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Phylogenomic insights into the spatio-temporal evolution of the Asian and Australasian
Bulbophyllum (Orchidaceae)

Thesis submitted by
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9th September 2019
for the degree of Doctor of Philosophy
in the College of Science and Engineering
James Cook University

Dedication

This thesis is dedicated to my brother Narayana, who left this world during my candidature.

Acknowledgments

I owe my gratitude to a great many people and institutions that have made this research possible. I acknowledge the first custodians of the land on which the James Cook University and Australian Tropical Herbarium now stand, the Yirrijandji, Gimuy-Yidinji and Djabugay people and all the first nations of the Wet Tropics Bioregion: the Bandjin, Bar-barrum, Djabugay, Girramay, Gugu Badhum, Gulngay, Jirrbal, Mamu, Ngadjon-jii, Nywaigi, Yalangi, Yidinji, Yirrijandji, Warrgamay, Wulgurukaba, Warrungnu peoples, the Wiri people of Eungella in the Central Mackay Coast Bioregion and the Ayapathu People of Kulla (McIlwraith Range) in the Cape York Peninsula Bioregion. For financial and in kind support, I thank the Australian Biological Resources Study BushBlitz program, Australian Government's National Environmental Resources Program, Bioplatforms Australia, Nature Conservancy and Tomas Foundation's Australian Conservation Taxonomy Award program, Hermon Slade Foundation, Wet Tropics Management Authority, Australian Orchid Foundation, Australian Biological Resources Study student travel grant scheme, Australasian Systematic Botany Society, Association for Tropical Biology and Conservation, Australian Tropical Herbarium and James Cook University.

For assistance in the field, I am grateful to colleagues and volunteers who worked with me, in many cases during multi-day treks and often under difficult conditions. For their company and support during these expeditions I am grateful to Stuart Worboys, Tapio Linderhaus, Kayleen Bransgrove, Scott Large, Lewis Roberts, Alicia Craig, Craig Costion, Bruce Gray, Jen Sanger, Steve Pearce, Dean Miller, Mark Ziemnicki, Paul Thuesen, Malcolm Brown and Nadiah Roslan. For collecting permits, I owe thanks to the QLD Parks and Wildlife Service and the Knungal Traditional Owners. For contributions of plant material, I must acknowledge contributions from the Manaaki Whenua - Landcare Research, Centre for Australian National Biodiversity Research, Australian National Botanical Gardens and Australian Tropical Herbarium collected by many including Clements, M.A., Field, A., Gray, B., Harrison, M., Jones, D.L., Kilgour, C.D., Lockyer, R., Mathieson, M.T., McCraith, G., Molloy, B.P.J., Reeve, T., Roberts, L., Sanger, J., Schulte, K., Taylor, J.M., Wallace, B.J. and Ziesing, P.D. For tuition, support and advice in the laboratory, I am grateful to Mel Harrison, Jafar Jabiri and Todd McLay and for support with analytical methods I thank, Todd McLay, Lars Nauheimer, Issac Overcast and Deren Eaton.

For providing a supportive, encouraging and enjoyable environment to carry out this research I am grateful to the staff and students from the Australian Tropical Herbarium, in particular my fellow postgraduate comrades Yumiko Baba, Caroline Puente- Lelievre, Sook Ngoh Phoon, Kayleen Bransgrove, Janet Gagul, Rismita Sari, Kamolphat Atsawawaranut, Lizzie Roebble, Lizzy Joyce and Nick Weigner. This research would not have been possible without the patient guidance of my research advisors Katharina Nargar, Mark Clements and Darren Crayn and I thank them for their endless encouragement and support throughout my candidature. I must also express my gratitude to my friends and family for their patience and support throughout this journey. Most of all I am grateful to my partner, Scott Large, for his endless love, encouragement, support and patience that made it possible to complete this research.

Publication plan

Each data chapter of this thesis (Chapters two-five) has been prepared for submission to a high impact peer reviewed journal. Chapter three is modified from the manuscript “Evolutionary relationships and spatio-temporal evolution of the hyperdiverse orchid genus *Bulbophyllum*: phylogenomic insights from the Australasian lineage” by Simpson, L., Clements, M.A., Crayn, D.M., Paule, J., Nargar, K. which is in review for publication in the journal Molecular Phylogenetics and Evolution. The remaining data chapters are in preparation for submission. Target journals include Molecular Phylogenetics and Evolution, Journal of Biogeography and Diversity and Distributions.

Statement of the contribution of others

My PhD research tuition has been supported by James Cook University, and the Australian Government's National Environmental Resources Program. The Australian Tropical Herbarium has been the host institution for this research, providing an office and laboratory facilities throughout my candidature.

Chapter one: Review of literature summarised in chapter one was carried out by Simpson, L. The chapter was authored by Simpson, L. with feedback from Nargar, K., Crayn, D and Clements, M.

Chapter two: Research concept was developed by Simpson, L. Nargar, K. and Clements, M. Clements, M. provided plant material for analysis and taxonomic expertise in identifying samples, making section assignments and selecting representative samples. DNA extractions were carried out by Simpson, L. and DNA sequencing was carried out by the Australian Genome Research Facility. Bioinformatics and analysis were carried out by Simpson, L. with guidance from Nargar, K. and Nauheimer, L. The chapter was authored, and all figures created by Simpson, L. with feedback from Nargar, K., Crayn, D and Clements, M. Financial support for genetic sequencing was provided by Bioplatforms Australia and the Hermon Slade Foundation. Dissemination of research results at scientific conferences was supported by the Australian Orchid Foundation and the Australian Biological Resources Study student travel grant scheme, with in kind support provided by the Australian Systematic Botany Society through waiver of conference fees.

Chapter three: Research concept was developed by Simpson, L. Nargar, K. and Clements, M. Clements, M. and Harrison, M. provided plant material for analysis. DNA extractions and PCR amplifications were carried out by Simpson, L. Library preparation for Illumina sequencing and all DNA sequencing was carried out by the Australian Genome Research Facility. Bioinformatics and analysis were carried out by Simpson, L. with guidance from Nargar, K. ITS-ETS sequences for 25 samples were generated by Orel, H. Scoring of morphological characters was provided by Clements, M. Genome size measurements were carried out by Paule, J. The chapter was authored, and all figures created by Simpson, L. with contributions from Nargar, K., Crayn, D. and Clements, M. Paule, J. and Orel, H. Financial support for genetic sequencing was provided by the Australian Biological Resources Study BushBlitz program, Bioplatforms Australia and the Hermon Slade Foundation. Dissemination of research results at scientific conferences was

supported by the Australian Orchid Foundation and the Australian Biological Resources Study student travel grant scheme, with in kind support provided by the Australian Systematic Botany Society through waiver of conference fees.

Chapter four and Chapter five: Research concept was developed by Simpson, L. Samples were collected by Simpson, L. accompanied in the field by Stuart Worboys, Tapio Linderhaus, Kayleen Bransgrove, Scott Large, Lewis Roberts, Alicia Craig, Craig Costion, Bruce Gray, Jen Sanger, Steve Pearce, Dean Miller, Mark Ziemicki, Paul Thuesen, Malcolm Brown and Nadiyah Roslan. DNA was extracted by Simpson, L. Restriction associated library preparation and sequencing was carried out by the Australian Genome Research Facility. Bioinformatics and analysis were carried out by Simpson, L. with advice from Nargar, K., Crayn, D., McLay, T., Overcast, I., Eaton, D. The chapter was authored, and all figures created by Simpson, L. with feedback from Nargar, K. and Crayn, D. Financial support for field work was provided by the Wet Tropics Management Authority. Financial support for genetic analysis was provided by the Australian Government's National Environmental Resources Program and the Nature Conservancy and Tomas Foundation's Australian Conservation Taxonomy Award program. Dissemination of research results at scientific conferences was supported by the Wet tropics Management Authority, with in kind support provided by the Australian Systematic Botany Society and the Association for Tropical Biology and Conservation through waiver of conference fees.

Chapter six: was authored by Simpson, L. with feedback from Crayn, D. and Nargar, K.

Abstract

Bulbophyllum Thouars is a hyper-diverse group (> 2000 species) of epiphytic and lithophytic orchids with a pantropical distribution. Previous molecular phylogenetic studies have focused on the Afrotropical and Neotropical taxa. Relationships among the Asian and Australasian taxa, which constitute the majority of species within the genus (ca. 1700 species), have been little studied and remain poorly understood. The present work, which comprises four studies, sought to resolve relationships within *Bulbophyllum* at different phylogenetic levels to improve understanding of evolutionary patterns and processes in the genus.

To reconstruct broad-level infrageneric relationships, 145 *Bulbophyllum* species were sampled, including 122 species from Asia and Australasia representing 60 sections (ca. 63%), and a representative sampling of the Afrotropical and Neotropical *Bulbophyllum* clades. Genome skimming with high throughput sequencing was carried out for 53 *Bulbophyllum* samples and 70 plastid coding regions and the ribosomal regions (ITS1, 5.8s, ITS2) were used in subsequent phylogenomic analysis, with the inclusion of 25 nuclear ribosomal ITS sequences generated with Sanger sequencing and 67 ITS accessions from GenBank. Maximum likelihood and Bayesian analyses resolved three highly supported clades within the genus: an Asian clade, an Australasian clade and an Afrotropical/Neotropical clade. Eleven sections were identified as polyphyletic: *Cirrhopetalum*, *Desmosanthes*, *Rhytionanthos*, *Hoplandra*, *Leopardinae*, *Macrocaulia*, *Polymeres* and *Sestochilus* or paraphyletic: *Lemniscata*, *Beccariana*, and *Stenochilus*. Molecular divergence time analyses placed the origin of *Bulbophyllum* in the early Oligocene, ca. 33.3 Ma. Crown diversification was estimated to have commenced in the late Oligocene and a rapid divergence between the three major lineages was inferred between ca. 24–23.4 Ma. Ancestral area reconstructions based on maximum likelihood using two models (DEC, DIVA) placed the origins of the Australasian clade in Australasia, the Afrotropical/Neotropical clade in the Afrotropics and the Asian clade in tropical Asia. Within the Asian clade, divergence among sections took place mainly throughout the Miocene and Pliocene. Multiple independent long-distance dispersals were inferred from tropical Asia: at least eight to temperate Asia, at least three to Australasia and at least six to the South Pacific. Within the Tropical Asian region at least ten independent dispersal events were inferred westward across Wallacea to Papuasia.

To elucidate interspecific relationships within the Australasian clade, 53 *Bulbophyllum* samples representing 34 species, including all described species within sect. *Adelopetalum* and closely

related species of sect. *Minutissima*, were analysed. Maximum likelihood and Bayesian analyses were carried out on a five-locus data set including two protein-coding regions (*ycf1*, *matK*) and three ribosomal regions (ITS1, 5.8s and ITS2). To further resolve relationships between the major clades retrieved, Sanger sequences were combined with sequences generated from genome skimming of 25 species representing major lineages, to produce plastid and nuclear supermatrices including 70 plastid coding regions and seven ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). Maximum likelihood and Bayesian analyses reconstructed a highly supported Australasian clade including section *Adelopetalum* together with Australasian members of sect. *Minutissima*. Within this clade six highly supported lineages were found, and interspecific relationships were well resolved. Molecular divergence time estimation and ancestral area reconstructions based on 70 plastid coding regions placed the origin of the Australasian clade in Australia in the late Oligocene, ca. 23.7 Ma, and inferred that subsequent diversification of the clade occurred predominantly on the Australian continent during the Miocene. Retraction and fragmentation of eastern Australian rainforests during this time were identified as likely drivers of lineage divergence within the Australasian clade. At least four independent long-distance dispersal events were inferred from the Australian continent eastwards from the mid-Miocene onwards: to Lord Howe Island, Norfolk Island, New Zealand, and New Caledonia. Ancestral character state reconstructions were undertaken using maximum likelihood for 18 floral and vegetative characters. Synapomorphies were identified for four highly supported clades within the Australasian lineage, while four characters were shown to be highly homoplasious within the clade. The phylogenetic analyses point towards a hybrid origin for *B. pygmaeum* (sect. *Minutissima*) and *B. weinthalii* (sect. *Adelopetalum*).

