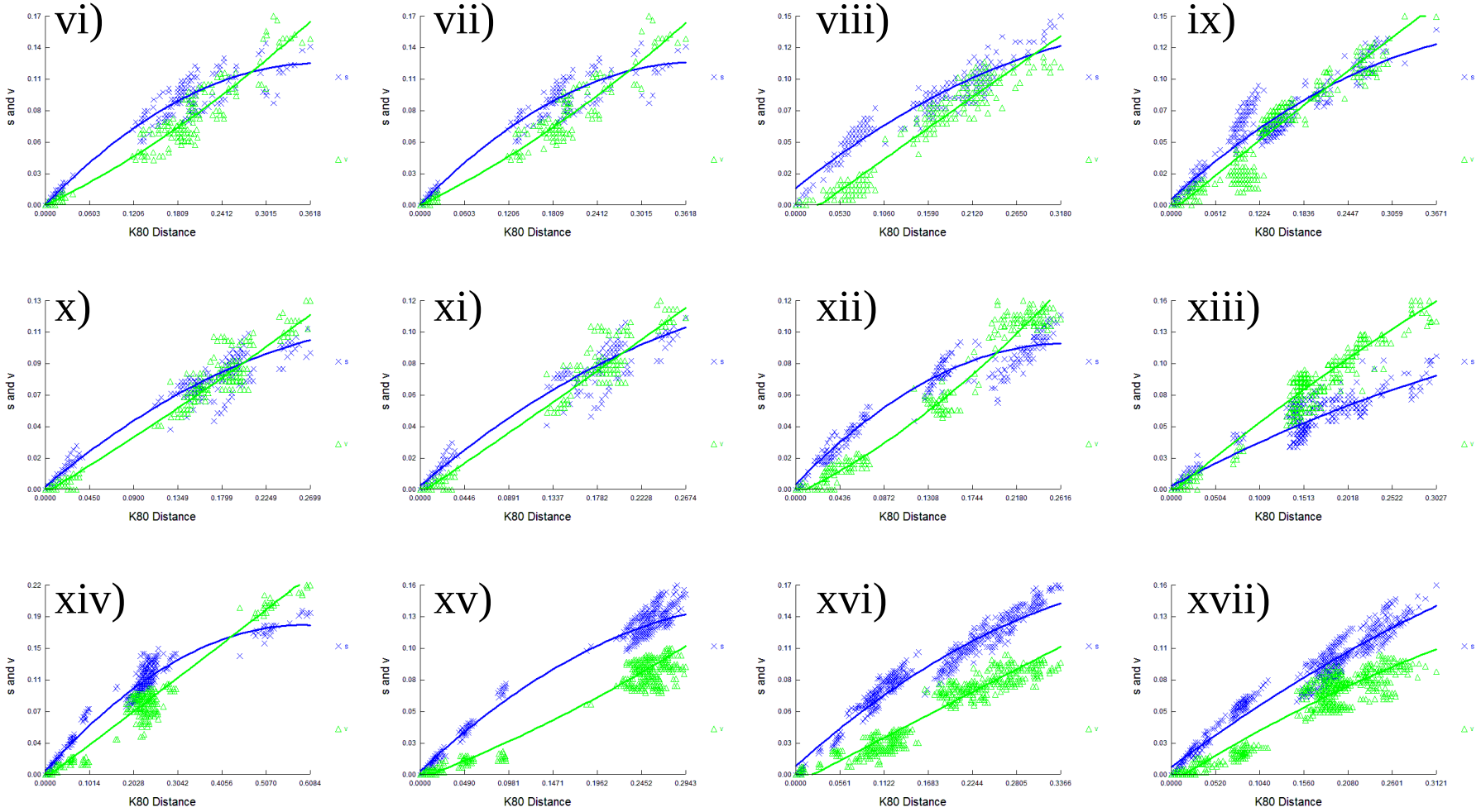
TEL 247	HMJ 271	Lestagella	McGregor	Breede	South Africa	-	-34.028	19.9048	√													
TEL 248	HMJ 274	Lithogloea	Boesmans McGregor	Breede	South Africa	-	-34.0416	19.9604	√	√												
TEL 249	HMJ 275	Species C	Boesmans McGregor	Breede	South Africa	-	-34.0416	19.9604		√	√											
TEL 250	HMJ 274	Lestagella	Boesmans McGregor	Breede	South Africa	-	-34.0416	19.9604		√	√											X
TEL 251	HMJ 275	Lithogloea	Boesmans McGregor	Breede	South Africa	-	-34.0416	19.9604	√													
TEL 252	HMJ 280	Lestagella	Tulbagh secret falls	Berg	South Africa	-	-33.1866	19.1337		√	√											
TEL 253	HMJ 277	Lestagella	Tulbagh secret falls	Berg	South Africa	-	-33.1855	19.1261	√	√	√	√			X		X					X
TEL 254	HMJ 277	Lestagella	Tulbagh secret falls	Berg	South Africa	-	-33.1855	19.1261	√													
TEL 255	HMJ 278	Nadinetella	Tulbagh secret falls	Berg	South Africa	-	-33.1855	19.1261	√	√	√											X
TEL 256	HMJ 277	Nadinetella	Tulbagh secret falls	Berg	South Africa	-	-33.1855	19.1261	√													
TEL 257	HMJ 278	Nadinetella	Tulbagh secret falls	Berg	South Africa	-	-33.1855	19.1261	√													
TEL 258	HMJ 285	Ephemerellina	Beaverlac- Ratel	Berg	South Africa	-	-32.8898	19.0821	√	√		√	√	X	X	X						X
TEL 259	HMJ 286	Nadinetella	Beaverlac- Ratel	Berg	South Africa	-	-32.9125	19.0706	√	√	√	√	√	X						X		X
TEL 260	HMJ 286	Nadinetella	Beaverlac- Ratel	Berg	South Africa	-	-32.9125	19.0706	√													
TEL 261	HMJ 288	Ephemerellina	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√											X
TEL 262	HMJ 288	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√											X
TEL 263	HMJ 288	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√											X
TEL 264	HMJ 288	Ephemerellina	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833														
TEL 265	HMJ 293	Lestagella	Jonkershoek	Eerste	South Africa	-	-33.9949	18.9792	√	√	√											X
TEL 266	**HMJ 292**	**Lithogloea**	**Jonkershoek**	**Eerste**	South Africa	-	-33.9949	18.9792	√	√	√											X
TEL 267	HMJ 288	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√											X
TEL 268	HMJ 294	Lithogloea	Jonkershoek	Eerste	South Africa	-	-34.3302	18.9567	√	√	√											X
TEL 269	HMJ 288	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√	√	√	X						X		X
TEL 270	HMJ 288	Nadinetella	Jonkershoek	Eerste	South Africa	-	-33.9983	18.9833	√	√	√	√	√	X						X		X
TEL 271	HMJ 298	Lestagella	Kogelberg	Palmiet	South Africa	-	-34.2879	18.9358		√	√			X								
TEL 272	HMJ 294		Kogelberg	Palmiet	South Africa	-																
TEL 273	HMJ 294		Kogelberg	Palmiet	South Africa	-																
TEL 274	HMJ 294		Kogelberg	Palmiet	South Africa	-																
TEL 275	HMJ 294		Kogelberg	Palmiet	South Africa	-																
TEL 276	HMJ 294	Species B	Kogelberg	Palmiet	South Africa	-	-34.3302	18.9567	√	√	√	√	√	X	X	X						X
TEL 277	HMJ 300	Lithogloea	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411		√												
TEL 278	HMJ 300		Hottentots H	Palmiet	South Africa	-																
TEL 279	HMJ 299		Hottentots H	Palmiet	South Africa	-																
TEL 280	HMJ 299	Ephemerellina	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411	√	√	√	√	√	X	X	X						X
TEL 281	HMJ 300	Lestagella	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411	√				√	X								X
TEL 282	HMJ 300	Lestagella	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411	√													
TEL 283	HMJ 299		Hottentots H	Palmiet	South Africa	-																
TEL 284	HMJ 299	Lithogloea	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411	√	√	√	√	√	X						X		X
TEL 285	HMJ 301	Species A	Hottentots H	Palmiet	South Africa	-	-34.0559	19.0411	√													
TEL 286	GEN 248C	Ephemerythidae	kzn		South Africa	-	25.3333	30.85				√	√									
TEL 287	GEN 248C	Ephemerythidae	kzn		South Africa	-	25.3333	30.85					√									
TEL 288	ZIM 77G		zimbabwe		Zimbabwe	-			√			√	√									

TEL 289	ZIM 71J		zimbabwe	Zimbabwe	-			√	√		
GENBANK		Manohyphella	Madagascar	Madagascar	GQ118336			√			**X**
GENBANK		Manohyphella	Madagascar	Madagascar	GQ118296	√					**X**
GENBANK		Manohyphella	Madagascar	Madagascar	GQ118259		√				**X**

Appendix 4B

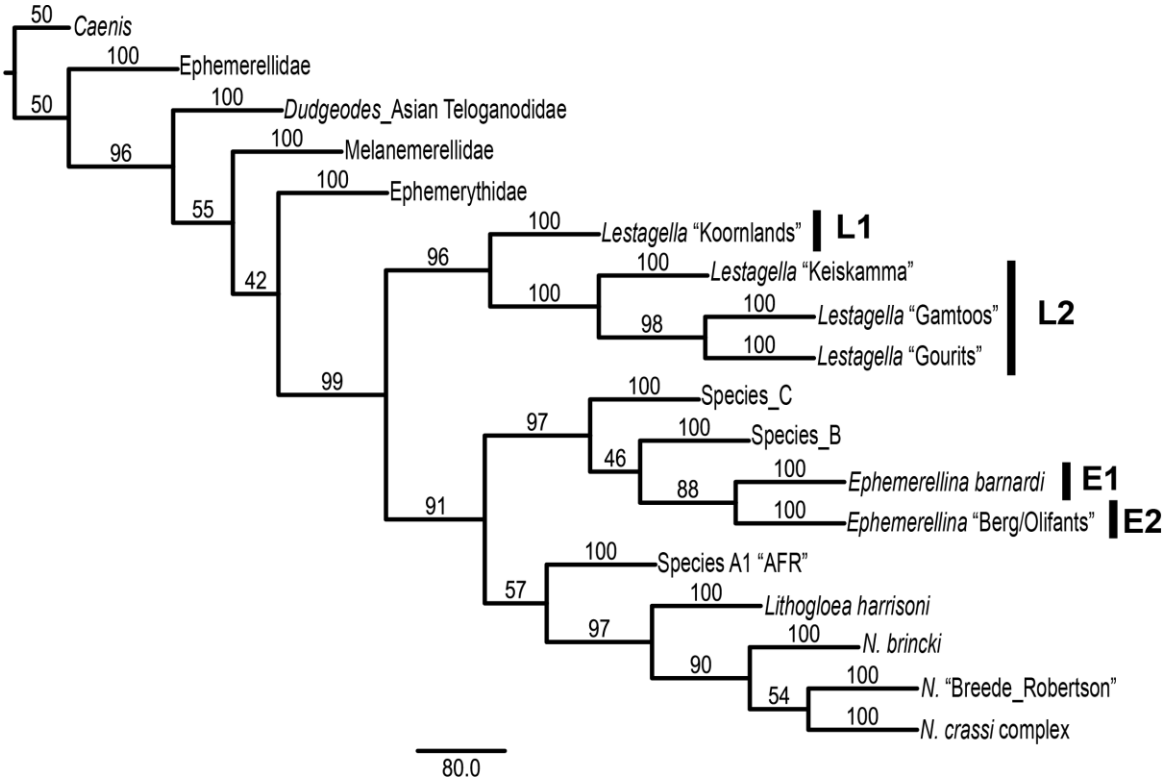


Appendix Figure 4B. Dambe saturation plots showing transversions (green) and transitions (blue) comparing all genera for each gene, (i) 12S, (ii) 16S, (iii) 28S, (iv) COI and (v) H3.

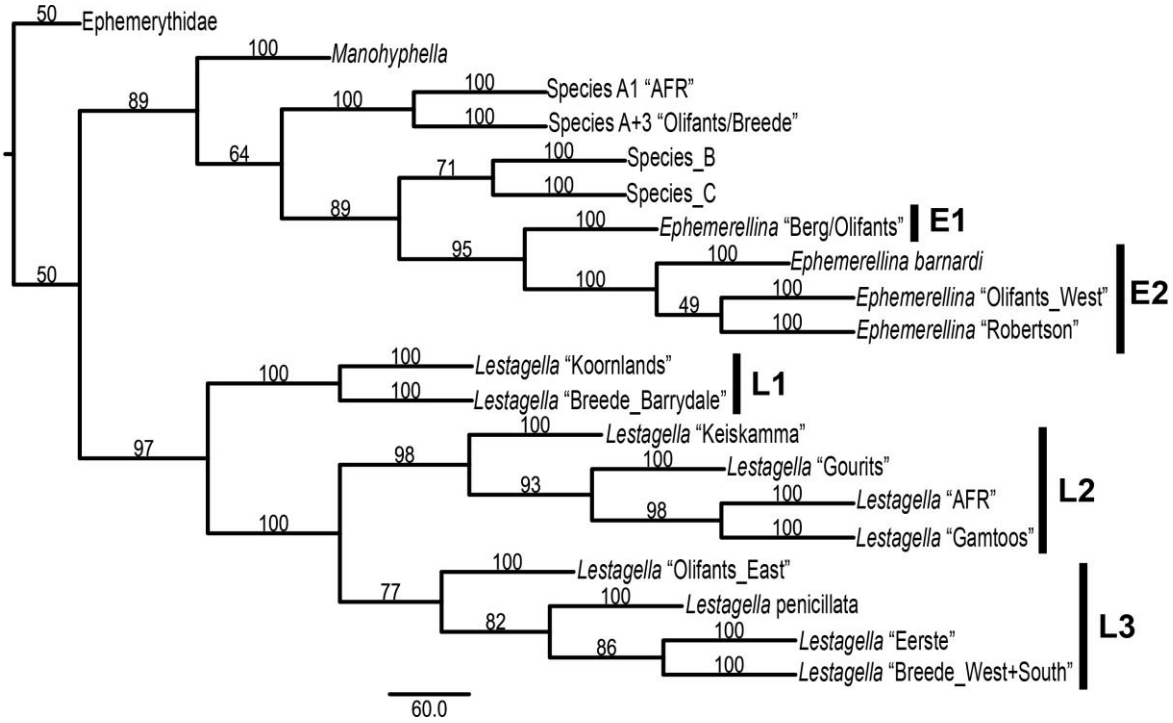


Appendix Figure 4B. Dambe saturation plots showing tansversions (green) and transitions (blue) within main clades for each gene, (vi) 12S "Ephem clade", (vii) 12S "Ephem clade" without SpC, (viii)12S "Lest clade", (ix) 12S "Nad/Lith clade", (x) 16S "Ephem clade", (xi) 16S "Ephem clade" without SpC, (xii) 16S "Lest clade", (xiii) 16S "Nad/Lith clade", (xiv) COI "Ephem clade", (xv) COI "Ephem clade" without SpC, (xvi) COI "Lest clade", (xvii) COI "Nad/Lith clade". Note removing the Species C group from the COI alignment reduced the saturation, there seems to be a large amount of substitutions in the group in the COI gene, possibly as a result of a pseudogene.