Two phylogeographic studies were carried out to assess intraspecific genetic diversity and structure of two *Bulbophyllum* species endemic to Australia's tropical mountaintops that are potentially threatened by climate change: *B. lageniforme* and *B. newportii*. For each species 1–14 (mean=8) individuals per population were sampled (total 65 samples for *B. lageniforme* and 91 for *B. newportii*) from 13 mountaintops spanning the distribution of both species (Wet Tropics, Central Mackay Coast and Cape York Peninsula bioregions). Genomic data was generated based on double-digest restriction site-associated DNA (ddRAD) sequencing. Three genomic datasets were generated for each species, with varying levels of filtering stringency allowing for different proportions of loci with missing data. These resulted in datasets ranging from 17379 to 4146 RAD loci for *B. lageniforme* and 7398 to 1582 RAD loci for *B. newportii*.

Maximum likelihood, coalescent and distance-based phylogenetic reconstructions and Bayesian clustering analyses revealed structured genetic diversity within *B. newportii* that is consistent with multiple refugial populations and divergence across biogeographic disjunctions such as the Black Mountain Corridor, Herbert River Valley, Normanby Basin, and Burdekin Basin. In contrast, *B. lageniforme* showed low genetic diversity and population structure that is consistent with the current distribution resulting from relatively recent expansion from a single refugial population. These two studies confirmed that subtle differences in niche breadth and habitat preference are important factors in predicting responses to environmental change, even among closely related species. Sampling strategies to preserve the genetic diversity within each species were provided for ex-situ conservation efforts.

The four studies presented in this thesis advance the understanding of evolutionary relationships within *Bulbophyllum* at different phylogenetic levels. Broad-scale phylogenetic analyses provided insights into intersectional relationships within the Asian clade and identified a highly supported Australasian clade within the genus. Within the latter, interspecific relationships were resolved. These studies provided the first reconstruction of the spatio-temporal evolution of the genus in the Asian and Australasian region and an important phylogenetic framework for future studies on this hyper-diverse group. The phylogeographic studies provide reconstructions of infraspecific diversity within two *Bulbophyllum* species endemic to the Australian tropical mountaintops based on large genomic datasets. The results revealed species responses to past climatic changes, important for effective conservation planning to mitigate against loss of diversity under future climate changes.

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Abbreviations

AIC: Akaike information criterion

BMC: Black Mountain Corridor

CANB: Australian National Biodiversity Research

CBG: Australian National Botanical Gardens

cds: coding sequence (refers to protein coding sequence)

CHR: Manaaki Whenua - Landcare Research (New Zealand)

CNS: Australianum Tropical Herbarium

DEC: dispersal, extinction cladogenesis (refers to the dispersal, extinction, cladogenesis model for reconstructing ancestral ranges).

DIVALIKE: dispersal, vicariance like (refers to the dispersal, vicariance like model for reconstructing ancestral ranges implemented in the program BioGeoBEARS)

DNA: deoxyribonucleic acid

HUEFS: State University of Feira de Santana (Brazil)

LDD: long-distance dispersal

Ma: Megannum (refers to one million years ago)

ML: Maximum likelihood

MRCA: most recent common ancestor

NGS: Next generation sequencing

PCR: polymerase chain reaction

rel. prop.: relative probability

RAD seq: Restriction associated DNA sequencing

SNP: single nucleotide polymorphism

WU: University of Vienna (Austria)

Chapter one: Background and general introduction

Orchidaceae comprises one of the most species rich families of flowering plants, with more than 25000 species, its diversity is rivalled only by the Asteraceae (Chase et al., 2015).

Orchidaceae are epiphytic, lithophytic or terrestrial herbs that occupy a global distribution across every continent except Antarctica. The rich diversity found within the family provides rich opportunities for studying evolutionary processes, however, interpreting complex morphological diversity has also presented a significant challenge to resolving evolutionary relationships with Orchidaceae. Nevertheless, with advances in the generation of molecular data considerable progress has been made toward resolving broad relationships among the sub-families, tribes and genera and the study of evolutionary processes within the family (Chomicki et al., 2015; Givnish et al., 2015; 2016; Perez-Escobar and Chomicki et al., 2017). Still, evolutionary relationships within some of the most diverse groups, such as *Bulbophyllum* Thouars, remain poorly understood.

1.1. Systematics and biology of Bulbophyllinae (Orchidaceae)

Subtribe Bulbophyllinae Schltr. is a highly diverse group of epiphytic and lithophytic orchids with a pantropical distribution, extending into the temperate zones of northeast Asia and Australasia. The subtribe harbours exceptional diversity, with species estimates ranging between 1867 to 2200 species (Pridgeon et al., 2014; Chase et al., 2015; WCSP 2018). The most recent taxonomic revision recognises all species of the subtribe within a single mega-diverse genus, *Bulbophyllum* Thouars, rendering the genus the second largest flowering plant genus (after *Astragalus* L., Fabaceae), and the most diverse genus within Orchidaceae (Frodin, 2004).

1.1.1. Morphology

The genus *Bulbophyllum* s.l. is characterised by plants having: 1) a creeping rhizome, bearing single-noded pseudobulbs (very rarely pseudobulbs absent) with usually one to two (rarely up to twelve) apical leaves; 2) a peduncle arising from the base of the pseudobulb or the rhizome; and 3) flowers having a fleshy and mostly mobile lip attached to a distinct column foot (Pridgeon et al., 2014). Notwithstanding these uniting characters, within the genus there is exceptional morphological diversity in both floral and vegetative characteristics. Plants range in size from minute with highly reduced leaves, such as *B. minutissimum* F.Muell. which has pseudobulbs measuring a few millimetres in diameter to large plants, such as *B. fletcherianum*

J.G.Fowler which has leaves over a metre long (Jones and Clements, 2001). Inflorescences range from single to many flowers (Pridgeon et al., 2014).

1.1.2. Distribution

Bulbophyllum inhabits all floristic continents defined by Brummitt (2001), except Eurasia and the Antarctic. Species counts for each floristic continent are: tropical Asia (1562 species), Africa (305), temperate Asia (152), the Pacific (49), Southern America (88), Australasia (35), and Northern America (7) (WCSP, 2018). The main centres of diversity are found in tropical Asia in the floristic regions of Malesia (667) and Papuasias (658) and in the Afrotropics in the western Indian ocean region, on the islands of Madagascar and the Mascarenes (218) (Brummitt, 2001; WCSP, 2018). More detail on the distribution of the genus in the Asian, Australasian and Pacific regions will be provided in Chapter two.

1.1.3. Ecology

Bulbophyllum species occur in a diverse range of habitats including perhumid rainforest, montane and mangrove forests, savannah woodland and high elevation scrub (Pridgeon et al., 2014). Generally, plants grow as epiphytes, occurring on all parts of the phorophyte from the base of tree trunks to the outermost twigs high in the canopy. Many species are also found as lithophytes or terrestrials, with only a small number of species predominantly lithophytes or terrestrials (Pridgeon et al., 2014). Like many orchids, *Bulbophyllum* species often occupy localised geographic ranges and form highly specialised relationships with phorophytes (Jones and Clements, 2001; Zolts, 2013), mycorrhizal fungi (Smith and Read, 2008), and pollinators (Bartareau, 1994; Tan and Nishida, 2000; Humeau et al., 2011; Tan et al., 2002). At least ten families of flies (Diptera) have been recorded to pollinate *Bulbophyllum* flowers (Tan et al., 2002; Bartareau, 1994; Humeau et al., 2011; Teck and Hong, 2011; 2012; Pridgeon et al., 2014).

1.1.4. Conservation

Many *Bulbophyllum* species are threatened by loss of habitat and pollinators, habitat fragmentation and degradation (Romand-Monnier and Chadburn, 2013; Gale et al., 2013; Hu, 2017; Simo et al., 2018; Gale et al., 2018). Threats to habitat have been particularly pronounced in regions harbouring the highest diversity of *Bulbophyllum* species, such as the islands of Madagascar and New Guinea. Deforestation over the past 50 years has presented significant challenges for the conservation of endemic flora in Madagascar and clearing of tropical forests across Asia and New Guinea is occurring at unprecedented and accelerating rates (Harper et al., 2007; Global Forest Watch, 2014). Many *Bulbophyllum* species exhibit high

endemism to tropical mountaintops, habitats that are considered highly vulnerable to a warming climate (Pounds et al., 1999; Still et al., 1999; Foster et al., 2001; Pimm, 2009; Ponce-Reyes et al., 2012). The specialised ecological relationships and the epiphytic or lithophytic habit increases the vulnerability of orchids to environmental changes (Fay, 2018).

1.1.5. Taxonomy

Due to the broad geographic distribution and significant ecological, morphological and species diversity, resolving evolutionary relationships in *Bulbophyllum* has been challenging and this is reflected in their complex taxonomic history (Table 1.1). The genus belongs to the largest of the five orchid subfamilies, the Epidendroideae, and is placed in the tribe Dendrobieae together with another large orchid genus, *Dendrobium* (Chase et al., 2015). The name *Bulbophyllum* is derived from the Latin *bulbus* (bulb-like) and Greek *phyllon* (leaf) and was proposed by the French Botanist L.A. du Petit-Thouars (1882) for material collected and described in a treatise on the natural history of the orchids of Madagascar, Mauritius and Reunion. The collection included 12 species, of which *B. nutans* was later designated as the type for the genus (Alrich and Higgins, 2001). Traditionally, several genera were recognised in addition to the large genus *Bulbophyllum* (Lindley, 1840; Reichenbach, 1861; Bentham and Hooker, 1883; Pfitzer, 1889; Schlechter, 1913; Holttum, 1953; Seidenfaden 1973; 1979; Vermeulen, 1987; Dressler, 1993; Garay et al., 1994; Szlachetko and Margonska, 2001; Clements and Jones, 2002). For example, Dressler's treatment of Orchidaceae (1993) recognised 14 additional genera to *Bulbophyllum*, namely *Chaseella* Summerh., *Codonosiphon* Schltr., *Dactylorhynchus* Schltr., *Drymoda* Lindl., *Genyorchis* Schltr., *Hapalochilus* Schltr., *Jejosephia* A.N.Rao & Mani, *Monomeria* Lindl., *Monosepalum* Schltr., *Pedilochilus* Schltr., *Saccoglossum* Schltr., *Sunipia* Lindl., *Tapeinoglossum* Schltr. and *Trias* Lindl. Molecular phylogenetic studies showed many genera recognised within Bulbophyllinae were nested within *Bulbophyllum* s.s. (Gravendeel et al., 2004; Fischer et al., 2007; Smidt et al., 2011). Recent treatments have sunk all genera into the large genus *Bulbophyllum* (Chen and Vermeulen, 2009; Vermeulen and O'Byrne, 2011; Pridgeon et al., 2014; Chase et al., 2015; Vermeulen et al., 2014). Infrageneric classification of *Bulbophyllum* has also been the subject of frequent revision, with the most recent infrageneric treatment recognising 97 sections within the genus (Vermeulen, 1993; Vermeulen and O'Byrne, 2011; Pridgeon et al., 2014; Vermeulen et al., 2015).

Table 1.1 Taxonomic revision within the subtribe Bulbophyllinae (Orchidaceae). Studies restricted to geographic regions are indicated with an asterisk.

Year	Author	Genera
1830-1840	Lindley	8
1861	Reichenbach	8
1883	Bentham and Hooker	9
1889	Pfitzer	12
1913	Schlechter	17
1950	Holttum	7
1979	Seidenfaden	7
1987	Vermeulen *Continental Africa	2
1993	Dressler	14
1994	Garay, Hamer & Siegerist	23
2001	Szlachetko and Margonska	19
2002	Clements and Jones *Australia and New Zealand	10
2009-2015	Chen and Vermeulen, 2009; Vermeulen and O'Byrne, 2011 *Sulawesi; Pridgeon et al., 2014; Vermeulen et al., 2014; Chase, 2015; Vermeulen et al., 2015 *Borneo	1

The treatment of *Bulbophyllum* at either the rank of subtribe or genus has significant taxonomic implications, with the later solution placing this group among the most diverse genera of flowering plants. Thus, robust evidence of evolutionary relationships must be utilised to inform and underpin a widely accepted taxonomy for *Bulbophyllum*. The decision to treat Bulbophyllinae at the rank of subtribe or genus remains beyond the scope of this thesis and here I refer to the group at the generic rank, *Bulbophyllum*, as has been proposed in the most recent revisions of the group. Cautiously, I make no assumption that treating Bulbophyllinae as a single genus is the most appropriate taxonomic solution for this diverse group and acknowledge that such a treatment is in contrast to the traditional view recognising several genera, which has been maintained until relatively recently.

1.2. Molecular phylogenetic studies of *Bulbophyllum*

Molecular phylogenetic studies have improved understanding of the evolutionary relationships within *Bulbophyllum*. So far, studies have largely focused on resolving relationships among the Neotropical and Afrotropical taxa, including representative sampling of species from these regions (Neotropics: 46%; continental Africa: 50%; Madagascar and the Mascarenes: 49% of species) and have utilised up to seven plastid and nuclear markers to reconstruct evolutionary relationships (Gravendeel et al., 2004; Smidt et al., 2011; Fisher et al., 2007; Gamisch et al., 2015; Gamisch and Comes, 2019). The research identified a strong biogeographic signal among major clades within the genus, resolving a highly supported Madagascan (BS 100, PP 1), continental African (BS 92, PP1), Neotropical (BS 95, PP 0.99–1) and an Asian/Australasian lineage which received lower support (BS <50, PP <0.90–0.97) (Gravendeel et al., 2004; Smidt et al., 2011; Fisher et al., 2007; Gamisch et al., 2015; Gamisch and Comes, 2019). The highest diversity of *Bulbophyllum* species is found within the Asian and Australasian region where 1675 species are recorded (WCSP, 2018). Molecular phylogenetic studies of this region have taken a narrower scope, focused on relationships among taxa from Peninsular Malaysia (Hosseini et al., 2012) or *Bulbophyllum* sections *Trias* (Wonnapijit et al., 2015) and *Cirrhopetalum* (Hu, 2018). So far, sampling of the Asian and Australasian taxa included in previous molecular phylogenetic analyses has been limited, including representatives of approximately 0.2–8.4% of species and 4.5–31% of sections (Gravendeel et al., 2004; Smidt et al., 2011; Fisher et al., 2007; Hu, 2018; Gamisch and Comes, 2019). Thus, previous molecular phylogenetics have only partially informed understanding of evolutionary relationships within *Bulbophyllum*, while evolutionary relationships among the Asian and Australasian taxa remain poorly understood. The recent infrageneric revision recognised 97 sections within *Bulbophyllum* and while taxonomic revision within the Afrotropical and Neotropical taxa has been based on robust phylogenetic evidence, relationships among the Asian and Australasian taxa have remained unclear in previous molecular phylogenetic studies. Taxonomic treatments of the Asian and Australasian taxa have been largely based on morphology and are yet to be tested using a robust molecular phylogenetic framework. Improved understanding of evolutionary relationships among the diverse Asian and Australasian *Bulbophyllum* is urgently required to underpin a robust taxonomy and inform conservation priorities, particularly in light of the increasing threat to habitat with accelerated clearing of tropical forests across Asia and New Guinea (Global Forest Watch, 2014; Fay, 2018).

Table 1.2 Study focus, sampling of species and genetic markers in molecular phylogenetic studies of Bulbophyllum

Year	Authors	No. species	No. markers	Geographic/taxonomic focus of sampling
2004	Gravendeel et al.	118	1	Asia
2011	Smidt et al.	41	3	Neotropics
2007	Fischer et al.	139	5	Madagascar and the Mascarenes
2012	Hosseini and Dadkhar	38	1	Peninsular Malaysia
2015	Gamisch et al.	33	5	Madagascar and the Mascarenes
2015	Wonnapijit and Sriboonlert	46	3	Section <i>Trias</i>
2018	Hu	112	4	Section <i>Cirrhopetalum</i> and allies
2019	Gamisch and Comes	320	1	Global

1.3. Phylogeography as a tool to inform conservation and challenges for plant phylogeography studies

Phylogeographic structure within species can provide important insights for conservation, revealing intraspecific genetic diversity, or lack thereof (Holland and Hadfield, 2002; Dufresnes et al., 2013; Zhao et al., 2019), appropriate units for conservation (Moritz, 2004) and historical processes, from which species responses to past environmental changes can be inferred (Newton et al., 1999; Hewitt, 2000; Sullivan et al., 2000; Lowe et al., 2010; Keppel et al., 2012). The field of phylogeography has been dominated by studies on fauna, particularly in Australia and more broadly across the southern hemisphere (Beheregaray, 2008). These studies have utilised rapidly evolving genes, such as mitochondrial DNA (mtDNA) that provide sufficient variation to resolve relationships among very closely related taxa (Beheregaray, 2008). Meanwhile, phylogeographic studies of plants have been limited by the lack of appropriate markers (Beheregaray, 2008). Plant organelle DNA (mtDNA and chloroplast DNA (cpDNA)) tend to evolve more slowly in plants than nuclear DNA (Wolfe et al., 1987) and consequently variation is generally insufficient for resolving relationships among closely related taxa, although with sufficient sequencing effort phylogeographic structure has been recovered from cpDNA markers (Demesure et al., 1996; Van der Merwe et al., 2014; McPherson et al., 2013).

Alternative methods that have been applied to plant phylogeographic studies include Amplified Fragment Length Polymorphisms (AFLPs) (Mant et al., 2005; Lambertini et al., 2006; Jaros et al., 2016; Simpson et al., 2018), microsatellites (Cozzolino et al., 2003; Musacchio et al., 2006; Lemes et al., 2010; Charrier et al., 2014; Pinheiro et al., 2014) or a combination of approaches (Schönswetter et al., 2007; Meudt and Bayly, 2008). Advances in high throughput sequencing methods are providing exciting new opportunities for the field of plant phylogeography, enabling the generation of large genomic datasets with the potential to resolve relationships among closely related plant taxa.

1.3.1. Australia's tropical montane forests - a community at high risk to a warming climate

Australia's tropical montane forests harbour a unique assemblage of biodiverse mesic forest habitats distributed along the continent's eastern coastal ranges of the Cape York Peninsula, Wet Tropics and central Mackay coast bioregions (Nix, 1991; Metcalfe and Ford, 2009; Williams et al., 2011). These upland communities are among the few relic populations of Gondwanan derived rainforests that once covered the Australian continent, before the Miocene aridification that caused extensive contraction of Australia's mesic habitats (Martin, 2006; Hill et al., 2011). Under contemporary climatic conditions Australia's Wet Tropics has a monsoonal climate with a distinct winter dry season. The montane forests that occur above approximately 800m elevation experience cool, wet climatic conditions throughout the year as they intercept orographic clouds, providing moisture through cloud stripping. However, rising global temperatures are expected to lift the altitude of the orographic cloud base, critically reducing moisture availability, placing the montane forest communities at high risk from climate change (Still et al., 1999; Foster, 2001; Williams et al., 2012). Studies modelling the distribution of Australian Wet Tropics mountaintop endemics under future climate scenarios have predicted significant declines, with many species qualifying as critically endangered (Hilbert et al., 2001; Williams et al., 2003; Shoo et al., 2005a; 2005b; Costion et al., 2015; Roebler, 2018). To mitigate against extinction, ex-situ conservation action has been recommended (Costion et al., 2015) and ex-situ conservation programs are now underway (Mountner, 2019)

Responses to past climatic fluctuation in Australia's Wet Tropics have been reconstructed in phylogeographic studies of reptiles (Dolman and Moritz, 2006; Moussalli et al., 2009; Edwards and Melville, 2010; Bell et al., 2010), amphibians (Hoskin et al., 2011; Bell et al., 2012; Oza et

al., 2012), invertebrates (Hugall et al., 2002; Bell et al., 2004) and mammals (Bowyer et al., 2000). However, plant responses to environmental changes in Australia's Wet Tropics are largely understood from palaeogeography studies (Kershaw, 1994; Kershaw et al., 2005), and only limited studies of contemporary plant phylogeography (Rossetto et al., 2009; Jones et al., 2010; Burke et al., 2013; Simpson et al., 2018), reflecting a paucity of plant phylogeographic studies in tropical forests globally (Moritz et al., 2000; Beheregaray, 2008; Morris and Shaw, 2018). *Bulbophyllum* provides a model group to investigate the response of endemic epiphytes to past climatic shifts in Australia's tropical montane communities. More than half of Australian *Bulbophyllum* species occur in the Wet Tropics and 88% are endemic to the upland rainforests of the region (Jones, 2006). As epiphytes, *Bulbophyllum* species are effective indicators of environmental change due to the unique environmental requirements of epiphytic species, particularly for moisture availability (Zotz and Bader, 2009). So far, no studies have been published reconstructing intraspecific diversity within Australasian *Bulbophyllum* species.

1.4. Research aims and methodological approaches

The broad aim of this thesis is to improve understanding of evolutionary relationships within *Bulbophyllum*, both among and within species, with a focus on the diverse and poorly understood Asian and Australasian taxa, and to use the results to infer historical biogeography, identify phylogenetically informative morphological characters and inform taxonomy and conservation efforts. To achieve this aim, I have carried out studies that reconstruct the spatio-temporal evolution of *Bulbophyllum* at three hierarchical phylogenetic scales. The first study aims to reconstruct infrageneric relationships within *Bulbophyllum* to produce a phylogenetic framework and reconstruct the spatio-temporal evolution of the genus, with a focus on increasing sampling of the Asian and Australasian taxa. The second study focuses on species-level relationships within the Australasian *Bulbophyllum* section *Adelopetalum* and close allies, to reconstruct the spatio-temporal evolution of this group in Australasia and identify phylogenetically informative morphological traits. The third and fourth study aim to assess the phylogeographic structure within two *Bulbophyllum* species endemic to Australia's tropical montane rainforests, to infer responses to past climatic changes and to inform sampling strategies for ex situ conservation considering future climate change. To achieve these aims, I have employed a broad range of DNA sequencing and analytical methods including next-generation sequencing, phylogenetic and population genetic analyses, divergence dating and ancestral trait reconstruction.

1.4.1. New approaches in plant molecular phylogenetics and phylogeography

The use of molecular data in phylogenetic studies has revolutionised our understanding of evolutionary relationships. Within Orchidaceae, phylogenetic reconstructions based on molecular data have informed evolutionary relationships between subfamilies (Cameron, 1999; Freudenstein et al., 2000), tribes (Gorniak et al., 2010; Freudenstein and Chase, 2015) and among and within genera (Cox et al., 1997; Burke et al., 2008, Alvarez-Molina and Cameron, 2009; Clements et al., 2011; Cisternas et al., 2012; Inda et al., 2012; Gardiner et al., 2013; Xiang et al., 2013; Bone et al., 2015; Zou et al., 2015; Hu et al., 2016; Jin et al., 2017; Ng et al., 2018; Salazar et al., 2018). However, despite increased sampling of molecular regions, for many orchid groups, phylogenetic relationships remained at least partially unresolved (Goldman et al., 2001; Soliva et al., 2001; Bateman et al., 2009; Clements et al., 2011; Cisternas et al., 2012; Chochai et al., 2012; Gardiner et al., 2013). Within *Bulbophyllum*, phylogenetic relationships among the Asian and Australasian taxa have remained unclear in molecular phylogenetic studies based on up to seven nuclear and plastid markers (Gravendeel et al., 2004; Fisher et al., 2007; Smidt et al., 2011; Gamisch et al., 2015; Gamisch and Comes, 2019).

Recent developments in massively parallel high throughput sequencing have provided new opportunities for producing large quantities of genetic sequence data (Voelkerding et al., 2009). Increasingly plant molecular phylogenetic studies are utilising these ‘next-generation’ sequencing (NGS) methods to generate highly informative datasets, including hundreds to thousands of molecular markers (Soltis et al., 2010; Johnson et al., 2018). These datasets have the power to provide new insights into evolutionary relationships from deep-level relationships within the angiosperms (Moore et al., 2010; Jansen et al., 2007) to relationships among species (Parks et al., 2009), including complex groups such as polyploids (Griffin et al., 2011) or where hybridisation and introgression has occurred (Twyford and Ennos, 2012). NGS methods have also provided new opportunities in the field of plant phylogeography (McPherson et al., 2013; Van der Merwe et al., 2014; Jiang et al., 2019). Within Orchidaceae, phylogenetic studies employing NGS methods have clarified relationships across phylogenetic scales from subfamilies and tribes (Givnish et al., 2015; Niu et al., 2017; Deng et al., 2018) to among and within genera and also within species (Abreu et al., 2018; Nargar et al., 2018; Bogarin et al., 2018). So far, NGS methods have not been applied in phylogenetic studies of *Bulbophyllum* and present an exciting new opportunity to generate informative datasets that can advance understanding of evolutionary relationships, particularly among the poorly understood Asian and Australasian taxa. To select the most appropriate methods for my

studies, I have carefully considered several NGS methods, summarised below, assessing the investment required and the phylogenetic level at which they are most suitable.