Appendix 4C



Appendix Figure 4C(i). "Afr/SAm Group" Species tree estimation, using 4 genes COI/12S/28S/H3



Appendix Figure 4C(ii). "Afr/Mad Group" Species tree using 4 genes COI/12S/28S/H3

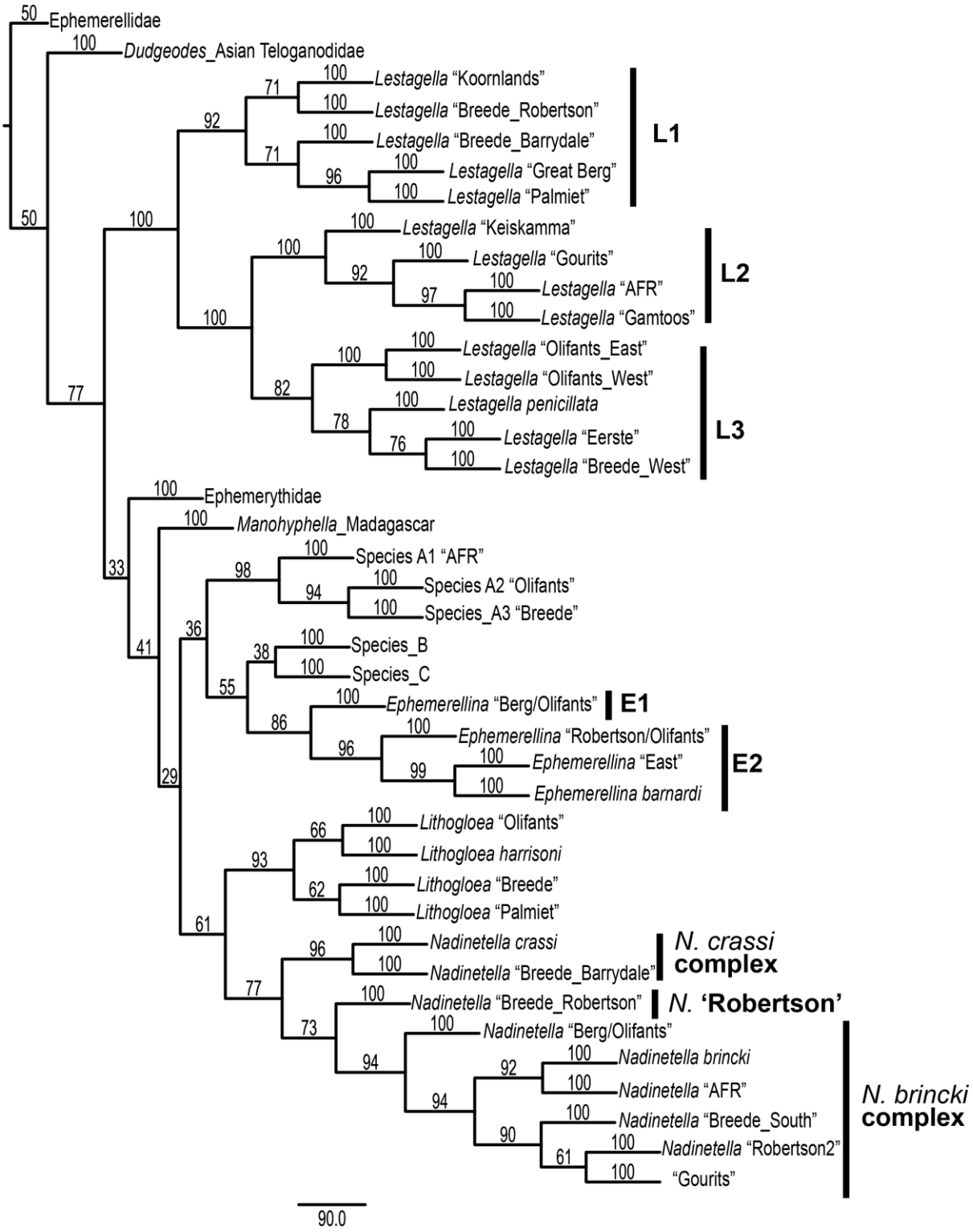
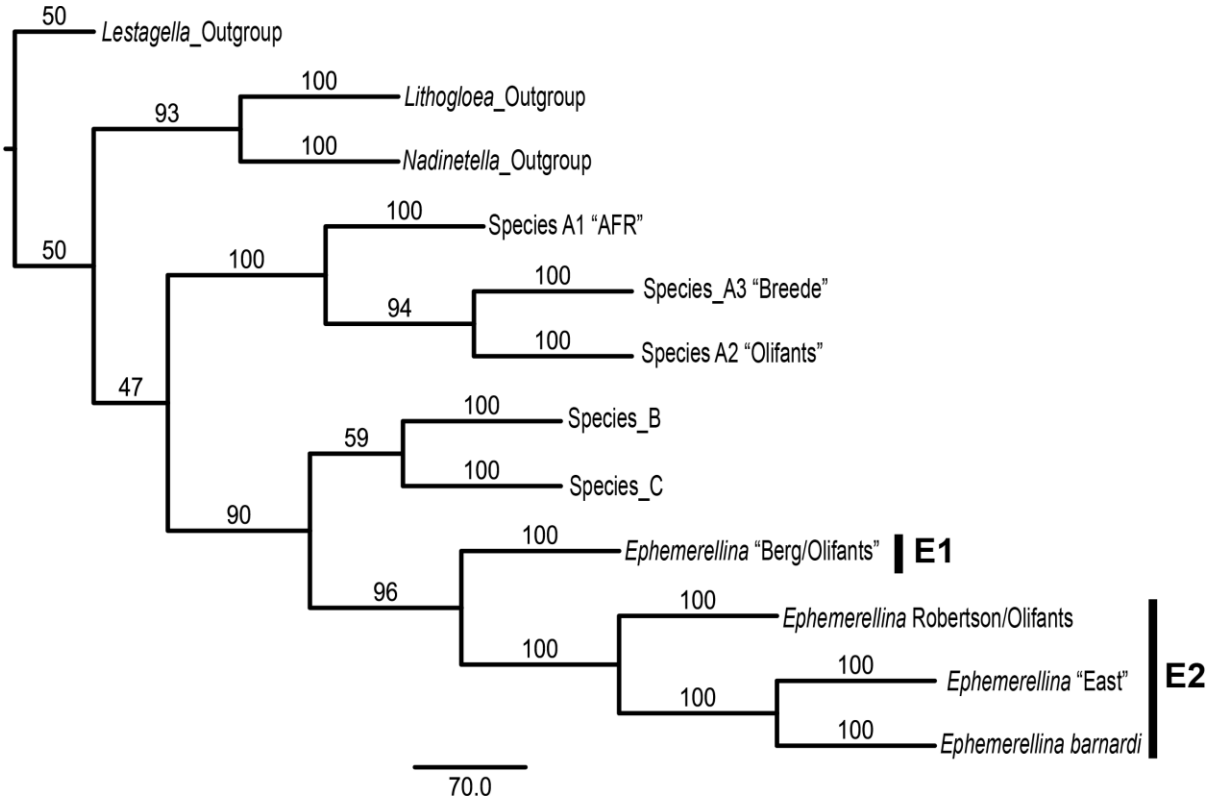
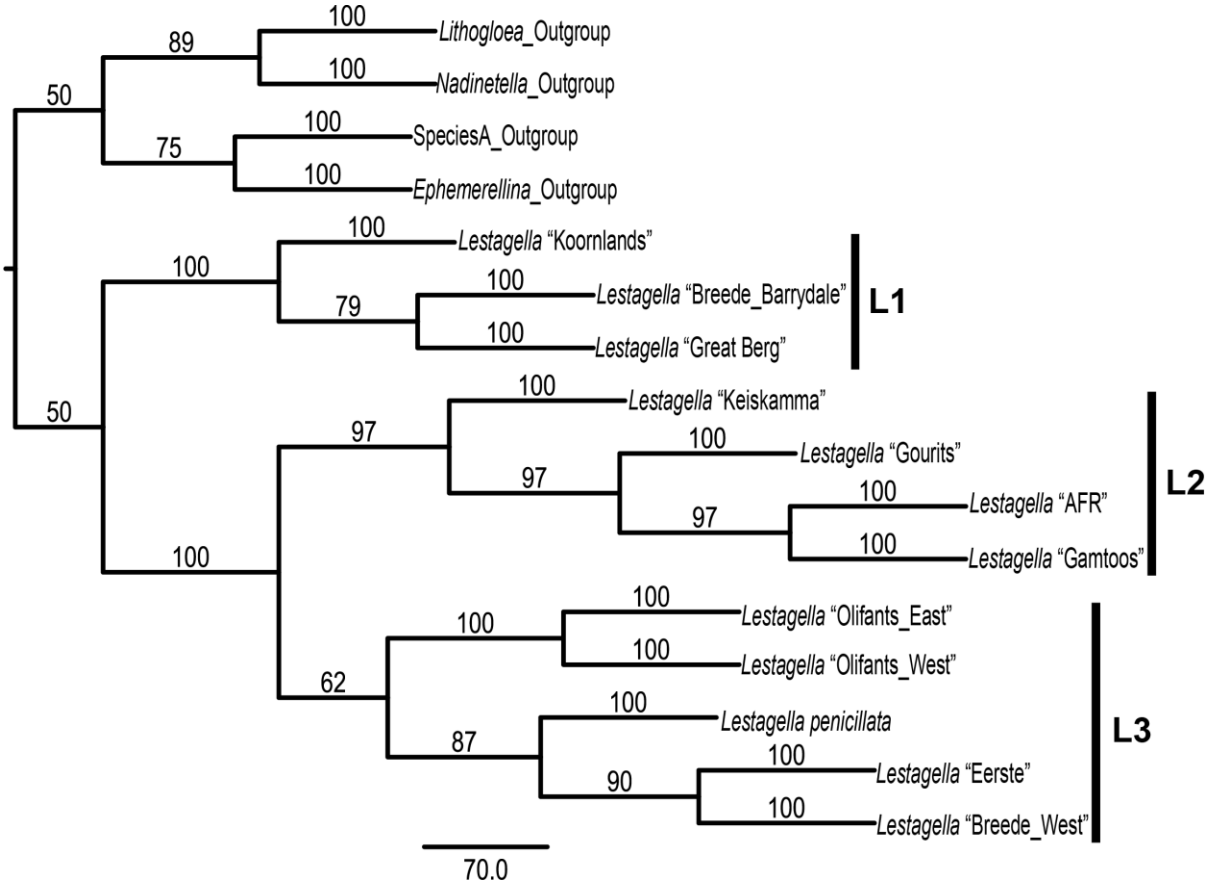


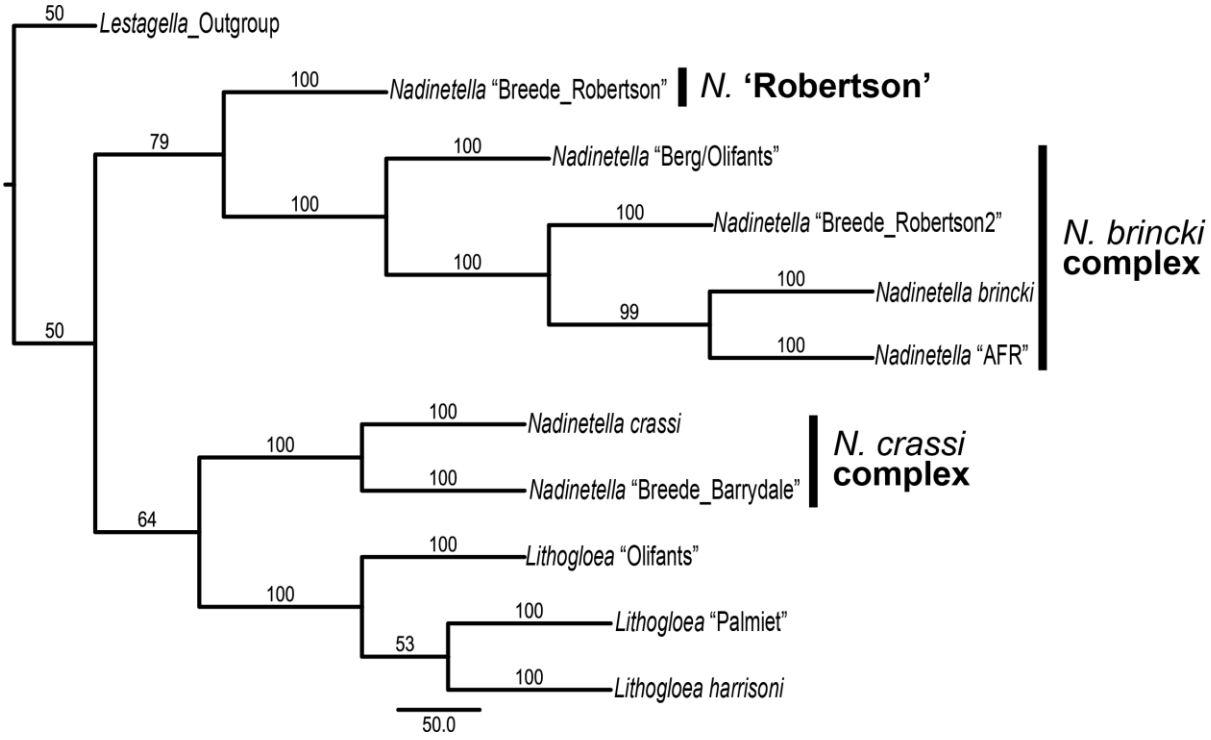
Figure 4C(iii). Species Tree estimation using only mitochondrial genes COI/16S/12S, analysis done using 115 taxa, including Manohyphella and Ephemerythidae



Appendix Figure 4C(iv). Species Tree Estimations of the ‘‘Ephem Clade’’, using four genes COI/16S/12S/H3.