1.4.1.1. Resolving broad-scale relationships

1.4.1.1.1. Genome skimming

Genome skimming methods utilise next generation sequencing platforms to sequence indiscriminately across a sample and involve a trade-off between sample number, genome size and sequencing depth that can be achieved during a sequencing run (Straub et al., 2012). The sequencing effort required to recover the large nuclear genome generally remains prohibitively expensive for most phylogenetic research dealing with many samples. However, the smaller, more abundant plastome, and high copy nuclear regions such as the nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) can be recovered with minimal sequencing effort (Straub et al., 2012). Genome skimming methods provide convenient approaches for generating molecular data that do not require prior marker development and have been utilised successfully to resolve relationships in molecular phylogenetic studies of plants (Givnish et al., 2010; Clement et al., 2014; Male et al., 2014; Stoughton et al., 2018), including orchids (Givnish et al., 2015). Limitations of genome skimming approaches must also be considered, as the utility of plastid markers for resolving evolutionary relationships with confidence varies among lineages and phylogenetic scales. For example, plastid markers may be generally suited for resolving relationships at higher taxonomic levels but due to the relatively slow evolution of the plastome, variation among more closely related lineages may be insufficient to resolve phylogenetic relationships. For example, broad level phylogenetic relationships reconstructed within Orchidaceae based on 75 plastid coding regions are largely well supported with most nodes receiving maximum support and yet relationships within the Epidendroideae are only partially well supported with several lineages receiving only moderate or low support (Givnish et al., 2015).

Nuclear ribosomal regions have been frequently utilised in plant phylogenetic research in particular the highly variable non transcribed spacer regions ITS1 and ITS2, with examples focused on Orchidaceae resolving relationships among (Cisternas et al., 2012) and within genera (Hu et al., 2016; Chochai et al., 2012). Resolution and support for phylogenetic relationships reconstructed from the nuclear ribosomal regions varies between taxonomic scales and among lineages and limitations due to issues of homology must also be considered. These include the possible presence of gene copies or paralogs resulting from gene duplication

events rather than speciation, where comparisons among taxa may be inadvertently made between paralogous regions (Poczai and Hyvonen, 2009). Further issues with homology surround the relatively high rate of mutation in the non-coding nuclear ribosomal intergenic spacer regions. These regions are highly variable and therefore can be highly informative for resolving relationships. However, over longer evolutionary time scales the variation in these regions can become saturated in which case the variation being compared among taxa may not be homologous and result in underestimating substitutions between highly divergent sequences (Alvarez and Wendel, 2003).

1.4.1.1.2. Exon capture

Exon capture, also referred to as target enrichment, methods use molecular ‘baits’ or ‘probes’ to isolate genetic regions of interest, such as low copy nuclear markers which cannot be recovered with shallow to modest genome skimming. Once probes have been developed, Exon capture provides a cost effective, reproducible technique for sequencing hundreds of molecular markers for large numbers of samples (Meyer and Kircher, 2010; Cronn et al., 2012,) and have been utilised to resolve relationships within the Ericaceae (Kadlec et al., 2017), Mimosoideae (Nicholls et al., 2015), Sarraceniaceae (Stephens et al., 2015), Arecaceae (Heyduk et al., 2016), Leguminosae (Sousa et al., 2015), Apocynaceae (Weitemier et al., 2014) and Orchidaceae (Bogarin et al., 2018). The uptake of target capture methods has been limited by the investment in whole genome or transcriptome sequencing that is required to design taxon-specific baits for sequence capture (Godden et al., 2012; Zimmer and Wen, 2012). Several bait sets designed for very broad applicability across diverse plant lineages are now commercially available (Mandel et al., 2014; Johnson et al., 2018). Nevertheless, for most groups’ taxon-specific marker development is required, which remains beyond the scope of many phylogenetic studies. Phylogenomic studies utilising target capture approaches may encounter issues when inadvertently targeting genes derived from a multi copy gene family resulting in the presence of paralogous genes. Marker development for target capture studies specifically targets single or low copy regions, however, the method used to differentiate multi and low or single copy gene regions can be limited by the reference database used for comparisons and/or the breadth of taxa included in the marker development process. This issue is likely to be particularly pronounced with the use of marker sets designed for broad taxonomic groups.

During my PhD candidature, the development of a custom target-sequence capture kit for *Bulbophyllum* was prohibitively cost and time intensive. Genome skimming is a cost and time efficient sequencing approach that offers promise for resolving relationships among and within sections. Hence, for this thesis I selected genome skimming as the most suitable high throughput sequencing approach to resolve broad-scale relationships in *Bulbophyllum*.

1.4.1.2. Resolving relationships at intraspecific level

1.4.1.2.1. Microsatellites

Microsatellite methods target highly repetitive genetic regions for resolving relationships in plant population level studies (Echt et al., 1998; Dayanandan et al., 2002; England et al., 2002; Zhou et al., 2003) including orchids (Cozzolino et al., 2003; Pillon et al., 2006; Soliva and Widmer, 2007; Pessoa et al., 2012) and specifically, *Bulbophyllum* (Hu et al., 2017). NGS methods are increasing efficiency in the initial investment required to identify polymorphic micro-satellite regions (Kim et al., 2015; Trapnell et al., 2015; Pimentel et al., 2018). However, the analysis of microsatellite data is limited to distance-based approaches and is not suitable to parsimony and likelihood phylogenetic methods (Karp et al., 1996; Koopman, 2005). Further, due to the low number of microsatellite markers included in such studies, inferences based on these markers have often remained of limited value.

1.4.1.2.2. Restriction associated DNA sequencing

Restriction associated DNA (RAD) sequencing methods (Miller et al., 2007; Baird et al., 2008; Peterson et al., 2012; Wang et al., 2012; Toonen et al., 2013) provide a genomic method suitable for resolving plant relationships at shallow phylogenetic scales, such as among closely related species or within a species. RAD sequencing methods have been successfully utilised to resolve plant relationships at interspecific (Hipp et al., 2014; Eaton et al., 2017) and intraspecific phylogenetic scales (Cavender-Bares et al., 2015; Hou et al., 2015). RAD sequencing methods utilise restriction enzymes to isolate thousands to hundreds of thousands of restriction fragments from across the genome. By utilising restriction enzymes RAD sequencing methods require no a priori sequence information and can therefore be applied to non-model organisms with no to relatively minimal taxon-specific optimisation (Davey et al., 2010). RAD sequencing approaches offer an advance on amplified fragment polymorphism methods (AFLPs) (Vos et al., 1995) by generating sequence data containing single nucleotide polymorphisms (SNPs) that can be utilised in parsimony and likelihood phylogenetic analyses (Leache and Oaks, 2017).

RAD sequencing methods have been used to generate large genomic datasets to resolve relationships at shallow taxonomic scales (Wang et al. 2013; Boucher et al., 2016) including within orchids (Bateman et al., 2018), complex groups that have undergone ancient hybridisation or introgression (Eaton and Ree, 2013; Escudero et al., 2014; Eaton et al., 2015) and have provided insights into plant responses to past environmental changes (Jiang et al., 2019). Bioinformatic pipelines have been developed that process large volumes of sequence data and mitigate against inherent bias that have been associated with RAD sequencing methods (Catchen et al., 2013; Eaton, 2014).

1.4.1.3. *Methodological approach for resolving interspecific relationships*

The first two studies of this thesis reconstruct phylogenetic relationships in *Bulbophyllum* at the species level and above. In preliminary analyses I have utilised three standard molecular markers to infer phylogenetic relationships and identify major lineages to inform further sampling. Given that previous molecular phylogenetic studies of *Bulbophyllum* were unable to resolve relationships among the Asian and Australasian taxa utilising standard molecular markers, I have also employed genome skimming methods to sequence plastid coding regions and high repeat nuclear regions such as the nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS), without the need for time and cost intensive marker development required for exon capture methods.

To reconstruct phylogenetic relationships, I have utilised maximum likelihood (Stamatakis, 2014) and Bayesian (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) phylogenetic methods. These methods incorporating models of nucleotide evolution have come to dominate phylogenetic studies, preferred over maximum parsimony methods, which have been shown to be statistically inconsistent (Felsenstein 1978). To assess the suitability of models of nucleotide substitution for my phylogenetic analyses, I have carried out model tests on a preliminary nuclear and plastid dataset (Nylander, 2004), selecting the most appropriate nucleotide substitution model based on the Akaike information criterion (AIC) (Akaike, 1974).

1.4.1.4. *Methodological approach for resolving intraspecific relationships*

The third and fourth study of this thesis reconstructs relationships within two *Bulbophyllum* species endemic to Australia's upland rainforests. To resolve intraspecific relationships, I have utilised the RAD sequencing method: double-digest RAD sequencing or ddRAD (Peterson et al., 2012), in preference over microsatellite markers, due to the minimal investment required in taxon-specific optimisation and the highly informative datasets produced by RAD sequencing

methods, which outperform microsatellites markers (Thrasher et al., 2018; Jeffery et al., 2016). To account for the reticulate nature of evolutionary relationships among closely related taxa I used analytical methods suitable for reconstructing relationships among closely related species. Among closely related taxa discordance among gene trees is frequently observed due to processes such as recombination, introgression and incomplete lineage sorting (Degnan and Rosenberg, 2009). To visualise discordance among RAD loci I have reconstructed distance based phylogenetic networks (Hudson, 1998). To reconstruct phylogenetic relationships, I used ML phylogenetic reconstruction based on concatenated datasets of RAD loci (Stamatakis, 2014). Additionally, I have considered several coalescent based methods, which allow for heterogeneity in the evolutionary histories among genes (Degnan and Rosenberg, 2009). Bayesian coalescent methods (Edwards et al., 2007; Heled and Drummond, 2010; Yang and Rannala, 2014) require substantial computational resources and consequently are unfeasible for large datasets sampling hundreds of taxa and thousands of loci (McCormack et al., 2013). More efficient methods include summary coalescent methods (Liu et al., 2010; Mirarab and Warnow, 2015), which estimate the species tree using summary statistics from individual gene trees. However, these methods do not directly utilise the genetic variation to reconstruct relationships. In my analyses I utilised a likelihood-based method that estimates the species tree directly from SNP data, which provides an efficient method of analysis even for very large datasets and has been shown to perform well in comparisons to Bayesian and summary based coalescent methods (Chifman and Kubatko, 2014; Chou et al., 2015). To identify genetic admixture within individuals and infer introgression among groups, I have also used a Bayesian clustering method, which is widely applied in population genetic and phylogeographic analyses (Pritchard et al., 2000; Falush et al., 2003).

1.4.2. Insights from divergence dating analyses into temporal evolution within Orchidaceae

Significant insights into the temporal evolution of lineages have been gained from divergence dating methods that utilise variation in the genetic sequences, to infer time since coalescence, based on the molecular clock hypothesis (Zuckerkandl and Pauling, 1965; Arbogast et al., 2002; Kumar, 2005). Divergence dating analyses have estimated the Orchidaceae to have evolved in the early Cretaceous, ca. 112 Ma, and have reconstructed temporal evolution within the family, and among and within genera (Ramirez et al., 2007, Givnish et al., 2015; Andriananjamanantsoa et al., 2016; Perez-Escobar and Chomicki et al., 2017; Nargar et al., 2018; Nauheimer et al., 2018; Pessoa et al., 2018). Divergence dating analyses of *Bulbophyllum*

estimated that the genus evolved in the early Oligocene, ca. 29.3 Ma, with crown divergence commencing in the mid Miocene, ca. 15.7 Ma (Gamisch and Comes, 2019). Within *Bulbophyllum*, studies have largely focused on reconstructing the temporal evolution within the *Cirrhopetalum* alliance (Hu, 2018) and the Malagasy lineage including sections *Bifalcula*, *Calamaria* and *Humboldtiorchis* (Gamisch et al., 2015). To build on the previous studies of the genus, I applied divergence dating analyses based on a 70 plastid cds dataset, to reconstruct the temporal evolution among a broad representative sampling of the Asian and Australasian taxa. Divergence dating analyses incorporate priors for nucleotide substitution, clock rate and branching process among other parameters. Studies have shown that the application of branching process priors such as the pure birth (Yule, 1925) or birth death (Nee et al., 1994; Gernhard, 2008) models can have significant impacts on the inference of divergence dates among lineages (Condamine et al., 2015). However, these effects are thought to be more pronounced when estimating divergence ages among ancient clades with a potential pattern of high extinction, while studies focused on more recently evolved lineages have shown no effect when comparing divergence ages between the pure birth or birth death priors (Couvreur, 2010). Within the Orchidaceae, divergence date estimations have frequently been made without explicit statistical testing of the branching process and examples can be found selecting pure birth (Surveswaran et al., 2018; Nargar et al., 2018) or birth death models (Gamisch and Comes, 2019; Van der Neit et al., 2017), which are both generally accepted for analyses of interspecific relationships. Where statistical testing has been applied within Orchidaceae a birth death model has been favoured (Perez-Escobar et al., 2017, Bogarin et al., 2019).