Appendix Figure 4C(v). Species Tree Estimations of the "Lest Clade" using four genes COI/16S/12S/H3.



Appendix Figure 4C(vi). Species Tree Estimations of the "Nad/Lith Clade" using four genes COI/16S/12S/H3.

Appendix 4D. Barrier analysis results for *Ephemerellina* , *Lestagella, Lithogloea, Nadintella brincki* Complex and Nadinetella crassi Complex, Species A, with bootstrap support values

Ephemerellina Group		
Catchment(s) 1	**Catchment(s) 2**	**Bootstrap**
AFR	Gourits	73
Gourits	Breede E Barrydale	31
Breede E Barrydale	Koornlands	69
Koornlands	Robertson	100
Koornlands	Breede W Mcgregor	95
Robertson	Breede W Overberg,Breede W Franshoek,Breede W Molenaars,Breede W Limietberg	100
Breede W Mcgregor	Breede W Overberg,Breede W Franshoek,Breede W Molenaars,Breede W Limietberg	62
Robertson	Breede W Mcgregor	100
Robertson	Great Berg	100
Robertson	Olifants East	100
Olifants East	Great Berg	100
Great Berg	Breede W Overberg,Breede W Franshoek,Breede W Molenaars,Breede W Limietberg	100
Olifants East	Olifants West	39
Breede W Franshoek,Breede W Molenaars,Breede W Limietberg	Palmiet,Eerste	88
Palmiet	Palmiet	26
Eerste	Palmiet	44
Breede W Overberg	Palmiet	71

Appendix 4D. Cont.

Lestagella Group		
Catchment(s) 1	**Catchment(s) 2**	**Bootstrap**
Keiskamma	Gamtoos,AFR	100
AFR	Gourits	26
AFR	Gourits	26
Gourits	Breede E Barrydale	100
Breede E Barrydale	Koornlands,Gamtoos,Gourits	100
Koornlands,Gamtoos,Gourits	Breede E Robertson	100
Koornlands,Gamtoos,Gourits	Breede W McGregor,Breede W Overberg,AFR,Keiskamma,Breede W Hex,Breede W Molenaars,Breede W Limietbe	100
Breede E Robertson	Breede W McGregor,Breede W Overberg,AFR,Keiskamma,Breede W Hex,Breede W Molenaars,Breede W Limietbe	100
Breede E Robertson	Olifants East	100
Palmiet	Breede W Overberg,AFR,Keiskamma,Breede W Molenaars	100
Palmiet	Eerste	100
Eerste	Breede W Molenaars,Breede W Limietberg	100
Great Berg	Breede W Limietberg,Breede W Hex	100
Eerste	Table Mountain	100
Table Mountain	Great Berg	100
Olifants East	Breede W Hex	100
Olifants East	Great Berg	100
Olifants East	Olifants West	75
Olifants West	Great Berg	100

Appendix 4D. Cont.

Lithogloea Group		
Catchment(s) 1	**Catchment(s) 2**	**Bootstrap**
Breede E Barrydale	Breede W McGregor	26
Breede W McGregor	Breede W McGregor,Breede W Overberg	5
Breede W McGregor	Breede E Robertson	17
Breede W McGregor,Breede W Overberg	Breede E Robertson	20
Breede E Robertson	Breede W Limietberg	24
Breede W Limietberg	Breede W Limietberg	5
Breede W McGregor,Breede W Overberg	Breede W Limietberg	23
Breede W McGregor,Breede W Overberg	Breede W Franshoek,Breede W Molenaars	72
Breede W Franshoek	Breede W Molenaars	4
Breede W Franshoek,Breede W Molenaars	Breede W Limietberg	53
Breede W Franshoek,Breede W Molenaars	Breede W Limietberg	58
Breede W Limietberg	Olifants East,Olifants West	73
Olifants E	Olifants East	4
Olifants E,Olifants West	Olifants West	17
Breede W Franshoek	Palmiet	87
Breede W Franshoek,Breede W Molenaars	Eerste	99
Palmiet	Eerste	98

Appendix 4D. Cont.

Nadinetella brincki Complex		
Catchment(s) 1	**Catchment(s) 2**	**Bootstrap**
AFR	AFR	11
AFR	AFR	27
AFR	AFR	13
AFR	Gourits	100
Gourits	Breede East Robertson	100
Breede East Robertson	Breede W Overberg	100
Breede East Robertson	Great Berg Tulbagh	100
Breede East Robertson	Olifants East,Olifants West,Great Berg Beaverlac	100
Breede W Overberg	Palmiet	100
Breede W Overberg	Breede W Molenaars	100
Breede W Overberg	Eerste	100
Breede W Overberg	Great Berg Tulbagh	100
Olifants East,Olifants West,Great Berg Beaverlac	Great Berg Tulbagh	100
Breede W Molenaars	Great Berg Tulbagh	100
Breede W Molenaars	Eerste,Great Berg Beaverlac	73
Eerste,Great Berg Beaverlac	Palmiet	71

Nadnetella crassi Complex		
Catchment(s) 1	**Catchment(s) 2**	**Bootstrap**
Breede E Barrydale	Koornlands	20
Koornlands	Breede W McGregor	100
Breede W McGregor	Breede W Limietberg,Eerste	82
Breede W Limietberg,Eerste	Palmiet	98

Appendix 4D. Cont.

Species A Group Catchment(s) 1	Catchment(s) 2	Bootstrap
AFR Tsitsikamma	AFR Tsitsikamma	31
AFR Tsitsikamma	AFR Outeniqua	45
AFR Tsitsikamma	AFR Outeniqua	18
AFR Tsitsikamma	AFR Outeniqua	90
AFR Outeniqua	AFR Outeniqua	100
AFR Outeniqua	AFR Outeniqua	100
AFR Outeniqua	AFR Outeniqua	80
AFR Outeniqua	Gourits,Breede E Barrydale	100
Gourits,Breede E Barrydale	Breede W McGregor	100
Breede W McGregor	Olifants East	100
Breede W McGregor	Palmiet	71
Palmiet	Olifants East	100

Appendix 4E. Specimens used for each taxon group for the Isolation By Distances (IBD) analyses.

Location details for each specimen can be found in Appendix 4A

Ephemerellina		***Lestagella***			**Species B**
TEL_034_Ephemerellina	TEL_044_Ephemerellina	TEL_021_Lestagella	TEL_097_Lestagella	TEL_226_Lestagella	TEL_076_Species_B
TEL_039_Ephemerellina	TEL_045_Ephemerellina	TEL_022_Lestagella	TEL_098_Lestagella	TEL_227_Lestagella	TEL_114_Species_B
TEL_040_Ephemerellina		TEL_006_Lestagella	TEL_102_Lestagella	TEL_230_Lestagella	TEL_175_Species_B
TEL_041_Ephemerellina		TEL_001_Lestagella	TEL_103_Lestagella	TEL_246_Lestagella	TEL_276_Species_B
TEL_042_Ephemerellina		TEL_002_Lestagella	TEL_104_Lestagella	TEL_247_Lestagella	TEL_048_Species_B
TEL_043_Ephemerellina		TEL_003_Lestagella	TEL_120_Lestagella	TEL_250_Lestagella	TEL_194_Species_B
TEL_066_Ephemerellina		TEL_004_Lestagella	TEL_121_Lestagella	TEL_253_Lestagella	
TEL_067_Ephemerellina		TEL_005_Lestagella	TEL_122_Lestagella	TEL_254_Lestagella	
TEL_075_Ephemerellina		TEL_013_Lestagella	TEL_128_Lestagella	TEL_252_Lestagella	
TEL_148_Ephemerellina		TEL_020_Lestagella	TEL_129_Lestagella	TEL_265_Lestagella	
TEL_080_Ephemerellina		TEL_025_Lestagella	TEL_132_Lestagella	TEL_271_Lestagella	
TEL_090_Ephemerellina		TEL_017_Lestagella	TEL_133_Lestagella	TEL_281_Lestagella	
TEL_095_Ephemerellina		TEL_023_Lestagella	TEL_134_Lestagella	TEL_282_Lestagella	
TEL_105_Ephemerellina		TEL_024_Lestagella	TEL_138_Lestagella	TEL_049_Lestagella	
TEL_115_Ephemerellina		TEL_026_Lestagella	TEL_140_Lestagella	TEL_050_Lestagella	
TEL_118_Ephemerellina		TEL_028_Lestagella	TEL_144_Lestagella		
TEL_158_Ephemerellina		TEL_029_Lestagella	TEL_145_Lestagella		
TEL_159_Ephemerellina		TEL_030_Lestagella	TEL_149_Lestagella		
TEL_170_Ephemerellina		TEL_032_Lestagella	TEL_178_Lestagella		
TEL_171_Ephemerellina		TEL_033_Lestagella	TEL_184_Lestagella		
TEL_160_Ephemerellina		TEL_035_Lestagella	TEL_185_Lestagella		
TEL_161_Ephemerellina		TEL_036_Lestagella	TEL_186_Lestagella		
TEL_172_Ephemerellina		TEL_062_Lestagella	TEL_187_Lestagella		
TEL_173_Ephemerellina		TEL_063_Lestagella	TEL_188_Lestagella		
TEL_212_Ephemerellina		TEL_071_Lestagella	TEL_189_Lestagella		
TEL_213_Ephemerellina		TEL_072_Lestagella	TEL_190_Lestagella		
TEL_236_Ephemerellina		TEL_077_Lestagella	TEL_191_Lestagella		
TEL_237_Ephemerellina		TEL_082_Lestagella	TEL_192_Lestagella		
TEL_258_Ephemerellina		TEL_083_Lestagella	TEL_193_Lestagella		
TEL_261_Ephemerellina		TEL_085_Lestagella	TEL_221_Lestagella		
TEL_280_Ephemerellina		TEL_087_Lestagella	TEL_217_Lestagella		
TEL_010_Ephemerellina		TEL_092_Lestagella	TEL_223_Lestagella		

Nadinetella brincki complex		*Nadinetella crassi* complex	*Lithogloea*		Species A
TEL_011_Nadinetella	TEL_215_Nadinetella	tel100_Nadinetella	TEL_064_Lithogloea	TEL_047_Lithogloea	TEL_018_Species_A
TEL_012_Nadinetella	TEL_228_Nadinetella	tel101_Nadinetella	TEL_065_Lithogloea	TEL_198_Lithogloea	TEL_019_Species_A
TEL_007_Nadinetella	TEL_229_Nadinetella	tel110_Nadinetella	TEL_073_Lithogloea	TEL_204_Lithogloea	TEL_015_Species_A
TEL_014_Nadinetella	TEL_232_Nadinetella	tel112_Nadinetella	TEL_079_Lithogloea	TEL_209_Lithogloea	TEL_027_Species_A
TEL_016_Nadinetella	TEL_233_Nadinetella	tel153_Nadinetella	TEL_081_Lithogloea		TEL_031_Species_A
TEL_037_Nadinetella	TEL_234_Nadinetella	tel162_Nadinetella	TEL_070_Lithogloea		TEL_084_Species_A
TEL_038_Nadinetella	TEL_235_Nadinetella	tel163_Nadinetella	TEL_089_Lithogloea		TEL_130_Species_A
TEL_068_Nadinetella	TEL_241_Nadinetella	tel176_Nadinetella	TEL_091_Lithogloea		TEL_135_Species_A
TEL_074_Nadinetella	TEL_242_Nadinetella	tel177_Nadinetella	TEL_093_Lithogloea		TEL_137_Species_A
TEL_078_Nadinetella	TEL_256_Nadinetella	tel214_Nadinetella	TEL_099_Lithogloea		TEL_139_Species_A
TEL_069_Nadinetella	TEL_255_Nadinetella	tel215_Nadinetella	TEL_108_Lithogloea		TEL_150_Species_A
TEL_086_Nadinetella	TEL_257_Nadinetella	tel241_Nadinetella	TEL_109_Lithogloea		TEL_168_Species_A
TEL_088_Nadinetella	TEL_259_Nadinetella	tel242_Nadinetella	TEL_113_Lithogloea		TEL_169_Species_A
TEL_094_Nadinetella	TEL_260_Nadinetella	tel262_Nadinetella	TEL_116_Lithogloea		TEL_219_Species_A
TEL_096_Nadinetella	TEL_262_Nadinetella	tel267_Nadinetella	TEL_117_Lithogloea		TEL_220_Species_A
TEL_100_Nadinetella	TEL_263_Nadinetella	tel269_Nadinetella	TEL_154_Lithogloea		TEL_245_Species_A
TEL_101_Nadinetella	TEL_267_Nadinetella		TEL_155_Lithogloea		TEL_285_Species_A
TEL_106_Nadinetella	TEL_269_Nadinetella		TEL_164_Lithogloea		
TEL_110_Nadinetella	TEL_270_Nadinetella		TEL_165_Lithogloea		
TEL_111_Nadinetella	TEL_051_Nadinetella		TEL_174_Lithogloea		
TEL_112_Nadinetella	TEL_052_Nadinetella		TEL_218_Lithogloea		
TEL_119_Nadinetella	TEL_207_Nadinetella		TEL_222_Lithogloea		
TEL_124_Nadinetella	TEL_210_Nadinetella		TEL_224_Lithogloea		
TEL_125_Nadinetella			TEL_225_Lithogloea		
TEL_131_Nadinetella			TEL_231_Lithogloea		
TEL_136_Nadinetella			TEL_243_Lithogloea		
TEL_153_Nadinetella			TEL_244_Lithogloea		
TEL_166_Nadinetella			TEL_248_Lithogloea		
TEL_167_Nadinetella			TEL_251_Lithogloea		
TEL_162_Nadinetella			TEL_266_Lithogloea		
TEL_163_Nadinetella			TEL_268_Lithogloea		
TEL_176_Nadinetella			TEL_284_Lithogloea		
TEL_177_Nadinetella			TEL_277_Lithogloea		
TEL_214_Nadinetella			TEL_046_Lithogloea		

Appendix 5A. Dispersal, vicariance and extinction routes taken according to the three RASP analyses for each dataset. Area code provided on the far right, probabilities >0.70 in bold

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Afr/SAm Group						
BBM	**5**	**2**	**1**	**0**	**E->DE->D|E**	**0.939**
	3	2	1	0	E->CE->C|E	0.4812
	2	2	1	0	A->AB->A|B	0.4767
	1	3	1	1	C->->AE->A|E	0.0625
Total		9	4	1		
SDEC	**5**	**0**	**1**	**0**	**DE->D|E**	**0.9706**
	4	**1**	**0**	**0**	**E->E^E->DE^E->E|DE**	**0.722**
	3	0	1	0	CE->C|E	0.6399
	2	0	1	0	AB->A|B	0.3921
	1	2	1	0	CE->ABCE->AB|CE	0.1156
Total		3	4	0		
SDIVA	**5**	**0**	**1**	**0**	**DE->D|E**	**1**
	4	**1**	**0**	**0**	**E->E^E->DE^E->E|DE**	**1**
	3	**0**	**1**	**0**	**CE->C|E**	**1**
	2	**0**	**1**	**0**	**AB->A|B**	**1**
	1	2	1	0	BC->ABCE->AB|CE	0.2543
Total		3	4	0		

Area	Code
North East Africa	A
South America	B
Indonesia	C
Keiskamma	D
Western Cape	E

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Afr/Mad Group						
BBM	**5**	**2**	**1**	**0**	**D->CD->C|D**	**0.9217**
	3	**2**	**1**	**0**	**D->AD->A|D**	**0.8377**
	2	**2**	**1**	**0**	**D->BD->B|D**	**0.8448**
	Total	6	3	0		
SDEC	**5**	**0**	**1**	**0**	**CD->C|D**	**0.9969**
	4	**1**	**0**	**0**	**D->D^D->CD^D->D|CD**	**0.9678**
	3	0	1	0	AD->A|D	0.6208
	2	**0**	**1**	**0**	**BD->B|D**	**0.9458**
	1	2	0	0	AD->AD^D->ABD^D->BD|AI	0.3315
	Total	3	3	0		
SDIVA	**5**	**0**	**1**	**0**	**CD->C|D**	**1**
	4	**1**	**0**	**0**	**D->D^D->CD^D->D|CD**	**1**
	3	**0**	**1**	**0**	**AD->A|D**	**1**
	2	**0**	**1**	**0**	**BD->B|D**	**1**
	1	2	0	0	AD->AD^D->ABD^D->BD|AI	0.5239
	Total	3	3	0		

Area	Code
North East Africa	A
Madagascar	B
Keiskamma	C
Western Cape	D

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Ephem Clade						
BBM	**18**	**2**	**1**	**0**	**B->BG->B|G**	**0.8845**
	16	2	1	0	E->BE->E|B	0.5109
	15	2	1	0	A->AE->E|A	0.3584
	14	2	1	0	G->AG->A|G	0.0851
	13	2	1	0	B->BG->B|G	0.434
	12	2	1	0	A->AB->A|B	0.3069
	11	2	1	0	H->AH->H|A	0.5393
	10	2	1	0	C->CF->C|F	0.4334
	9	2	1	0	C->CH->C|H	0.0896
	8	2	1	0	E->EI->I|E	0.6658
	7	2	1	0	E->CE->E|C	0.1232
	6	2	1	0	E->DE->E|D	0.6441
	4	2	1	0	F->FG->F|G	0.4444
	3	2	1	0	C->CF->C|F	0.2871
	2	2	1	0	E->CE->C|E	0.1471
	1	3	1	1	F->->EG->E|G	0.0207
Total		33	16	1		

Area	Code
Afromontane Rivers	A
Gourits	B
Palmiet	C
Berg	D
Olifants	E
Breede West	F
Breede Barrydale	G
Breede Koornlands	H
Breede Robertson	I

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Ephem Clade						
SDEC						
	18	**0**	**1**	**0**	**BG->B|G**	**0.9931**
	17	**1**	**0**	**0**	**BG->BG^B->B|BG**	**0.8317**
	16	1	1	0	BE->BEG->E|BG	0.4612
	15	1	1	0	AE->ABE->BE|A	0.201
	14	4	1	0	G->AEFG->AE|FG	0.0044
	13	**0**	**1**	**0**	**BG->B|G**	**0.7697**
	12	1	1	0	AG->ABG->A|BG	0.2977
	11	1	1	0	GH->AGH->H|AG	0.1406
	10	**0**	**1**	**0**	**CF->C|F**	**0.9888**
	9	2	1	0	FH->CFGH->CF|GH	0.0742
	8	0	1	0	EI->I|E	0.5667
	7	2	1	0	EF->EFHI->EI|FH	0.0204
	6	**0**	**1**	**0**	**DE->E|D**	**1**
	5	2	0	0	E->E^E->DEF^E->DE|EF	0.0289
	4	**0**	**1**	**0**	**FG->F|G**	**1**
	3	1	1	0	FG->CFG->C|FG	0.0962
	2	3	1	0	F->EFG->FG|E	0.0011
	1	1	1	1	EF->F->FG->F|G	0.0003
Total		20	16	1		

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Ephem Clade						
SDIVA						
	18	**0**	**1**	**0**	**BG->B|G**	**1**
	17	1	0	0	BG->BG^B->B|BG	0.515
	16	1	1	0	BE->BEG->E|BG	0.2717
	15	1	1	0	AE->ABE->BE|A	0.2125
	14	4	1	0	G->AEFG->AE|FG	0.1264
	13	**0**	**1**	**0**	**BG->B|G**	**1**
	12	1	1	0	AG->ABG->A|BG	0.3863
	11	2	1	1	BH->H->AGH->H|AG	0.1285
	10	**0**	**1**	**0**	**CF->C|F**	**1**
	9	3	1	1	CG->C->BCFH->CF|BH	0.0424
	8	**0**	**1**	**0**	**EI->I|E**	**1**
	7	2	1	0	EG->CEGI->EI|CG	0.0171
	6	**0**	**1**	**0**	**DE->E|D**	**1**
	5	2	0	0	E->E^E->DEG^E->DE|EG	0.0586
	4	**0**	**1**	**0**	**FG->F|G**	**1**
	3	1	1	0	CG->CFG->C|FG	0.5235
	2	1	1	0	EG->CEG->CG|E	0.0434
	1	1	0	0	EG->EG^G->EG|G	0.0154
Total		20	15	2		

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Lest Clade						
BBM	11	2	1	0	B->BC->B|C	0.4057
	10	2	1	0	D->BD->D|B	0.2704
	9	2	1	0	A->AD->A|D	0.5029
	8	2	1	0	E->EI->E|I	0.423
	7	2	1	0	F->EF->E|F	0.276
	6	2	1	0	H->FH->H|F	0.5002
	5	2	1	0	H->AH->H|A	0.1937
	4	2	1	0	J->GJ->J|G	0.4558
	3	2	1	0	K->JK->J|K	0.3066
	2	2	1	0	K->HK->K|H	0.0814
	1	2	1	0	I->IK->K|I	0.1167
Total		22	11	0		
SDEC	**11**	**0**	**1**	**0**	**BC->B|C**	**0.9804**
	10	1	1	0	CD->BCD->D|BC	0.3938
	9	1	1	0	AD->ACD->A|CD	0.0828
	8	2	1	0	I->EI->E|I	0.2466
	7	2	1	0	I->FI->I|F	0.0582
	6	2	1	0	H->HI->H|I	0.044
	5	1	1	0	AH->ADH->H|AD	0.0034
	4	0	1	0	GJ->J|G	0.4982
	3	1	1	0	JK->GJK->GJ|K	0.175
	2	4	1	0	K->AHJK->JK|AH	0.0011
	1	1	1	1	IJ->I->IK->K|I	0.0003
Total		15	11	1		

Area	Code
Keiskamma	A
Gamtoos	B
Afromontane Rivers	C
Gourits	D
Eerste	E
Liesbeek	F
Berg	G
Olifants	H
Breede West	I
Breede Barrydale	J
Breede Koornlands	K

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Lest Clade						
SDIVA	**11**	**0**	**1**	**0**	**BC->B|C**	**1**
	10	1	1	0	CD->BCD->D|BC	0.5
	9	1	1	0	AD->ACD->A|CD	0.1667
	8	**0**	**1**	**0**	**EI->E|I**	**1**
	7	1	1	0	FI->EFI->EI|F	0.495
	6	2	1	1	EH->H->FHI->H|FI	0.1212
	5	2	1	0	AH->ADEH->EH|AD	0.0051
	4	**0**	**1**	**0**	**GJ->J|G**	**1**
	3	1	1	0	JK->GJK->GJ|K	0.5
	2	3	1	1	IJ->J->AHJK->JK|AH	0.0013
	1	3	0	1	BJ->J->J^I^J->IJ^I^J->IJ|IJ	0.0007
Total		14	10	3		

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability	Area	Code
Nad/Lith Clade							Afromontane Rivers	A
BBM	21	2	1	0	G->CG->G|C	0.5271	Gourits	B
	20	2	1	0	D->DG->D|G	0.3062	Eerste	C
	19	2	1	0	A->AD->D|A	0.3681	Palmiet	D
	18	2	1	0	J->BJ->B|J	0.468	Berg	E
	17	2	1	0	G->GJ->G|J	0.3419	Olifants	F
	16	2	1	0	G->AG->G|A	0.2023	Bereede West	G
	15	2	1	0	E->EF->E|F	0.4266	Breede Barrydale	H
	14	3	1	1	J->->EG->E|G	0.0594	Breede Koornlands	I
	12	**2**	**1**	**0**	**D->CD->D|C**	**0.7461**	Breede Robertson	J
	11	2	1	0	D->DG->G|D	0.4547		
	9	2	1	0	H->HI->I|H	0.5516		
	8	2	1	0	H->DH->H|D	0.2		
	7	2	1	0	H->HJ->H|J	0.1261		
	6	2	1	0	H->HJ->H|J	0.3543		
	5	2	1	0	G->GH->G|H	0.3174		
	4	2	1	0	G->DG->G|D	0.5789		
	2	2	1	0	F->FG->G|F	0.5094		
	1	2	1	0	H->FH->F|H	0.1286		
Total		37	18	1				

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Nad/Lith Clade						
SDEC	**21**	**0**	**1**	**0**	**CG->G|C**	**0.7368**
	20	1	1	0	CG->CDG->D|CG	0.3589
	19	1	1	0	AG->ACG->CG|A	0.3919
	18	**0**	**1**	**0**	**BJ->B|J**	**0.7391**
	17	1	1	0	GJ->BGJ->G|BJ	0.3997
	16	2	0	0	G->G^G->AGJ^G->GJ|AG	0.1265
	15	**0**	**1**	**0**	**EF->E|F**	**0.9799**
	14	1	1	0	FG->EFG->EF|G	0.0865
	13	1	1	0	GJ->FGJ->J|FG	0.031
	12	**0**	**1**	**0**	**CD->D|C**	**0.9719**
	11	**1**	**1**	**0**	**DG->CDG->G|CD**	**0.883**
	10	1	0	0	D->D^D->DG^D->D|DG	0.53
	9	**0**	**1**	**0**	**HI->I|H**	**0.8272**
	8	1	1	0	DH->DHI->HI|D	0.1548
	7	4	1	0	G->DGHJ->DH|GJ	0.0019
	6	**0**	**1**	**0**	**HJ->H|J**	**0.9213**
	5	1	1	0	GH->GHJ->G|HJ	0.3892
	4	1	1	0	DG->DGH->GH|D	0.2239
	3	1	0	0	G->G^G->DG^G->DG|G	0.2647
	2	0	1	0	FG->G|F	0.3615
	1	1	0	0	FG->FG^G->FG|G	0.0032
Total		18	17	0		

Appendix 5A. Cont.