Initial divergence dating analyses carried out in chapter two of this thesis do not include explicit testing of branching priors and clock models. The broadly accepted uncorrelated relaxed log normal clock (Drummond et al., 2006; 2012) was selected to account for rate heterogeneity among lineages and the birth death branching prior was selected as a suitable model, being favoured in statistical comparisons within other orchid lineages (Perez-Escobar et al., 2017; Bogarin et al., 2019). In the absence of statistical testing potential bias due to the selected priors cannot be ruled out. However, divergence dating analyses carried out in chapter two are focused on resolving relationships within *Bulbophyllum* and previous divergence dating analyses have estimating diversification within the genus has taken place during the Neogene (Gamisch and Comes, 2019). Therefore, the effect of the branching prior is likely to be low if not insignificant given this study is focused on relatively recently diverging

rather than ‘ancient’ lineages. Given the evidence for the widespread heterogeneity among substitution rates among lineages across many different taxonomic groups it is likely that the relaxed clock would be favoured over a strict clock. In chapter three statistical testing of branching and clock priors is carried out with favoured models supporting those utilised in chapter two and identified in previous studies of orchids.

In divergence time estimation, calibration of the substitution rate is of critical importance and requires a priori knowledge of the divergence date between two lineages in the phylogeny. In historical biogeographic studies, divergence dating analyses are usually calibrated with dated fossils (Givnish et al., 2015; Chomicki et al., 2015; Chaw et al., 2004; Sauquet et al., 2012; Bremer et al., 2004). So far, no orchid fossils have been confidently assigned to *Bulbophyllum* therefore I have utilised a reliable, well documented macrofossil of a related orchid, *Dendrobium winikaphyllum* (Conran et al., 2009). The fossil provided a calibration point within the closely related genus *Dendrobium*, which forms a sister group relationship with *Bulbophyllum* (Gorniak et al., 2010) and has been previously utilised to calibrate divergence dating analyses of orchids (Givnish et al., 2015; Chomicki et al., 2015; Burke et al., 2013; Gustafsson et al., 2010). I have assigned the fossil calibration to the stem node of the Australasian *Dendrobium* clade, based on clearly defined morphological characteristics shared between the fossil and the extant Australasian *Dendrobium* lineage (Doyle and Donoghue et al., 1993; Conran et al., 2009). Additionally, I have selected outgroups to allow the application of three secondary calibrations taken from a family-wide divergence dating analysis based on four plastid and nuclear markers (*matK*, *rbcl*, ITS and *trnL-trnF*), calibrated with four orchid fossils (Chomicki et al., 2015).

1.4.3. Applications of ancestral trait reconstruction in Orchidaceae

Ancestral trait reconstruction utilises the measured characteristics of extant taxa to infer the phenotypic, biogeographic or ecological traits for ancestral nodes of a phylogenetic tree, providing a means to test hypotheses of evolutionary change in a phylogenetic framework. These methods are frequently applied to dated phylogenies to provide a time-calibrated reconstruction of the evolutionary history of lineages, providing insights into evolutionary processes and the drivers of evolutionary change (Joy et al., 2016).

1.4.3.1. Historical biogeography of *Bulbophyllum*

The application of divergence dating, and ancestral range reconstruction has provided new insights into the spatio-temporal evolution of the Orchidaceae around the world. Studies have reconstructed historical biogeography of orchids in the Americas (Salazar et al., 2018, Perez-Escobar and Chomicki et al., 2017), Africa (Micheneau et al., 2008), Asia (Xiang et al., 2016, and Australasia (Nauheimer et al., 2018; Nargar et al., 2018) and across continents (Guo et al., 2012; Givnish et al., 2016; Surveswaran et al., 2018). *Bulbophyllum* provides an ideal model group for studying spatio-temporal evolution, with exceptional species diversity and a near-global distribution. Molecular phylogenetic studies have revealed a clear biogeographic signal among highly supported continental African, Madagascan and Neotropical clades, suggesting taxa in each region have diversified in situ (Fischer et al., 2007; Smidt et al., 2011). The Afrotropical/Neotropical clade was composed of a Malagasy lineage and continental African lineages, with the Neotropical group nested within the Afrotropical clade. Based on these relationships a single long-distance dispersal (LDD) event was postulated from continental Africa to the Neotropics (Smidt et al., 2011). Ancestral range estimations carried out during a global study of *Bulbophyllum* placed the origin of the genus in the Asian-Pacific region, with subsequent LDD to the Afrotropics and Neotropics giving rise to the continental African, Madagascan and Neotropical lineages (Gamisch and Comes, 2019). The study was focused on global patterns of spatio-temporal evolution and recognised a broad Asian-Pacific biogeographic region. Consequently, the spatio-temporal evolution of *Bulbophyllum* within the Asian and Australasian region remained poorly understood. Hypotheses of predominantly eastern or western Malesian lineages have been proposed based on unpublished analyses (Pridgeon et al., 2014), however, these hypotheses are yet to be tested against comprehensive molecular datasets. Ancestral range reconstruction methods provide an exciting opportunity to reconstruct the spatio-temporal evolution of this diverse and broadly distributed genus.

Increasingly complex models of range evolution have been applied in ancestral range reconstruction analyses. The dispersal-vicariance (DIVA) (Ronquist, 1997) and dispersal-extinction cladogenesis (DEC) (Ree et al., 2005; Ree and Smith, 2008) models have been widely applied in historical biogeography studies, while Bayesian methods utilising continuous-time Markov modelling have been developed for more complex analyses including a large number of areas (Ronquist and Huelsenbeck, 2003; Landis et al., 2013). Models have also incorporated founder speciation or jump dispersal events (Matzke, 2013b; Matzke, 2014), however the appropriate application of models incorporating founder speciation is debated (Ree and

Sanmartín, 2018). I have utilised ancestral reconstruction methods applied to chronograms reconstructed from a 70 plastid cds dataset to reconstruct the spatio-temporal evolution within the genus, with a focus on the Asian and Australasian taxa. I have designated taxa to geographic regions based on the floristic continents defined in the World Geographic Scheme for Defining Plant Distributions (Brummitt, 2001). I have selected two models of range evolution frequently used in studies of historical biogeography for comparison, the dispersal-vicariance model (Ronquist, 1997) and a dispersal-extinction cladogenesis model (Ree et al., 2005; Ree and Smith 2008) and among these models of range evolution, identified the most appropriate based on AIC statistics. I have excluded models incorporating founder event speciation events from my analyses, based on recent concerns regarding the appropriate application of these models (Ree and Sanmartin, 2018).

1.4.3.2. Ancestral character reconstruction

The application of ancestral character reconstruction within Orchidaceae has provided insights into evolution of floral and vegetative morphological traits (Yukawa and Uehara, 1996; Raskoti et al., 2016; Nargar et al., 2018), pollination syndrome (Schiestl and Cozzolino, 2008; Inda et al., 2012; Johnson et al., 2013; Breitenkopf et al., 2015), mycoheterotrophy (Motomura et al., 2010), chromosomes (Oliveira et al., 2015) and photosynthetic pathways (Motomura et al., 2008; Silvera et al., 2009). So far, molecular phylogenetic studies of *Bulbophyllum* have applied these methods to reconstruct the evolution of floral morphology, pollination syndrome and niche within a Madagascan lineage (Fischer et al., 2007; Gamisch et al., 2015; 2016) and to explore the evolution of morphological traits and photosynthetic pathways within the *Cirrhopetalum* alliance (Hu, 2018).

Cladistic studies of Asian and Australasian *Bulbophyllum* sections *Adelopetalum*, *Lepanthanthe*, *Macrouris*, *Pelma*, *Peltopus*, and *Uncifera* have reconstructed species relationships based on morphological synapomorphies (Vermeulen, 1993). However, the homology of the characters examined has not yet been tested in a molecular phylogenetic framework. To build on previous studies of the *Bulbophyllum* section *Adelopetalum*, in this thesis I will carry out ancestral trait reconstruction to test the homology of morphological traits and identify phylogenetically informative characters within section *Adelopetalum* and related taxa. Ancestral states have frequently been reconstructed in a parsimony framework, minimising state changes given the observed tree and character state distribution (Maddison et al., 1984). However, the assumptions of maximum parsimony are frequently violated (Maddison 1994, Collins et al., 1994; Yang et al., 1995; Cunningham et al., 1997; Zhang and Nei, 1997).

Alternatively, many studies adopt a maximum likelihood (ML) approach that determines the probability of the observed character states based on a stochastic model of evolution (Schluter et al., 1997; Pagel, 1999). To estimate the relative probabilities of morphological traits at each ancestral node, I have carried out character reconstructions using a likelihood approach (Schluter et al., 1997; Pagel, 1999), with the Mk 1 model, that assumes all changes between alternative character states are equally probable (Lewis, 2001).

1.5. Thesis outline

In Chapter two, I examine broad-level infrageneric relationships within *Bulbophyllum* with focus on the phylogenetic placement of Asian and Australasian sections within the genus. I sampled 145 *Bulbophyllum* species, including 122 species from the Asian and Australasian region, representing ca. 63% of Asian and Australasian sections and carried out maximum likelihood and Bayesian phylogenetic analyses based on 70 plastid coding regions and three nuclear ribosomal regions (ITS1, 5.8s, ITS2). To reconstruct spatio-temporal evolution, divergence dates were estimated with a relaxed-clock analysis, carried out in a Bayesian framework and ancestral ranges were reconstructed in a likelihood framework, applying both dispersal-vicariance and dispersal, extinction, cladogenesis models of range evolution.

In Chapter three, I reconstruct interspecific relationships among and within *Bulbophyllum* sections *Adelopetalum* and *Minutissima* to circumscribe the Australasian *Bulbophyllum* lineage and resolve interspecific relationships within this group. I sampled 34 *Bulbophyllum* species, including one to three representatives for all described species within section *Adelopetalum* and Australasian and Asian representatives of sect. *Minutissima*. Phylogenetic relationships were reconstructed with maximum likelihood and Bayesian analyses based on supermatrices of 70 plastid coding regions and seven nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). To reconstruct the spatio-temporal evolution I estimated divergence dates with a relaxed-clock analysis carried out in a Bayesian framework and reconstructed ancestral ranges in a likelihood framework, applying both dispersal-vicariance and dispersal, extinction, cladogenesis models of range evolution. To identify phylogenetically informative and homoplasious characters I carried out ancestral character reconstructions for 19 morphological characters.

In Chapter four, I examine infraspecific genetic diversity within the Australian Wet Tropics montane rainforest endemic species *Bulbophyllum lageniforme*, which is potentially threatened by climate change, to infer evolutionary processes that have been influenced by past climatic changes. To reconstruct phylogeographic patterns within *B. lageniforme*, I carried

out distance, maximum likelihood and coalescent based phylogenetic analyses and Bayesian population clustering analyses on restriction associated DNA genomic datasets, generated for 65 samples collected from seven mountaintops spanning the distribution of the species in the Wet Tropics Bioregion. To inform ex situ conservation efforts, sampling strategies were identified that conserve the genetic diversity present within the species.

In Chapter five, I investigate intraspecific genetic diversity within the Australian tropical upland forest endemic species, *Bulbophyllum newportii*, which is potentially threatened by climate change, to infer evolutionary processes that have been influenced by past climatic changes. To reconstruct phylogeographic patterns within *B. newportii*, I carried out distance, maximum likelihood and coalescent based phylogenetic analyses and Bayesian population clustering analyses on restriction associated DNA genomic datasets, generated for 91 samples, collected from 13 mountaintops spanning the distribution of the species in the Wet Tropics, Cape York Peninsula and Central Mackay Coast Bioregions. To inform ex situ conservation efforts, sampling strategies were identified that conserve the genetic diversity present within the species.

In Chapter six, I provided a synthesis of the research outcomes and directions for further investigation.