	Node	Dispersal	Vicariance	Extinction	Event Route	Probability
Nad/Lith Clade						
SDIVA	**21**	**0**	**1**	**0**	**CG->G|C**	**1**
	20	1	1	0	CG->CDG->D|CG	0.3525
	19	1	1	0	AG->ACG->CG|A	0.1462
	18	**0**	**1**	**0**	**BJ->B|J**	**1**
	17	1	1	0	GJ->BGJ->G|BJ	0.499
	16	2	0	0	G->G^G->AGJ^G->GJ|AG	0.0593
	15	**0**	**1**	**0**	**EF->E|F**	**1**
	14	1	1	0	FG->EFG->EF|G	0.1066
	13	1	1	0	GJ->FGJ->J|FG	0.119
	12	**0**	**1**	**0**	**CD->D|C**	**1**
	11	**1**	**1**	**0**	**DG->CDG->G|CD**	**0.516**
	10	1	0	0	D->D^D->DG^D->D|DG	0.2663
	9	**0**	**1**	**0**	**HI->I|H**	**1**
	8	1	1	0	DH->DHI->HI|D	0.1413
	7	2	1	0	GH->DGHJ->DH|GJ	0.0116
	6	**0**	**1**	**0**	**HJ->H|J**	**1**
	5	1	1	0	GJ->GHJ->G|HJ	0.5
	4	1	1	0	DG->DGJ->GJ|D	0.2367
	3	1	0	0	G->G^G->DG^G->DG|G	0.2542
	2	0	1	0	FG->G|F	0.3208
	1	2	0	0	G->G^G->FGH^G->FG|GH	0.0241
Total		17	17	0		

Appendix 5B

The following two pages contain Appendix Figures 5B (i) to (v) BEAST analysis using the clock rate obtained from the tectonic calibration for (i) “Afr/SAm Group”, (ii) “Afr/Mad Group”, (iii) “Ephem Clade”, (iv) “Lest Clade” and (v) “Nad/Lith Clade”. The trees obtained from these BEAST analyses were used in the RASP anlayses.

(i)

Caenis_sp
tel055_Ephemerellidae
tel060_Dudgeodes_ulmeri
tel144_Lestagella_Amatole
tel184_Lestagella_Gamtoos
tel185_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel281_Lestagella_Hottentots
tel156_SpeciesC_Barrydale
tel157_SpeciesC_Barrydale
tel239_newgenus_McGregor
tel276_newgenus_Kogelberg
tel212_Ephemerellina_Barrydale
tel280_Ephemerellina_Hottentots
tel258_Ephemerellina_Beaverlac
tel214_Nadinetella_Barrydale
tel269_Nadinetella_Jonkershoek
tel232_Nadinetella_Dassieshoek
tel259_Nadinetella_Beaverlac
tel270_Nadinetella_Jonkershoek
tel225_Lithogloea_Robertson
tel284_Lithogloea_Hottentots
tel220_Lithogloea_Barrydale
tel008_Ephemerythus_Zambia
tel142_Melanemerella

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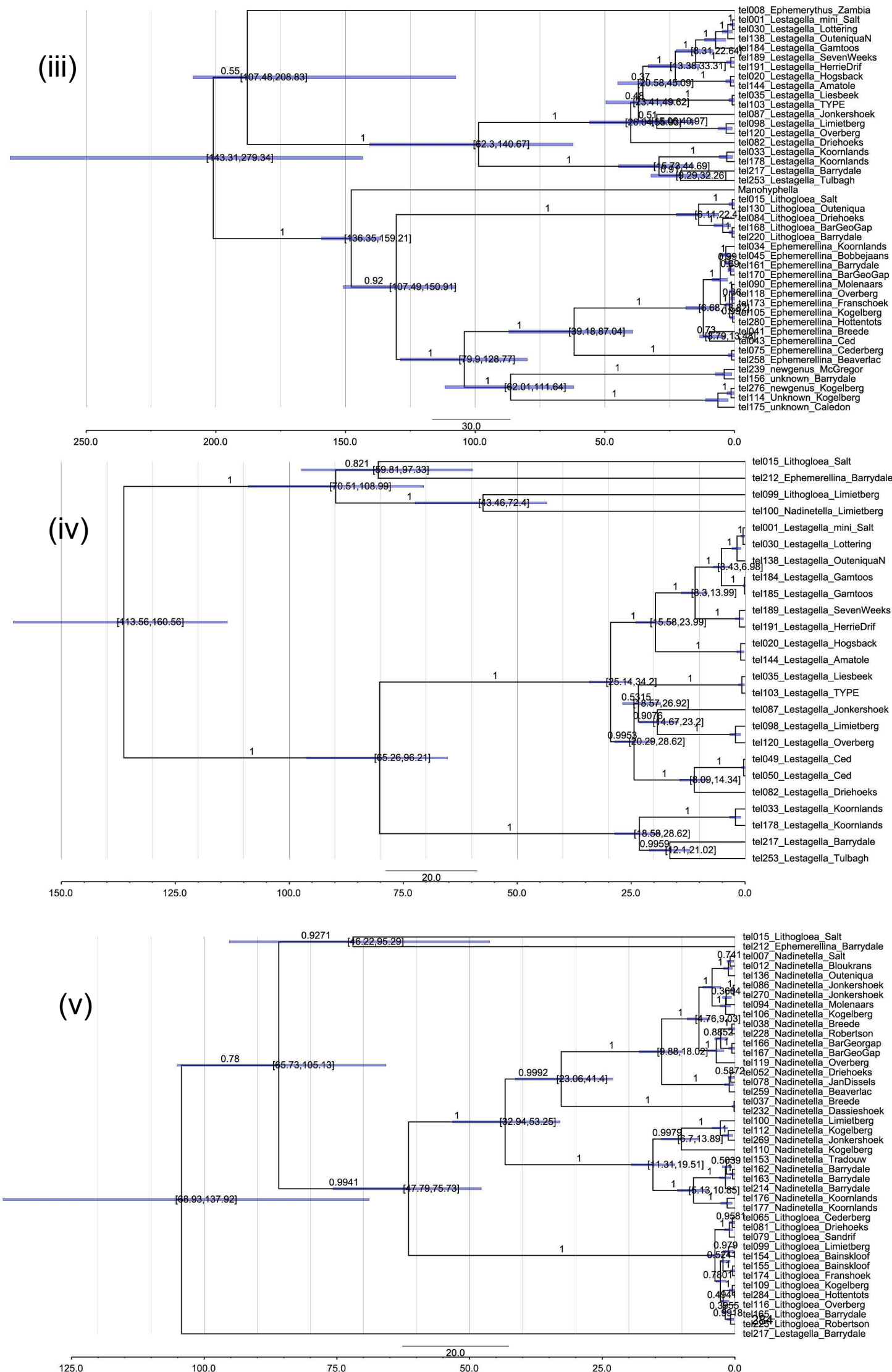
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(ii)

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tel030_Lestagella_Lottering
tel138_Lestagella_OuteniquaN
tel184_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel191_Lestagella_HerrieDrif
tel020_Lestagella_Hogsback
tel144_Lestagella_Amatole
tel035_Lestagella_Liesbeek
tel103_Lestagella_TYPE
tel087_Lestagella_Jonkershoek
tel098_Lestagella_Limietberg
tel120_Lestagella_Overberg
tel082_Lestagella_Driehoeks
tel033_Lestagella_Koornlands
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel253_Lestagella_Tulbagh
Manohyphella
tel015_Lithogloea_Salt
tel130_Lithogloea_Outeniqua
tel084_Lithogloea_Driehoeks
tel168_Lithogloea_BarGeoGap
tel220_Lithogloea_Barrydale
tel034_Ephemerellina_Koornlands
tel045_Ephemerellina_Bobbejaans
tel161_Ephemerellina_Barrydale
tel170_Ephemerellina_BarGeoGap
tel090_Ephemerellina_Molenaars
tel118_Ephemerellina_Overberg
tel173_Ephemerellina_Franschoek
tel105_Ephemerellina_Kogelberg
tel280_Ephemerellina_Hottentots
tel041_Ephemerellina_Breede
tel043_Ephemerellina_Ced
tel075_Ephemerellina_Cederberg
tel258_Ephemerellina_Beaverlac
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tel156_unknown_Barrydale
tel276_newgenus_Kogelberg
tel114_Unknown_Kogelberg
tel175_unknown_Caledon

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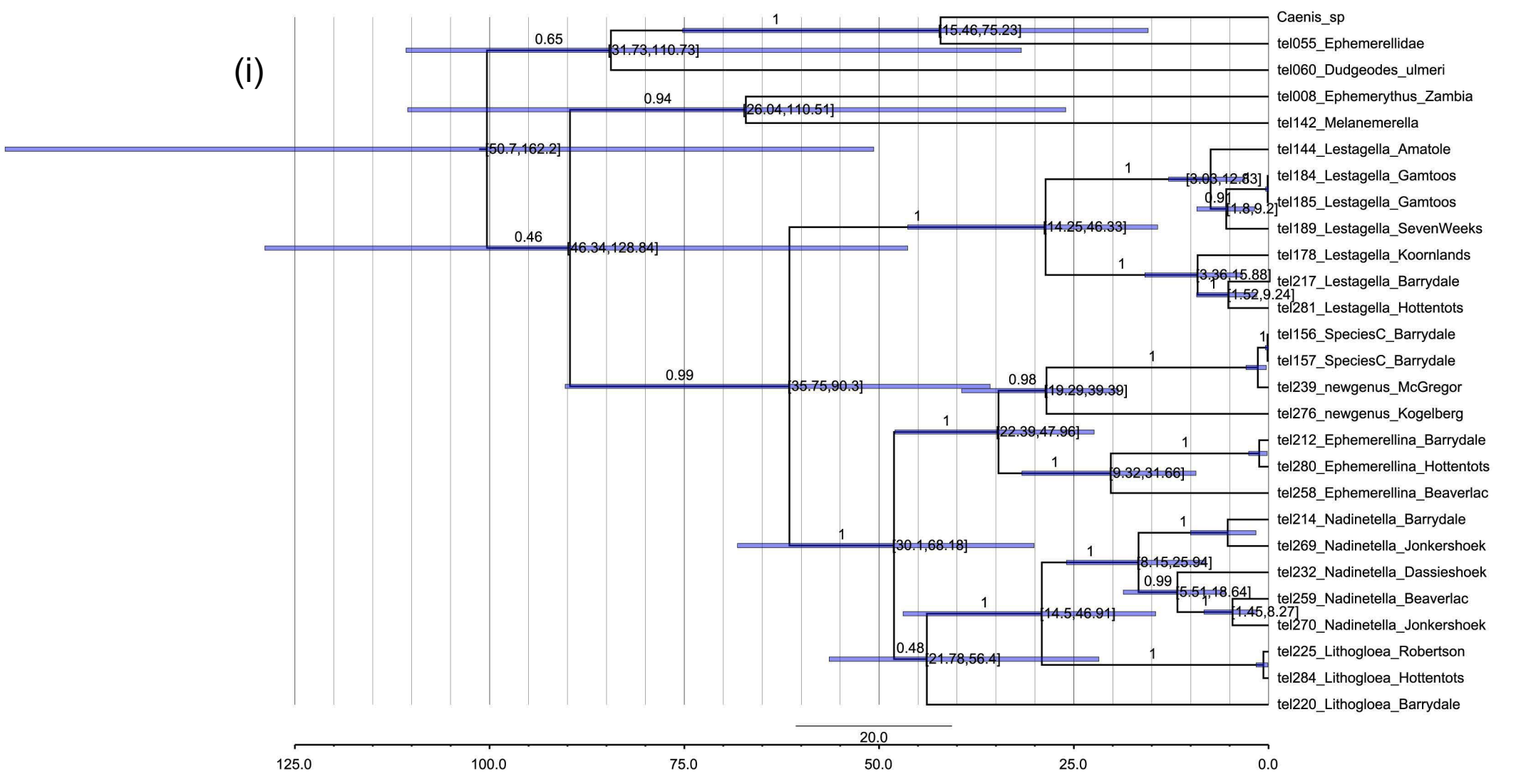
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(iii)
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tel184_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel191_Lestagella_HerrieDrif
tel020_Lestagella_Hogsback
tel144_Lestagella_Amatole
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tel103_Lestagella_TYPE
tel087_Lestagella_Jonkershoek
tel098_Lestagella_Limietberg
tel120_Lestagella_Overberg
tel082_Lestagella_Driehoeks
tel033_Lestagella_Koornlands
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel253_Lestagella_Tulbagh
Manohyphella
tel015_Lithogloea_Salt
tel130_Lithogloea_Outeniqua
tel084_Lithogloea_Driehoeks
tel168_Lithogloea_BarGeoGap
tel220_Lithogloea_Barrydale
tel034_Ephemerellina_Koornlands
tel045_Ephemerellina_Bobbejaans
tel161_Ephemerellina_Barrydale
tel170_Ephemerellina_BarGeoGap
tel090_Ephemerellina_Molenaars
tel118_Ephemerellina_Overberg
tel173_Ephemerellina_Franschoek
tel105_Ephemerellina_Kogelberg
tel280_Ephemerellina_Hottentots
tel041_Ephemerellina_Breede
tel043_Ephemerellina_Ced
tel075_Ephemerellina_Cederberg
tel258_Ephemerellina_Beaverlac
tel239_newgenus_McGregor
tel156_unknown_Barrydale
tel276_newgenus_Kogelberg
tel114_Unknown_Kogelberg
tel175_unknown_Caledon
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[143.31,279.34]
[62.3,140.67]
[436.35,159.21]
0.92
[407.49,150.91]
[39.18,87.04]
[79.9,128.77]
[62.01,111.64]
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250.0
200.0
150.0
100.0
50.0
0.0
(iv)
tel015_Lithogloea_Salt
tel212_Ephemerellina_Barrydale
tel099_Lithogloea_Limietberg
tel100_Nadinetella_Limietberg
tel001_Lestagella_mini_Salt
tel030_Lestagella_Lottering
tel138_Lestagella_OuteniquaN
tel184_Lestagella_Gamtoos
tel185_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel191_Lestagella_HerrieDrif
tel020_Lestagella_Hogsback
tel144_Lestagella_Amatole
tel035_Lestagella_Liesbeek
tel103_Lestagella_TYPE
tel087_Lestagella_Jonkershoek
tel098_Lestagella_Limietberg
tel120_Lestagella_Overberg
tel049_Lestagella_Ced
tel050_Lestagella_Ced
tel082_Lestagella_Driehoeks
tel033_Lestagella_Koornlands
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel253_Lestagella_Tulbagh
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[8.09,14.34]
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125.0
100.0
75.0
50.0
25.0
0.0
(v)
tel015_Lithogloea_Salt
tel212_Ephemerellina_Barrydale
tel007_Nadinetella_Salt
tel012_Nadinetella_Bloukrans
tel136_Nadinetella_Outeniqua
tel086_Nadinetella_Jonkershoek
tel270_Nadinetella_Jonkershoek
tel094_Nadinetella_Molenaars
tel106_Nadinetella_Kogelberg
tel038_Nadinetella_Breede
tel228_Nadinetella_Robertson
tel166_Nadinetella_BarGeorgap
tel167_Nadinetella_BarGeoGap
tel119_Nadinetella_Overberg
tel052_Nadinetella_Driehoeks
tel078_Nadinetella_JanDissels
tel259_Nadinetella_Beaverlac
tel037_Nadinetella_Breede
tel232_Nadinetella_Dassieshoek
tel100_Nadinetella_Limietberg
tel112_Nadinetella_Kogelberg
tel269_Nadinetella_Jonkershoek
tel110_Nadinetella_Kogelberg
tel153_Nadinetella_Tradouw
tel162_Nadinetella_Barrydale
tel163_Nadinetella_Barrydale
tel214_Nadinetella_Barrydale
tel176_Nadinetella_Koornlands
tel177_Nadinetella_Koornlands
tel065_Lithogloea_Cederberg
tel081_Lithogloea_Driehoeks
tel079_Lithogloea_Sandrif
tel099_Lithogloea_Limietberg
tel154_Lithogloea_Bainskloof
tel155_Lithogloea_Bainskloof
tel174_Lithogloea_Franshoek
tel109_Lithogloea_Kogelberg
tel284_Lithogloea_Hottentots
tel116_Lithogloea_Overberg
tel165_Lithogloea_Barrydale
tel225_Lithogloea_Robertson
tel217_Lestagella_Barrydale
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0.78
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[32.94,53.25]
0.9941
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0.741
0.3604
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0.8852
[9.88,18.02]
0.5872
0.9979
[5.7,13.89]
[11.31,19.51]
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[5.13,10.85]
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0.979
0.524
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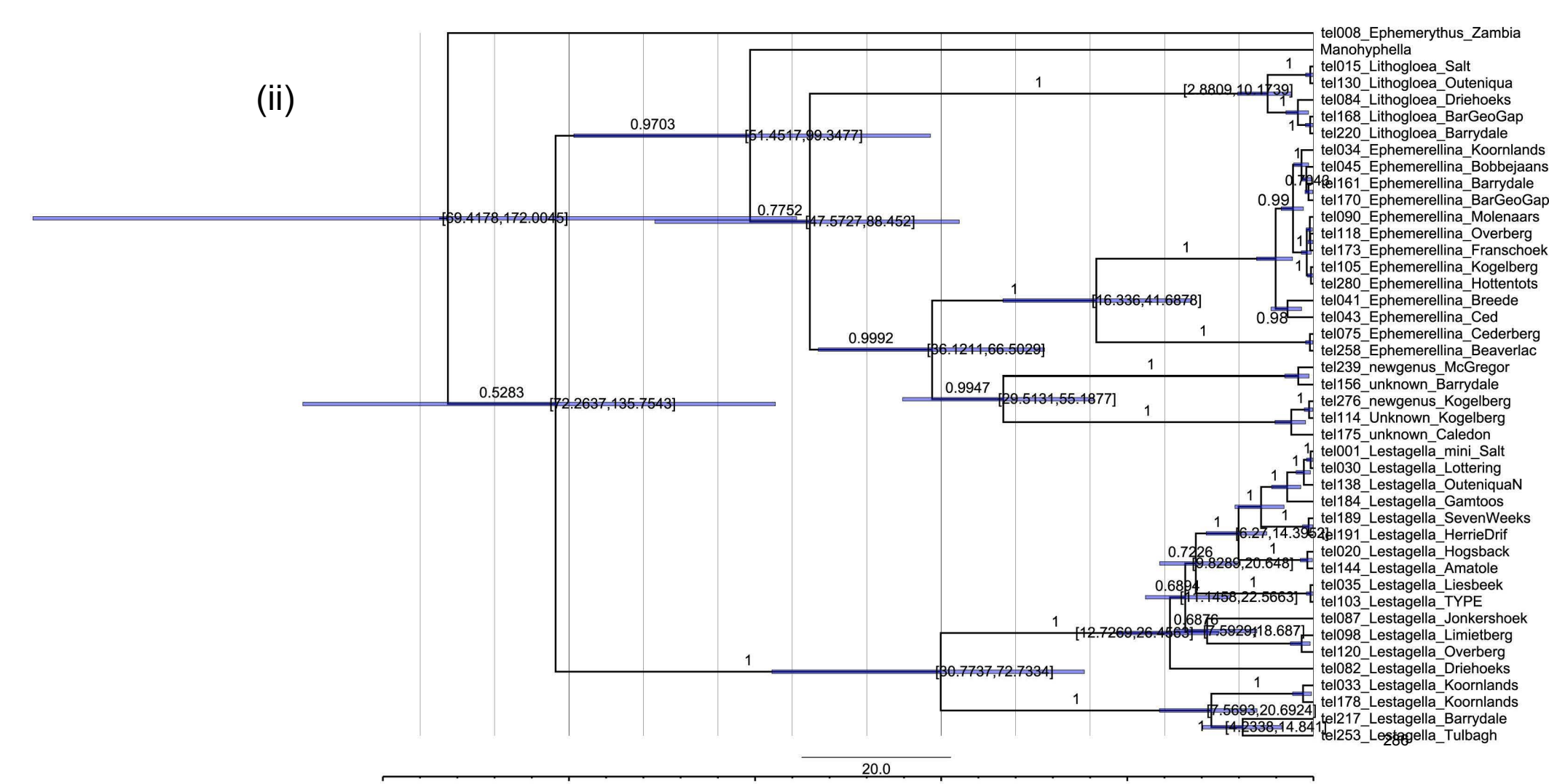
Appendix 5C

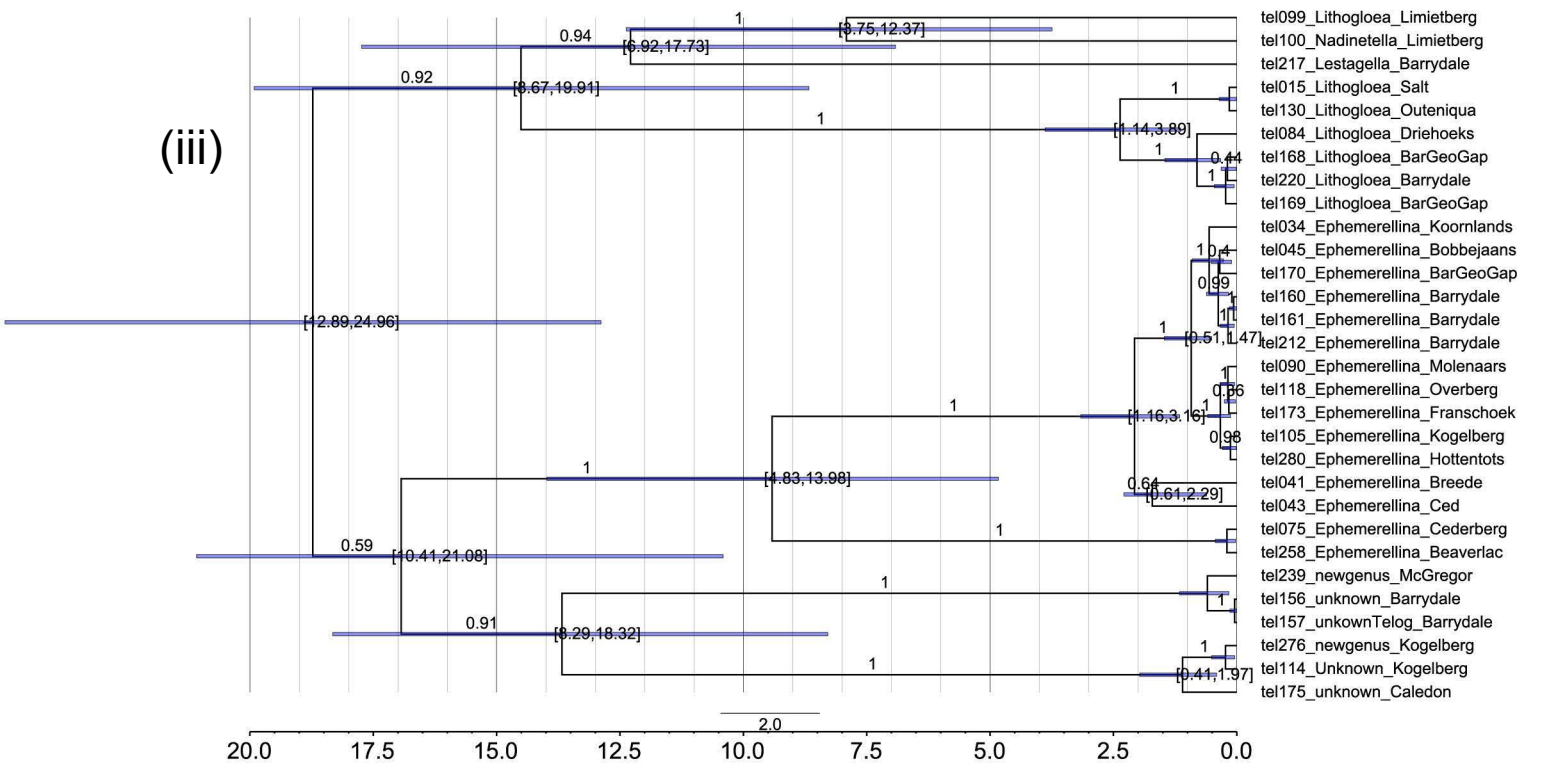
The following two pages contain Appendix Figures 5C (i) to (v) BEAST analysis using the published clock rate (divergence rate of 2.69 % per million years for combined COI/16S) obtained from Papadopoulou et al. (2010) for (i) “Afr/SAm Group”, (ii) “Afr/Mad Group”, (iii) “Ephem Clade”, (iv) “Lest Clade” and (v) “Nad/Lith Clade”. These trees are for comparative purposes (with Appendix 5B and RASP analyses).

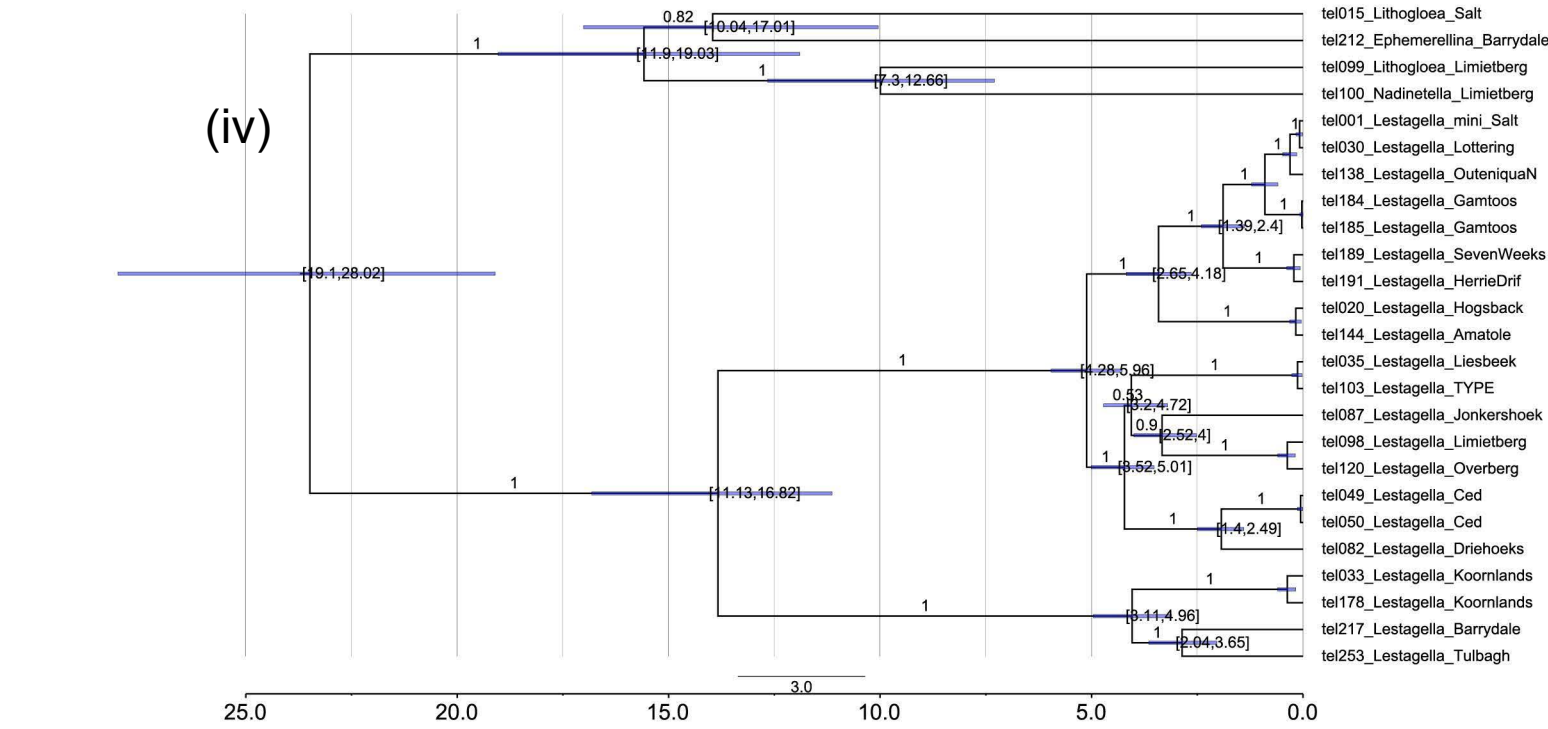
(i)