Chapter two: Phylogenomic insights into the spatio-temporal evolution of the Asian and Australasian *Bulbophyllum*

2.1. Introduction

The biogeography of the Malay Archipelago, where the south east Asian and Australasian plates coalesce, has been of interest since the early observations of Müller (1846) and Wallace (1860) and remains the focus of ongoing study (Lohman et al., 2011; Crayn et al., 2015; Tomas et al., 2017; Huang and Knowles, 2018; Morley, 2018; Torre et al., 2019; Hausdorf, 2019). Wallace observed “South America and Africa, separated by the Atlantic, do not differ so widely as Asia and Australia” (Wallace, 1860, p. 174). Asia and Australasia each have a complex geological history (Zahirovic et al., 2016), culminating in the collision of the Asian and Australasian plates in the late Oligocene, ca. 23 Ma (Hall et al., 2011). The collision of the two plates initiated a period of dramatic geological change in the region forming a complex archipelago, with major geological uplift occurring in New Guinea at the northern edge of the Australasian plate during the mid-Miocene, approximately 12 Ma (Hall et al., 2011). The Asian and Australasian landmasses have been permanently separated by a series of deep-water straights. However, rising and falling sea levels associated with the glacial cycles during the Pliocene and Pleistocene have periodically flooded, exposed, fused and sundered islands with each other and the continental landmasses on both the Asian and Australasian plates (Voris, 2000). The Malay Archipelago is exceptional biodiverse; covering only 4% of the planet's land area, the region harbours 25% of terrestrial species (Corlett, 2009). Disparate biogeographic patterns have been observed between various plant and animal groups and several biogeographic barriers have been identified in the region marking major disjunctions (Wallace, 1863; Weber, 1902; Mayr, 1944; Huxley, 1968; Lydekker, 2011). However, studies have shown that for many plant taxa, these barriers are semi-permeable (Sniderman and Jordan, 2011; Richardson et al., 2012; Caryn et al., 2015; Yap et al., 2018). *Bulbophyllum* is broadly distributed and exceptionally diverse in the Asian and Australasian region and thus provides a model group for studying patterns of spatio-temporal evolution within the Asian and Australasian region. *Bulbophyllum* occurs in each of the four floristic continents identified in this area: temperate Asia (164 species), tropical Asia (1768 species), the Pacific (55 species) and Australasia (36 species) (Brummitt, 2001; WCSP, 2018). Centres of diversity are found in the Malay Peninsula and

Archipelago in the floristic regions of Malaysia (667 species) and Papuaia (658 species) (Brummitt, 2001; WCSP, 2018).

Previous molecular phylogenetic studies have identified a strong biogeographic signal among major clades within *Bulbophyllum*. Phylogenetic analyses resolved highly supported Madagascan (BS 100, PP 1), continental African (BS 92, PP1) and Neotropical lineage (BS 95, PP 0.99–1) (Fisher et al., 2007; Smidt et al., 2011; Gamisch et al., 2015; Gamisch and Comes, 2019). The Neotropical and continental African clades were resolved in a highly supported sister group (BS 99, PP 0.99–1) and together with the Madagascan clade formed a highly supported Afrotropical/Neotropical clade (BS 95, PP 0.85–0.98) (Gravendeel et al., 2004; Fisher et al., 2007; Smidt et al., 2011; Gamisch et al., 2015; Gamisch and Comes, 2019). While the Asian and Australasian *Bulbophyllum* taxa constitute the bulk of species diversity within the genus (>1600 species), so far sampling has been limited relative to the diversity found in this region. Previous molecular phylogenetic studies have included between 0.2–7% of species and 4.5–31% of sections, with the Australasian taxa represented by at most eight species (Gamisch and Comes, 2019). Phylogenetic analyses resolved the Asian and Australasian taxa in a clade, reconstructed in a sister group relationship to the Afrotropical/Neotropical clade (Fischer et al., 2007; Smidt et al., 2011; Gamisch et al., 2015). The Asian/Australasian clade received moderate support in analyses based on the nuclear ribosomal ITS region (PP <0.90–0.97), (Gamisch et al., 2015; Gamisch and Comes, 2019), but remained unsupported (BS <50, PP <0.50) in analyses based on four plastid loci and the nuclear ribosomal ITS region (Fisher et al., 2007).

Recent infrageneric revision of *Bulbophyllum* recognises 97 sections within the genus, including 67 Asian and Australasian sections (Pridgeon et al., 2014). The infrageneric revision presented a summary of sectional relationships within the genus reconstructed from nuclear ribosomal ITS sequences, which included a broader representative sampling of the Asian and Australasian sections (62%), citing unpublished analyses (Pridgeon et al., 2014). The Asian and Australasian taxa were resolved in a clade with two lineages, one comprising predominantly western Asian sections and the other predominantly eastern Asian sections, suggesting biogeography has shaped evolutionary relationships among *Bulbophyllum* species in the region. However, taxon names, vouchers and provenance details and support for relationships were not provided for detailed interpretation of this analysis (Pridgeon et al., 2014). Overall, evolutionary relationships among the Asian and

Australasian taxa have remained poorly understood in previous molecular phylogenetic studies due to sampling limitations and poorly supported phylogenetic relationships. A robust phylogenetic framework is required to understand phylogenetic relationships and spatio-temporal evolution within *Bulbophyllum* in the Asian and Australasian region.

Recent advances in next generation sequencing methods present new opportunities for generating large molecular datasets to resolve evolutionary relationships in complex plant groups (Voelkerding et al., 2009; Soltis et al., 2010; Johnson et al., 2018). Genome skimming is a next generation sequencing method that can efficiently generate sequence data to reconstruct the abundant plastome, and high copy nuclear regions such as the nuclear ribosomal regions (Straub et al., 2012). A plastome is the circular genome contained within plastid organelles with a standard size between 120–160 kilo base pairs in length in land plants, while examples of smaller plastid genomes are found within mycoheterotrophic orchids (Yuan, et al., 2018; Delannoy et al., 2011). Within the plastome, gene order is relatively conserved but substitution and indel mutations are relatively frequent, providing a useful resource for reconstructing evolutionary relationships in plant lineages (Wicke et al., 2011). Numerous copies of the plastome are present in most plant cells and therefore modest next generation sequencing effort can yield sufficient coverage of sequence reads to reconstruct the plastid genome. In previous studies of *Bulbophyllum*, that were based on sequences of 1–7 plastid and nuclear regions only, relationships among major clades and the Asian and Australasian taxa have remained unclear, limited by the phylogenetic information present in these few markers. Genome skimming methods provide a convenient opportunity to target the plastome and highly repetitive nuclear regions and generate large molecular datasets that may clarify evolutionary relationships within *Bulbophyllum*.

Aims of this study are to:

1. examine the potential of plastome-scale sequence datasets to reconstruct evolutionary relationships in *Bulbophyllum*;
2. clarify infrageneric relationships within *Bulbophyllum* s.l. with a focus on the Asian and Australasian taxa, and
3. reconstruct the spatio-temporal evolution of the genus.

2.2. Methods

2.2.1. Sampling

In total, 156 orchid samples, representing 148 species were sampled in this study, with sequence data generated for 91 samples and complemented with 63 accessions from GenBank. From *Bulbophyllum*, 145 species were included. From the Asian and Australasian region, 122 species were sampled, representing 60 sections. The sampling included approximately 63% of 67 Asian and Australasian sections recognised in the recent infrageneric revision of the genus (Pridgeon et al., 2014). However, the revision does not document species circumscribed or sections synonymised within the recognised sections. Therefore, section assignments based on Pridgeon et al. (2014) could not be applied directly to samples used in this study. Alternatively, section assignments were determined based on published treatments (Wight, 1851; Reichenbach, 1861; Bentham and Hooker, 1883; Pfitzer, 1888; 1889; Ridley, 1898; 1908; Cogniaux, 1902; Schlechter, 1911; 1912; 1913; Smith, 1914; 1933; Summerhayes, 1935; Seidenfaden, 1979; Averyanov, 1994; Garay et al., 1994; Vermeulen, 1993; 2001; 2002; Vermeulen and O'Byrne, 2008; Smidt et al. 2011). A complete sampling of Australasian species was achieved. From the Afrotropical/Neotropical clade, 15 representatives were included, with representative sampling from the Malagasy (six species), continental African (five species) and Neotropical (four species) lineages informed by previous phylogenetic studies (Fischer et al., 2007; Smidt et al., 2011). The outgroup included representatives from *Dendrobium* (six species), which is sister to *Bulbophyllum*, as well as tribes Malaxideae (two species), Arethuseae (one species), Nervilleae (one species), and Neottieae (one species) based on previous molecular phylogenetic studies (Gorniak et al., 2010; Chomicki et al., 2015; Givnish et al., 2015). Details of the plant material studied, voucher information, and GenBank accessions are provided in Table 1.

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Table 2.1 Plant material with collection details. Sample ID indicates Australian Tropical Herbarium DNA ID (CNS) or for accessions sourced from GenBank, the GenBank accession ID. Samples for which data was generated during this study are indicated with an asterisk.

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. absconditum</i> subsp. <i>absconditum</i>	<i>Pelma</i> (Finet) Schltr. (1913)	CANB: Clements, M.A. 11242	G05247*
<i>B. acuminatum</i> (Ridl.) Ridl.	<i>Ephippium</i> Schltr. (1913)		JF428117
<i>B. affine</i> Wall. ex Lindl.	<i>Stenochilus</i> J.J.Sm. (1914)		EF195916
<i>B. alexandrae</i> Schltr.	<i>Kinethrix</i> Schltr. (1910)		EF195952
<i>B. alkmaarense</i> J.J.Sm.	<i>Coelochilus</i> Schltr. (1913)	CANB: McCraith, G. 076c	G05454*
<i>B. alsiosum</i> Ames	<i>Beccariana</i> Pfitz. (1888)		EF195917
<i>B. ambrosia</i> (Hance) Schltr.	<i>Sestochilus</i> (Breda) Benth. & Hook.f. (1883)		KC568306
<i>B. andersonii</i> (Hook.f.) J.J.Sm.	<i>Cirrhopetalum</i> (Lindl.) Rchb.f. (1861)		JN619417
<i>B. antenniferum</i> (Lindl.) Rchb.f.	<i>Hyalosema</i> (Rolfe) Schltr. (1911)	CANB: Reeve, T. 638	G05294*
<i>B. aphanopetalum</i> Schltr.	<i>Peltopus</i> Schltr. (1913)	CANB: Ziesing, P. 152	G05241*
<i>B. argyropus</i> (Endl.) Rchb.f.	<i>Adelopetalum</i> (Fitzg.) J.J. Verm. (1993)	CANB: Orchid Research Group 5613	G00333*
<i>B. armeniacum</i> J.J.Sm.	<i>Stachysanthes</i> (Blume) Averyanov (1994)		JF428112
<i>B. baladeanum</i> J.J.Sm.	<i>Hoplandra</i> J.J. Verm. (2008)	CANB: Clements, M.A. 11187	G05443*

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. baileyi</i> F.Muell.	<i>Stenochilus</i>		G03904
<i>B. baronii</i> Ridl.	<i>Ploiarium</i> Schltr. (1925)	CANB: Orchid Research Group 6242	G06040*
<i>B. biflorum</i> Teijsm. & Binn.	<i>Biflorae</i>		EF195919
<i>B. biflorum</i> Teijsm. & Binn.	<i>Biflorae</i> Garay, Hamer & Siegrist (1994)	CANB: Orchid Research Group 5887	G05442*
<i>B. boonjee</i> B.Gray & D.L.Jones	<i>Adelopetalum</i>	CANB: Jones, D.L. 4226	G03563*
<i>B. bowkettiae</i> F.M. Bailey	<i>Polymeres</i> (Bl.) J.J. Verm. & P. O'Byrne	CANB: Jones, D.L. 8697	G01618*
<i>B. bracteatum</i> (Fitzg.) F.M.Bailey	<i>Adelopetalum</i>	CNS: Simpson, L. 497	G01553*
<i>B. burfordiense</i> Garay, Hamer & Siegerist	<i>Hyalosema</i>		AY273716
<i>B. cambodianum</i> (Christenson) J.J.Verm., Schuit. & de Vogel	<i>Trias</i> (Lindl.) J.J.Verm., Schuit. & de Vogel (2014)	CANB: Orchid Research Group 3077	G05275*
<i>B. campos-portoi</i> Brade	<i>Napellii</i> Rchb.f. (1861)		GQ339721
<i>B. careyanum</i> (Hook.) Spreng.	<i>Racemosae</i> Benth. & Hook.f. (1883)		KF866244
<i>B. cariniflorum</i> Rchb.f. in W.G.Walpers	<i>Lemniscata</i>		KF866243
<i>B. caudatisepalum</i> Ames & C. Schweinf.	<i>Stachysanthes</i> (Blume) Avery. (1994)		JF428108

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Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. cauliflorum</i> Hook.f.	<i>Desmosanthes</i> (Blume) J.J.Sm. (1933)	CANB: Wallace, B.J. 20/91	G05243*
<i>B. cheiropetalum</i> Ridl.	<i>Epicranthes</i> (Blume) Benth. & Hook.f. (1883)		JF428109
<i>B. ciliatum</i> (Blume) Lindl.	<i>Hoplandra</i>	CANB: Orchid Research Group 4316	G05450*
<i>B. cirrhosum</i> L.O.Williams	<i>Bulbophyllaria</i> (Rchb.f.) Griseb. (1864)		GQ339684
<i>B. clandestinum</i> Lindl.	<i>Oxysepala</i>		JF706719
<i>B. clandestinum</i> Lindl.	<i>Oxysepala</i> (Wight) Rchb.f (1861)	CANB: Orchid Research Group 3080	G05272*
<i>B. corythium</i> N.Hallé	<i>Adelopetalum</i>	CANB: Clements, M.A. 11238	G00370*
<i>B. cruciatum</i> J.J.Sm.	<i>Codonosiphon</i> Schltr. (1911)	CBG: 8600516	G01056*
<i>B. cruentum</i> Garay, Hamer & Siegerist	<i>Macrobulbon</i> Schltr. (1912)	CANB: Orchid Research Group 5524	G06039*
<i>B. cumingii</i> (Lindl.) Rchb.f.	<i>Cirrhopetalum</i>		EF195923
<i>B. dayanum</i> Rchb.f.	<i>Acrochaene</i> (Lindl.) J.J. Verm. Schuit. & de Vogel (2014).		JF428119
<i>B. dearei</i> (Rchb.f.) Rchb.f.	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)		EF195924

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. digoelense</i> J.J.Sm.	<i>Intervallatae</i> Ridl. (1897)	CANB: Orchid Research Group 6246	G05276*
<i>B. dischidiifolium</i> J.J.Sm.	<i>Polyblepharon</i> Schltr. (1913)	CBG: 9013189	G05278*
<i>B. drymoglossum</i> Maxim.	<i>Stachysanthes</i> (Blume) Averyanov (1994)		AB786897
<i>B. echinolabium</i> J.J.Sm.	<i>Lepidorhiza</i> Schltr. (1911)	CANB: McCraith, G. 165	G05237*
<i>B. elassoglossum</i> Siegerist	<i>Polymeres</i>	CANB: Clements, M.A. 11502	G05456*
<i>B. elisae</i> (F.Muell.) Benth.	<i>Adelopetalum</i>	CNS: Simpson, L. 499	G01549*
<i>B. ericssonii</i> Kraenzl.	<i>Beccariana</i>	CANB: Orchid Research Group 6981	G05214*
<i>B. evasum</i> T.E.Hunt & Rupp	<i>Brachystachyae</i> Benth. & Hook.f. (1883),	CANB: Gray, B. 8744	G01247*
<i>B. exaltatum</i> Lindl.	<i>Didactyle</i> (Lindl.) Cogn (1902)		GQ339714
<i>B. exiguum</i> F.Muell.	<i>Adelopetalum</i>	CNS: Schulte, K. 121	G00234*
<i>B. falcatum</i> (Lindl.) Rchb.f.	<i>Megaclinium</i> (Lindl.) Summerh (1921)	HUEFS: E.C. Smidt 772	GQ339683
<i>B. fenestratum</i> J.J.Sm.	<i>Cirrhopetalum</i>	CANB: Orchid Research Group 7179	G05207*
<i>B. flavescens</i> (Blume) Lindl.	<i>Stachysanthes</i>		JF428102
<i>B. flavescens</i> (Blume) Lindl.	<i>Stachysanthes</i> (Blume) Avery. (1994)	CANB: Clements, M.A. 11548	G05280*

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Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. foetidum</i> Schltr.	<i>Beccariana</i>	CANB: Clements, M.A. 6431	G05873*
<i>B. fonsflorum</i> J.J.Verm.	<i>Macrouris</i> Schltr. (1913)	CANB: Clements, M.A. 9599	G00332*
<i>B. forrestii</i> Seidenf.	<i>Rhytionanthos</i> (Garay, Hamer & Siegerest) J.J.Verm., Schuit. & de Vogel (2014)	CANB: Orchid Research Group 1271	G06037*
<i>B. gadgarrense</i> Rupp	<i>Oxysepala</i>	CNS: Harrison, M. <i>s.n.</i>	G01602*
<i>B. gerlandianum</i> Kraenzl.	<i>Stenochilus</i> J.J.Sm. (1914)		EF195926
<i>B. globuliforme</i> Nicholls	<i>Minutissima</i> Pftiz. (1888)	CANB: McCraith, G. 86	G00392*
<i>B. gracillimum</i> (Rolfe) Rolfe	<i>Ephippium</i>	CNS: Field, A. <i>s.n.</i>	G01627*
<i>B. grandimesense</i> B.Gray & D.L.Jones	<i>Oxysepala</i>	CNS: Harrison, M. <i>s.n.</i>	G01589*
<i>B. guttulatum</i> (Hook.f.) N.P.Balacr.	<i>Brachyantha</i>		KF866245
<i>B. guttulatum</i> (Hook.f.) N.P.Balacr.	<i>Brachyantha</i> Rchb.f. (1861)	CANB: Orchid Research Group 5587	G05250*
<i>B. hamatipes</i> J.J.Sm.	<i>Leopardinae</i> Benth. & Hook.f. (1883)		EF195929
<i>B. hamelinii</i> W.Watson	<i>Alcistachys</i> Schltr. (1924)		EF195979
<i>B. hapalanthos</i> Garay	<i>Lichenophylax</i> Schltr. (1924)		EF195980
<i>B. helenae</i> (Kuntze) J.J.Sm.	<i>Rhytionanthos</i>		KF866240

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. hexarhopalon</i> Schltr.	<i>Epicranthes</i>	CANB: Clements, M.A. 7854	G00340*
<i>B. humblotii</i> Rolfe	<i>Humblotiorchis</i> Schltr. (1925)	WU: FS1008	EF195986
<i>B. inconspicuum</i> Maxim.	<i>Plumata</i> J.J.Verm., Schuit, & de Vogel (2014)		AB786895
<i>B. intertextum</i> Lindl.	<i>Cocoina</i> Pfitz. (1889)		EF195930
<i>B. intricatum</i> Seidenf.	<i>Racemosae</i>	CANB: G. McCraith 83	G05202*
<i>B. japonicum</i> (Makino) Makino	<i>Cirrhopetlaum</i>		AB786894
<i>B. johnsonii</i> T.E.Hunt	<i>Polymeres</i>	CNS: Harrison, M. s.n.	G01620*
<i>B. kaniense</i> Schltr.	<i>Macrouris</i>	CANB: Clements, M.A. 6249	G05254*
<i>B. kwangtungense</i> Schltr., Repert.	<i>Desmosanthes</i>		JN619414
<i>B. lageniforme</i> F.M.Bailey	<i>Adelopetalum</i>	CNS: Schulte, K. 95	G01630*
<i>B. lamingtonense</i> D.L.Jones	<i>Oxysepala</i>	CANB: Orchid Research Group 6945	G00812*
<i>B. lasianthum</i> Lindl.	<i>Beccariana</i>		JF428116
<i>B. lemniscatoides</i> Rolfe	<i>Lemniscata</i> Pfitz. (1888)	CANB: Orchid Research Group 7153	G05204*
<i>B. leopardinum</i> (Wall.) Lindl. ex Wall.	<i>Leopardinae</i>		JN619412
<i>B. lewisense</i> B.Gray & D.L.Jones	<i>Oxysepala</i>	CNS: Harrison, M. s.n.	G01607*

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Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. lilacinum</i> Ridl.	<i>Racemosae</i>		JF428111
<i>B. lilianae</i> Rendle	<i>Adelopetalum</i>	CNS: Schulte, K. 87	G01632*
<i>B. lindleyanum</i> Griff.	<i>Hirtula</i> Ridl. (1908), fide J.J.Verm. (2002)	CANB: Orchid Research Group 6237	G06035*
<i>B. linearifolium</i> King & Pantl.	<i>Stachysanthes</i> (Blume) Avery. (1994)		JF428103
<i>B. lineolatum</i> Schltr.	<i>Brachypus</i> Schltr. (1913)	CANB: Clements, M.A. 9580	G05262*
<i>B. lobbii</i> Lindl.	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)	CANB: Orchid Research Group 5166	G05229*
<i>B. lobbii</i> subsp. <i>siamense</i>	<i>Sestochilos</i>		EF195942
<i>B. longiflorum</i> Thouars	<i>Cirrhopetalum</i>	CANB: Orchid Research Group 5502	G06022*
<i>B. longissimum</i> (Ridl.) J.J.Sm.	<i>Cirrhopetaloides</i> Garay, Hamer & Siegerist (1994)	CANB: Orchid Research Group 5525	G06041*
<i>B. lupulinum</i> Lindl.	<i>Oreonastes</i> G.A.Fischer & J.J.Verm. (in prep.)		EF195932
<i>B. macphersonii</i> Rupp	<i>Polyblepharon</i>	CANB: Orchid Research Group 5589	G00953*
<i>B. macraei</i> (Lindl.) Rchb.f.	<i>Macrostylidia</i> Garay, Hamer & Siegerist (1994)	CANB: Clements, M.A. 12421	G06045*
<i>B. macranthum</i> Lindl.	<i>Stenochilus</i>		EF195933

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. macranthum</i> Lindl.	<i>Stenochilus</i> J.J.Sm. (1914)	CBG: 770881	G05452*
<i>B. marginatum</i> Schltr.	<i>Fruticicola</i> Schltr. (1913)		G03906*
<i>B. maxillare</i> (Lindl.) Rchb.f.	<i>Hoplandra</i>	CANB: Taylor, J.M. 284	G01299*
<i>B. maxillare</i> (Lindl.) Rchb.f.	<i>Hoplandra</i>	CANB: Jones, D.L. 3587	G05267*
<i>B. maximum</i> (Lindl.) Rchb.f.	<i>Megaclinium</i> (Lindl.) Summerh (1921)	CANB: Orchid Research Group 6250	G05289*
<i>B. membranaceum</i> Teijsm. & Binn.	<i>Scyphosepalum</i> Schltr. (1913)		JF428107
<i>B. minutissimum</i> (F.Muell.) F.Muell.	<i>Minutissima</i>	CNS: Simpson, L. 503	G01587*
<i>B. mirum</i> J.J.Sm.	<i>Plumata</i>	CANB: Orchid Research Group 5501	G06031*
<i>B. moniliforme</i> E.C.Parish & Rchb.f.	<i>Macrocaulia</i>	CANB: Clements, M.A. 9605	G03763*
<i>B. mutabile</i> (Blume) Lindl.	<i>Stachysanthes</i>		JF428101
<i>B. nagelii</i> L.O.Williams	<i>Furvescens</i> E.C.Smidt, Borba & Van den Berg (2011)		GQ339720
<i>B. nematopodum</i> F.Muell.	<i>Papulipetalum</i> Schltr. (1913)	CNS: Harrison, M. s.n.	G01612*
<i>B. newportii</i> (F.M.Bailey) Rolfe	<i>Adelopetalum</i>	CNS: Simpson, L. 495	G01565*
<i>B. nodosum</i> (Rolfe) J.J.Sm.	<i>Rhytionanthos</i>		KF866241
<i>B. ocellatum</i> Cootes & M.A.Clem.	<i>Polymeres</i>	CANB: Clements, M.A. 11603	G05293*

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Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. odoratissimum</i> (Sm.) Lindl. ex Wall.	<i>Desmosanthes</i>		KF866242
<i>B. odoratum</i> (Blume) Lindl.	<i>Stachysanthes</i> (Blume) Averyanov (1994)		JF428113
<i>B. omerandrum</i> Hayata	<i>Brachyantha</i>		JN619419
<i>B. oretopetalum</i> Garay, Hamer & Siegerist	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)		EF195936
<i>B. orientale</i> Seidenf.	<i>Racemosae</i>		JN619416
<i>B. ovalifolium</i> (Blume) Lindl.	<i>Macrocaulia</i> (Blume) Aver. (1994)		JF428104
<i>B. ovalifolium</i> (Blume) Lindl.	<i>Macrocaulia</i>	CANB: Orchid Research Group 6254	G05210*
<i>B. pallidiflorum</i> Schltr.	<i>Polymeres</i>	CANB: Clements, M.A. 11200	G05215*
<i>B. patens</i> King ex Hook.f.	<i>Stenochilus</i> J.J.Sm. (1914)		JF428100
<i>B. pecten-veneris</i> (Gagnep.) Seidenf.	<i>Cirrhopetalum</i>		JN619418
<i>B. pileatum</i> Lindl.	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)		EF195940
<i>B. putidum</i> (Teijsm. & Binn.) J.J.Sm.	<i>Cirrhopetaloides</i>	CANB: Wallace, B. 19/91	G05872*