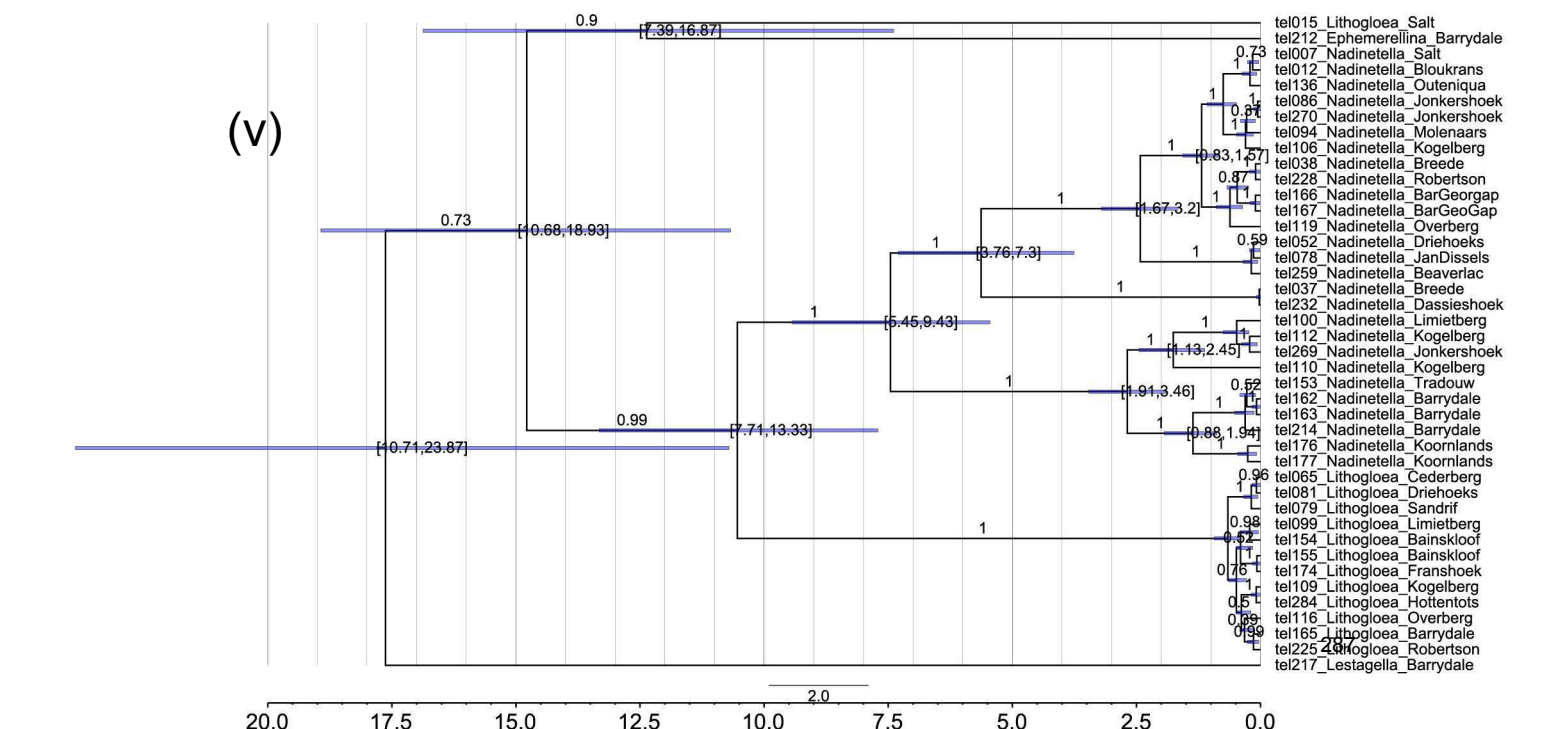

Caenis_sp
tel055_Ephemerellidae
tel060_Dudgeodes_ulmeri
tel008_Ephemerythus_Zambia
tel142_Melanemerella
tel144_Lestagella_Amatole
tel184_Lestagella_Gamtoos
tel185_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel281_Lestagella_Hottentots
tel156_SpeciesC_Barrydale
tel157_SpeciesC_Barrydale
tel239_newgenus_McGregor
tel276_newgenus_Kogelberg
tel212_Ephemerellina_Barrydale
tel280_Ephemerellina_Hottentots
tel258_Ephemerellina_Beaverlac
tel214_Nadinetella_Barrydale
tel269_Nadinetella_Jonkershoek
tel232_Nadinetella_Dassieshoek
tel259_Nadinetella_Beaverlac
tel270_Nadinetella_Jonkershoek
tel225_Lithogloea_Robertson
tel284_Lithogloea_Hottentots
tel220_Lithogloea_Barrydale
1
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0.94
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1
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1
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1
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1
1
0.99
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0.98
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1
[22.39,47.96]
1
1
[9.32,31.66]
1
1
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1
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0.99
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0.48
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1
20.0
125.0
100.0
75.0
50.0
25.0
0.0

(ii)


tel008_Ephemerythus_Zambia
Manohyphella
tel015_Lithogloea_Salt
tel130_Lithogloea_Outeniqua
tel084_Lithogloea_Driehoeks
tel168_Lithogloea_BarGeoGap
tel220_Lithogloea_Barrydale
tel034_Ephemerellina_Koornlands
tel045_Ephemerellina_Bobbejaans
tel161_Ephemerellina_Barrydale
tel170_Ephemerellina_BarGeoGap
tel090_Ephemerellina_Molenaars
tel118_Ephemerellina_Overberg
tel173_Ephemerellina_Franschoek
tel105_Ephemerellina_Kogelberg
tel280_Ephemerellina_Hottentots
tel041_Ephemerellina_Breede
tel043_Ephemerellina_Ced
tel075_Ephemerellina_Cederberg
tel258_Ephemerellina_Beaverlac
tel239_newgenus_McGregor
tel156_unknown_Barrydale
tel276_newgenus_Kogelberg
tel114_Unknown_Kogelberg
tel175_unknown_Caledon
tel001_Lestagella_mini_Salt
tel030_Lestagella_Lottering
tel138_Lestagella_OuteniquaN
tel184_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel191_Lestagella_HerrieDrif
tel020_Lestagella_Hogsback
tel144_Lestagella_Amatole
tel035_Lestagella_Liesbeek
tel103_Lestagella_TYPE
tel087_Lestagella_Jonkershoek
tel098_Lestagella_Limietberg
tel120_Lestagella_Overberg
tel082_Lestagella_Driehoeks
tel033_Lestagella_Koornlands
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel253_Lestagella_Tulbagh
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1
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1
1
1
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1
1
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0.9992
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125.0
100.0
75.0
50.0
25.0
0.0


(iii)
tel099_Lithogloea_Limietberg
tel100_Nadinetella_Limietberg
tel217_Lestagella_Barrydale
tel015_Lithogloea_Salt
tel130_Lithogloea_Outeniqua
tel084_Lithogloea_Driehoeks
tel168_Lithogloea_BarGeoGap
tel220_Lithogloea_Barrydale
tel169_Lithogloea_BarGeoGap
tel034_Ephemerellina_Koornlands
tel045_Ephemerellina_Bobbejaans
tel170_Ephemerellina_BarGeoGap
tel160_Ephemerellina_Barrydale
tel161_Ephemerellina_Barrydale
tel212_Ephemerellina_Barrydale
tel090_Ephemerellina_Molenaars
tel118_Ephemerellina_Overberg
tel173_Ephemerellina_Franschoek
tel105_Ephemerellina_Kogelberg
tel280_Ephemerellina_Hottentots
tel041_Ephemerellina_Breede
tel043_Ephemerellina_Ced
tel075_Ephemerellina_Cederberg
tel258_Ephemerellina_Beaverlac
tel239_newgenus_McGregor
tel156_unknown_Barrydale
tel157_unkownTelog_Barrydale
tel276_newgenus_Kogelberg
tel114_Unknown_Kogelberg
tel175_unknown_Caledon
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0.64
[0.61,2.29]
0.59
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20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0


(iv)
tel015_Lithogloea_Salt
tel212_Ephemerellina_Barrydale
tel099_Lithogloea_Limietberg
tel100_Nadinetella_Limietberg
tel001_Lestagella_mini_Salt
tel030_Lestagella_Lottering
tel138_Lestagella_OuteniquaN
tel184_Lestagella_Gamtoos
tel185_Lestagella_Gamtoos
tel189_Lestagella_SevenWeeks
tel191_Lestagella_HerrieDrif
tel020_Lestagella_Hogsback
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tel087_Lestagella_Jonkershoek
tel098_Lestagella_Limietberg
tel120_Lestagella_Overberg
tel049_Lestagella_Ced
tel050_Lestagella_Ced
tel082_Lestagella_Driehoeks
tel033_Lestagella_Koornlands
tel178_Lestagella_Koornlands
tel217_Lestagella_Barrydale
tel253_Lestagella_Tulbagh
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[3.52,5.01]
[11.13,16.82]
[1.4,2.49]
[3.11,4.96]
[2.04,3.65]
3.0
25.0
20.0
15.0
10.0
5.0
0.0


(v)
tel015_Lithogloea_Salt
tel212_Ephemerellina_Barrydale
tel007_Nadinetella_Salt
tel012_Nadinetella_Bloukrans
tel136_Nadinetella_Outeniqua
tel086_Nadinetella_Jonkershoek
tel270_Nadinetella_Jonkershoek
tel094_Nadinetella_Molenaars
tel106_Nadinetella_Kogelberg
tel038_Nadinetella_Breede
tel228_Nadinetella_Robertson
tel166_Nadinetella_BarGeorgap
tel167_Nadinetella_BarGeoGap
tel119_Nadinetella_Overberg
tel052_Nadinetella_Driehoeks
tel078_Nadinetella_JanDissels
tel259_Nadinetella_Beaverlac
tel037_Nadinetella_Breede
tel232_Nadinetella_Dassieshoek
tel100_Nadinetella_Limietberg
tel112_Nadinetella_Kogelberg
tel269_Nadinetella_Jonkershoek
tel110_Nadinetella_Kogelberg
tel153_Nadinetella_Tradouw
tel162_Nadinetella_Barrydale
tel163_Nadinetella_Barrydale
tel214_Nadinetella_Barrydale
tel176_Nadinetella_Koornlands
tel177_Nadinetella_Koornlands
tel065_Lithogloea_Cederberg
tel081_Lithogloea_Driehoeks
tel079_Lithogloea_Sandrif
tel099_Lithogloea_Limietberg
tel154_Lithogloea_Bainskloof
tel155_Lithogloea_Bainskloof
tel174_Lithogloea_Franshoek
tel109_Lithogloea_Kogelberg
tel284_Lithogloea_Hottentots
tel116_Lithogloea_Overberg
tel165_Lithogloea_Barrydale
tel225_Lithogloea_Robertson
tel217_Lestagella_Barrydale
0.9
[7.39,16.87]
0.73
[10.68,18.93]
[0.83,1.57]
[1.67,3.2]
[3.76,7.3]
[5.45,9.43]
[1.13,2.45]
[1.91,3.46]
0.99
[7.71,13.33]
[0.88,1.94]
[10.71,23.87]
2.0
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0

Appendix 6A. Details of imago and subimago specimens used for geometric morphometrics, including number and side of wings photographed for each specimen

Individual code	HMJ code	Sex	Genus / Species	Area	River	Catchment	Stage	Right FW	Left FW	Right HW	Left HW
1	Barnard '32	M	Lestagella penicillata	Table Mountain	Cape Peninsula	Cape Peninsula	A	X	X	X	.
2	5	F	Lestagella	Tsitsikamma	Elandsbos	Tsitsikamma	A	X	X	X	.
3	5	M	Lestagella	Tsitsikamma	Elandsbos	Tsitsikamma	A	X	X	X	X
4	5	F	Lestagella	Tsitsikamma	Elandsbos	Tsitsikamma	A	X	X	X	X
5	5	M	Lestagella	Tsitsikamma	Elandsbos	Tsitsikamma	A	X	X	X	X
6	17	M	Lestagella	Tsitsikamma	Lottering	Tsitsikamma	A	X	X	X	X
7	25	F	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
8	25	F	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
9	25	M	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
10	25	M	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
11	25	F	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
12	26	M	Lithogloea Spine Hairy	Tsitsikamma	Bloukrans	Tsitsikamma	A	X	X	X	X
13	27	F	Lestagella	Hogsback	Tyume	Keiskamma	A	X	X	X	X
14	27	M	Lestagella	Hogsback	Tyume	Keiskamma	A	X	.	X	X
15	27	M	Lestagella	Hogsback	Tyume	Keiskamma	A	X	X	X	X
16	50	M	Lestagella	Tsitsikamma	Salt	Tsitsikamma	A	X	.	.	.
17	50	F	Lestagella	Tsitsikamma	Salt	Tsitsikamma	S	X	X	.	.
18	56	F	Lestagella	Tsitsikamma	Salt	Tsitsikamma	A	X	.	.	.
19	56	F	Lestagella	Tsitsikamma	Salt	Tsitsikamma	A	X	X	.	.
20	80	F	Lestagella	Rondegat	Rondegat	Olifants	S	X	X	X	X
21	80	M	Lestagella	Rondegat	Rondegat	Olifants	A	X	X	X	X
22	80	M	Lestagella	Rondegat	Rondegat	Olifants	A	X	X	X	X
23	82	F	Nadinetella	Rondegat	Rondegat	Olifants	A	X	X	X	X
24	82	M	Nadinetella	Rondegat	Rondegat	Olifants	A	X	X	X	X
25	86	M	Nadinetella	Rondegat	Rondegat	Olifants	A	X	X	X	.
26	98	M	Lithogloea Spine Hairy	Driehoeks	Driehoeks	Olifants	A	X	X	X	X
27	98	M	Lestagella	Driehoeks	Driehoeks	Olifants	S	X	X	X	X

28	106	F	Lestagella	Jonkershoek	Swartboskloof	Eerste	A	X	X	X	X
29	106	M	Lestagella	Jonkershoek	Swartboskloof	Eerste	A	X	X	X	X
30	109	F	Nadinetella	Jonkershoek	Eerste	Eerste	A	X	X	X	.
31	109	M	Nadinetella	Jonkershoek	Eerste	Eerste	A	X	X	X	X
32	113	M	Lestagella	Molenaars	Molenaars	Breede	A	X	X	X	X
33	113	F	Lestagella	Molenaars	Molenaars	Breede	A	X	X	X	X
34	116	F	Lestagella	Bainskloof	Witte	Breede	A	X	X	X	X
35	116	M	Lestagella	Bainskloof	Witte	Breede	A	X	X	X	X
36	121	M	new genus	Kogelberg	Oudebos	Palmiet	A	X	X	X	X
37	121	F	Nadinetella	Kogelberg	Oudebos	Palmiet	A	X	.	X	X
38	121	F	new genus	Kogelberg	Oudebos	Palmiet	S	X	X	X	X
39	121	F	Ephemerellina	Kogelberg	Oudebos	Palmiet	A	.	.	X	X
40	130	M	Ephemerellina	Kogelberg	Dwars	Palmiet	A	.	.	X	X
41	130	F	Ephemerellina	Kogelberg	Dwars	Palmiet	A	.	.	X	X
42	133	F	Lithogloea Spine Hairy	Greyton	Gobos	Breede	S	X	X	X	X
43	133	F	Lithogloea Smooth	Greyton	Gobos	Breede	A	X	X	X	X
44	145	F	Lestagella	Outeniqua	Touws	Outeniqua	A	X	X	X	.
45	163	M	Nadinetella	Tsitsikamma	Salt	Tsitsikamma	A	X	X	X	X
46	179	M	Lithogloea Smooth	Rondegat	Rondegat	Olifants	A	X	X	X	X
47	181	M	Lestagella	Table Mountain	Cape Peninsula	Cape Peninsula	A	X	.	X	X
48	181	M	Lestagella	Table Mountain	Cape Peninsula	Cape Peninsula	A	.	.	.	.
49	181	F	Lestagella	Table Mountain	Cape Peninsula	Cape Peninsula	A	X	X	X	X
50	181	M	Lestagella	Table Mountain	Cape Peninsula	Cape Peninsula	A	X	X	X	X
51	181	M	Lestagella	Table Mountain	Cape Peninsula	Cape Peninsula	A	X	X	X	X
52	206	F	Nadinetella	Bainskloof	Witte	Breede	A	X	X	X	X
53	223	F	Nadinetella	Barrydale	Huis	Breede	A	X	X	X	.
54	225	M	Nadinetella	Barrydale	Huis	Breede	A	X	X	X	X
55	264	F	Lithogloea Spine Hairy	Barrydale	Huis	Breede	S	X	X	X	X
56	266	M	Nadinetella	Robertson	DeHoop	Breede	A	X	X	X	X
57	266	M	Nadinetella	Robertson	DeHoop	Breede	A	X	.	X	X
58	266	F	Nadinetella	Robertson	DeHoop	Breede	A	X	X	X	X

59	266	M	Lestagella	Robertson	DeHoop	Breede	S	X	X	X	X
60	266	F	Lithogloea Smooth	Robertson	DeHoop	Breede	S	X	X	X	.
61	266	F	Lithogloea Smooth	Robertson	DeHoop	Breede	S	X	X	X	X
62	270	M	Nadinetella	Robertson	Dassieshoek	Breede	S/A	X	.	X	X
63	270	M	Ephemerellina	Robertson	Dassieshoek	Breede	S	X	.	X	X
64	272	M	Lithogloea Spine Hairy	McGregor	Olifantsdoornkloof	Breede	A	X	X	X	X
65	272	F	Lithogloea Spine Hairy	McGregor	Olifantsdoornkloof	Breede	A	X	X	X	X
66	273	M	Lestagella	McGregor	Olifantsdoornkloof	Breede	A	X	X	X	.
67	273	F	Lithogloea Smooth	McGregor	Olifantsdoornkloof	Breede	A	X	X	X	X
68	273	F	Lestagella	McGregor	Olifantsdoornkloof	Breede	A	X	X	X	X
69	273	M	new genus	McGregor	Olifantsdoornkloof	Breede	S	X	X	X	X
70	275	F	new genus	McGregor	Boesmans	Breede	S	X	.	X	.
71	276	M	Lestagella	Robertson	Boskloof	Breede	A	X	X	X	X
72	278	F	Nadinetella	Tulbagh	secretfalls	Berg	A	X	X	X	X
73	288	M	Nadinetella	Jonkershoek	Eerste	Eerste	A	X	X	X	X
74	293	M	Lestagella	Jonkershoek	Eerste	Eerste	A	X	X	X	X
75	293	M	Lestagella	Jonkershoek	Eerste	Eerste	A	X	X	X	.
76	293	M	Lestagella	Jonkershoek	Eerste	Eerste	A	X	X	X	.
77	294	M	Ephemerellina	Kogelberg	Oudebos	Palmiet	S	X	X	X	.
78	298	M	Lithogloea Smooth	Kogelberg	Oudebos	Palmiet	A	X	X	X	X
79	299	M	Ephemerellina	Hotentots Holland	Palmiet	Palmiet	A	.	.	X	X

Appendix 6B. Results of the Mahalanobis distance permutation tests from the CVAs for fore wing and hind wings, at species level. Also for Lestagella by catchment. Significant differences indicate differences in mean shapes between the respective groups

Forewing species perm test signifcance, * is significance, ns is Not significant

	E. barnardi	*L. sp1*	*L. sp2*	*L. sp3*	*L. penicillata*	*L. sp4*	*L. sp7*	*L. sp6*	*L. sp5*	*L. harrisoni*	*N. crassi*	*N. brincki*	*Species C*	*Species B*
L. sp1	*													
L. sp2	*	*												
L. sp3	ns	*	*											
L. penicillata	*	*	ns	*										
L. sp4	*	*	*	*	*									
L. sp7	ns	*	*	*	*	ns								
L. sp6	*	*	*	*	*	*	*							
L. sp5	*	*	*	ns	ns	*	*	ns						
L. harrisoni	*	*	*	*	*	*	*	*	ns					
N. crassi	*	*	*	*	*	*	*	*	ns	*				
N. brincki	*	*	*	*	*	*	*	*	*	*	*			
Species C	ns	*	*	ns	ns	*	ns	*	ns	*	*	*		
Species B	ns	*	*	ns	ns	*	ns	*	ns	*	*	*	ns	
Species A	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Appendix 6B. Cont.

Hindwing species perm test signifcance, * is significance, ns is Not significant

	E. barnardi	*L. sp1*	*L. sp2*	*L. sp3*	*L. penicillata*	*L. sp4*	*L. sp7*	*L. sp6*	*L. sp5*	*L. harrisoni*	*N. crassi*	*N. brincki*	*Species C*	*Species B*
L. sp1	*													
L. sp2	*	*												
L. sp3	*	ns	*											
L. penicillata	*	*	ns	*										
L. sp4	*	*	*	ns	*									
L. sp7	*	*	*	ns	*	ns								
L. sp6	*	*	*	*	*	*	*							
L. sp5	ns	ns	*	ns	ns	ns	ns	ns						
L. harrisoni	*	*	*	*	*	*	*	*	*					
N. crassi	*	*	*	*	*	*	*	*	ns	*				
N. brincki	*	*	*	*	*	*	*	*	*	*	*			
Species C	*	*	*	*	*	*	*	*	*	*	*	*		
Species B	*	*	*	*	*	*	*	*	ns	*	*	*	*	
Species A	*	*	*	*	*	*	*	*	ns	*	*	*	*	*

Appendix 6B. Cont.

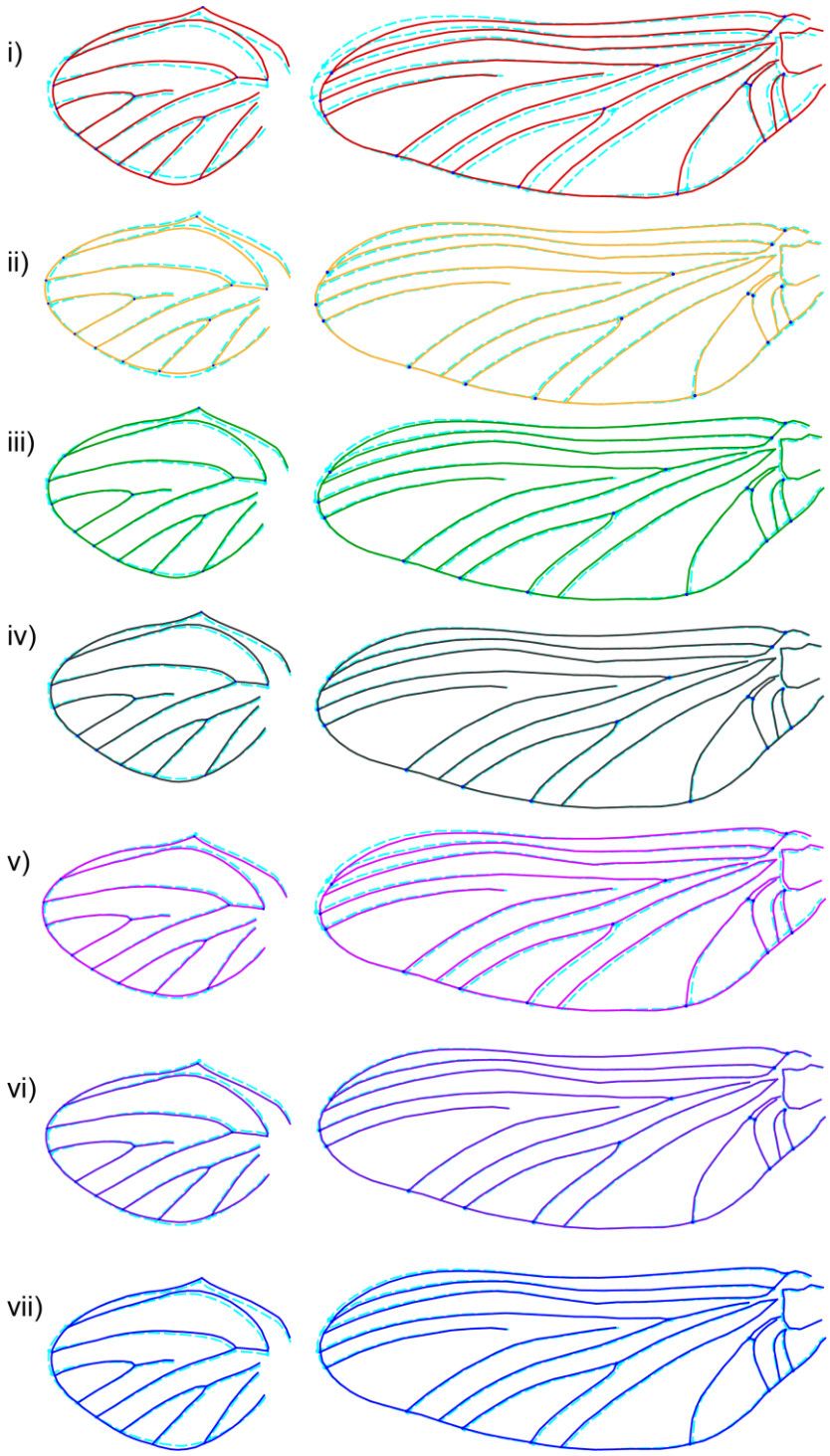
Forewing Lestagella catchment species perm test signifcance, * is significance, ns is Not significant

	bree	Eerste	Keiskamma	Cape Peninsula	Olifants	Outeniqua
Eerste	ns					
Keiskamma	*	*				
Cape Peninsula	*	*	*			
Olifants	*	ns	*	*		
Outeniqua	ns	*	ns	ns	ns	
Tsitsikamma	*	*	*	*	*	*

Hindwing Lestagella catchment species perm test signifcance, * is significance, ns is Not significant

	bree	Eerste	Keiskamma	Cape Peninsula	Olifants	Outeniqua
Eerste	*					
Keiskamma	*	*				
Cape Peninsula	*	*	*			
Olifants	*	*	*	*		
Outeniqua	*	ns	ns	*	ns	
Tsitsikamma	*	*	*	*	*	*

Appendix 6C



Appendix Figure 6C. Size-corrected wing shape variation between genera (coloured lines) compared to the average shape (light-blue dotted line), i) Ephemerellina, ii) Lestagella, iii) Lithogloea, iv) Nadinetella, v) Species A, vi) Species B, vii) Species C.