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. pygmaeum</i> (Sm.) Lindl.	<i>Minutissima</i>	CANB: Molloy, B.P.J. 134/99	G03988*
<i>B. radicans</i> F.M.Bailey	<i>Fruticicola</i>	CNS: Schulte, K. 80	G00214*
<i>B. radicans</i> F.M.Bailey	<i>Fruticicola</i>	CANB: Lockyer, R. 6	G05228*
<i>B. reptans</i> (Lindl.) Lindl. ex Wall.	<i>Repantia</i> J.J.Verm. (2001)		JN114441
<i>B. restrepia</i> (Ridl.) Ridl.	<i>Hoplandra</i>		JF428123
<i>B. rhopalophorum</i> Schltr.	<i>Sphaeracron</i> Schltr. (1913)	CBG: 9013094	G05261*
<i>B. roseopictum</i> J.J.Verm., Schuit. & de Vogel	<i>Sunipia</i> (Lindl.) J.J.Verm., in prep.	CANB: Clements, M.A 12085	G03907*
<i>B. sauguetiense</i> Schltr.	<i>Papulipetalum</i>	CANB: Clements, M.A. 7235	G05258*
<i>B. saurocephalum</i> Rchb.f.	<i>Saurocephalum</i> Schltr. (1912)	CANB: Clements, M.A. 11496	G05212*
<i>B. schillerianum</i> Rchb.f.	<i>Oxysepala</i>	CANB: Orchid Research Group 6328	G00374*
<i>B. schinzianum</i> Kraenzl.	<i>Ptiloglossum</i> Lindl. (1862)	CANB: Clements, M.A. 11554	G01642*
<i>B. shepherdii</i> (F.Muell.) Rchb.f.	<i>Oxysepala</i>		G00324*
<i>B. smitinandii</i> Seidenf.	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)		EF195943
<i>B. tianguii</i> K.Y.Lang & D.Luo	<i>Cirrhopetalum</i>		JN619415
<i>B. triaristella</i> Schltr.	<i>Micromonanthe</i> Schltr. (1913)	CANB: G.McCraith 080	G01202*

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Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>B. tripudians</i> C.S.P.Parish & Rchb.f.	<i>Tripudianthes</i> Seidenf. (1979)	CANB: Orchid Research Group 3081	G05249*
<i>B. tuberculatum</i> Colenso	<i>Adelopetalum</i>	CHR: 572204	CHR572204
<i>B. uniflorum</i> (Blume) Hassk.	<i>Beccariana</i>		JF428098
<i>B. ventriosum</i> H.Perrier	<i>Ploiarium</i> Schltr. (1916)		EF196075
<i>B. wadsworthii</i> Dockrill	<i>Oxysepala</i>	CNS: Kilgour, C.D. 1143	G00574*
<i>B. wakoi</i> Howcroft	<i>Beccariana</i>	CANB: Orchid Research Group 7176	G05222*
<i>B. wallichii</i> Rchb.f.	<i>Tripudianthes</i> Seidenf. (1979)		JN619411
<i>B. weinthalii</i> R.S. Rogers	<i>Adelopetalum</i>	CANB: Orchid Research Group 7186	G03564*
<i>B. windsorensense</i> B.Gray & D.L.Jones	<i>Oxysepala</i>	CNS: Harrison, M. s.n.	G01609*
<i>Coelogyne flaccida</i> Lindl.		WIS: v0289172	n/a
<i>Dendrobium brunneum</i> Schuit. & Peter B.Adams		CANB: Orchid Research Group 5485	G00781
<i>Dendrobium cunninghamii</i> Lindl.		CANB: Molloy, B.P.J. 061/98	G02336
<i>Dendrobium fuscescens</i> (Griff.) Summerh.		CANB: Orchid Research Group 6920	G01021
<i>Dendrobium macropus</i> (Endl.) Rchb.f.		CANB: Ziesing, P.D. 345	G02316

Species name	<i>Bulbophyllum</i> section	Voucher details	Sample ID
<i>Dendrobium moniliforme</i> (L.) Sw.		CANB: Clements, M.A. 8262	G00882
<i>Dendrobium muricatum</i> Finet		CANB: Clements, M.A. 3093	G02350
<i>Dienia ophrydis</i> (J.Koenig) Seidenf.		CBG:9306410	G01303
<i>Neottia cordata</i> (L.) Rich.		CANB: Clements, M.A. 9865	G05314
<i>Nervilia concolor</i> (Blume) Schltr.		CANB: Roberts, L. ORG3787	G03442
<i>Oberonia complanata</i> (A.Cunn.) M.A.Clem. & D.L.Jones		CNS:135322	G00309

2.2.2. DNA extraction, amplification, sequencing and alignment

Total genomic DNA was extracted from ca. 10–20 mg silica dried leaf material. Extractions were carried out with commercial extraction kits (Qiagen DNeasy plant kit, Venlo, Netherlands; ChargeSwitch gDNA Plant Kit, Invitrogen, Carlsbad, USA) or using the CTAB method (Doyle and Doyle, 1990) with modifications as described in Weising et al. (2005).

2.2.2.1. Sanger sequencing

Nuclear ITS sequences were generated for 66 *Bulbophyllum* samples, including representatives for 87% of the Australasian species, and five *Dendrobium* species. The nuclear Internal Transcribed Spacer (ITS) was amplified with primers 17F and 26SER (Sun et al., 1994). PCR reactions were carried out in 20 µL volumes consisting of 2.5 µL PCR buffer (200mM Tris HCl pH 8.4, 500mM KCl), 0.5 µL MgCl (25mM), 0.5 µL each of forward and reverse primer (10µM), 0.5 µL dNTPs (10mM), 1 µL DMSO, 0.9 µL BSA, 0.4 µL KAPA Taq DNA polymerase (5U/µL) (Kapa Biosystems, Wilmington, USA), 10.7 µL H₂O and 2.5 µL gDNA (ca. 5 ng/µL). A touchdown PCR was carried out with an initial denaturation step at 94 °C for 2 min, followed by 7 cycles of 94 °C denaturation for 45 sec, 66 °C annealing for 45 sec (reducing 1 °C at each cycle) and 72 °C extension for 1 min and 30 sec, with a final extension at 72 °C for 5 min. PCR products were cleaned using exonuclease (ExoI) and alkaline phosphatase (FastAP) (Thermo Fisher Scientific, Waltham, USA) using 7.5 µL of PCR product, 0.25 µL of ExoI, 1 µL of FastAP and 1.25 µL of H₂O incubated for 15 mins at 37 °C, followed by 15 mins at 85 °C. Sequencing reactions were carried out using the amplification primers and sequencing was conducted on an AB3730xl 96-capillary sequencer (Australian Genome Research Facility, Brisbane, Australia). Sequences were assembled and edited in Geneious R10 (Kearse et al., 2012) and aligned using the MAFFT plug-in (Kato et al., 2002; 2005; 2013) including 67 *Bulbophyllum* sequences sourced from GenBank.

2.2.2.2. Illumina sequencing

To further clarify higher level phylogenetic relationships among Asian and Australasian sections, 64 orchid species were sampled for genome skimming to recover the high copy plastome and nuclear ribosomal regions. From *Bulbophyllum*, 50 Asian and Australasian species were sampled representing 39 sections (approximately 55% of Asian/Australasian sections recognised by Pridgeon et al., 2014). Libraries were constructed with 100–300 ng total DNA using the TruSeq Nano DNA LT library preparation kit (Illumina, San Diego, USA) for an insert size of 350 base pairs (bp) and paired-end reads following the manufacturer's protocol. Libraries were multiplexed 96 times and DNA sequencing with 125 bp paired-end reads was

carried out on an Illumina HiSeq 2500 platform at the Australian Genomic Research Facility, Melbourne (Australia). Reads were filtered for quality, trimmed of Illumina adapters and demultiplexed by the Australian Genomic Research Facility. Demultiplexed sequence reads were mapped in Geneious R10 (Kearse et al., 2012) to a reference plastid gene set from *Dendrobium officinale* (GenBank accession numbers KJ862886 and KC771275), *Anoectochilus roxburghii* (KP776980) and *Goodyera fumata* (KJ501999). For the nuclear ribosomal ITS region, sequence reads were assembled against the ITS region of *Dendrobium speciosum* (GenBank accession number KC811532). Mapping was carried out with the Geneious mapper using custom sensitivity, iterating up to five times, without trimming. Gaps were allowed with a maximum gap size of 75bp, a maximum of 25% per read, a maximum of 20 mismatches per read and ignoring words repeated more than 12 times. The quality of the assemblies was checked and edited manually where required. Consensus sequences were generated specifying the highest quality threshold and a minimum coverage of ten reads. Alignments of consensus sequences were carried out for each marker separately using MAFFT v.7.222 (Katoh et al., 2002; 2005; 2013) with the default settings. ITS regions were aligned together with sequences generated with Sanger sequencing and accessions sourced from GenBank and DRYAD (<https://doi.org/10.5061/dryad.n9r58>) for *Coelogyne flaccida* (Givnish et al., 2015). Alignments of all coding sequences were checked for regions that were poorly assembled and edited manually where required. Plastid coding regions *ndhA-K* and *psbG* were excluded due to poor coverage or assembly and satisfactory alignments were concatenated into a single plastid alignment. The nuclear ITS data set included 161 taxa and 1003bp with 842 variable sites (70%). The plastid dataset included 70 coding regions for 64 taxa and was 61420bp with 25244 variable sites (41%). Plastid coding regions included in the dataset were: *accD*, *atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *atpI*, *ccsA*, *cemA*, *clpP*, *infA*, *matK*, *orf42*, *petA*, *petB*, *petD*, *petG*, *petL*, *petN*, *psaA*, *psaB*, *psaC*, *psaI*, *psaJ*, *psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*, *rbcL*, *rpl2*, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl23*, *rpl32*, *rpl33*, *rpl36*, *rpoA*, *rpoB*, *rpoC1*, *rpoC2*, *rps2*, *rps3*, *rps4*, *rps7*, *rps8*, *rps11*, *rps12*, *rps14*, *rps15*, *rps16*, *rps18*, *rps19*, *ycf1*, *ycf2*, *ycf3*, *ycf4*, and *ycf68*.

2.2.3. Phylogenetic analysis

Phylogenetic relationships were carried out independently for the plastid and nuclear alignments using maximum likelihood (ML) and Bayesian inference. Reconstructions were carried out based on a GTR gamma substitution model identified among the top performing models in tests carried out in MrModeltest2 v.2.3 (Nylander, 2004) on preliminary plastid and nuclear datasets (Appendix 2.1). ML analyses were carried out using the program RAxML v.

8.2.4 (Stamatakis, 2014) applying the rapid hill climbing algorithm with a GTR gamma nucleotide substitution model. Bootstrap analyses were conducted with the same settings based on 100 bootstrap replicates. Bayesian analyses were carried out using MrBayes 3.2.5 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) applying a GTR gamma model with datasets partitioned into nuclear versus plastid regions. Three independent MCMC runs with three heated chains each were carried out for 5 million generations sampling trees every 10 000 generations. Convergence of independent runs was confirmed by the average standard deviation of split frequencies dropping below 0.01 for the plastid dataset and 0.03 for the nuclear dataset. Bayesian all compatible consensus trees were constructed with 2.5 million generations discarded as burn-in and posterior probability values of nodal support.

2.2.4. Divergence time estimation

Divergence times were estimated from the 70 plastid cds dataset applying a relaxed lognormal clock (Drummond et al., 2006) as implemented in BEAST2 v. 2.4.8 (Bouckaert et al., 2014) using an GTR gamma substitution model and a birth death prior on speciation and extinction, conditional on the root of the tree (Gernhard, 2008). Three secondary calibration points were applied based on the results of a family wide molecular clock analysis by Chomicki et al. (2015). To account for uncertainty in the ages determined in the analysis of Chomicki et al., these priors were set with a normal distribution with the mean and sigma set to replicate the 95% higher posterior density (HDP) intervals estimated in their study. The root age was set to 55.02 Ma (HDP: 42.0–68.0). The next secondary calibration point was applied to the last common ancestor of Dendrobiinae, Malaxideae, and Arethuseae and was set to 47.77 Ma (HDP: 36.4–59.1). Monophyly was constrained for this node consistent with relationships reconstructed and previous phylogenetic analyses (Chomicki et al. 2015; Givnish et al., 2015). The last secondary calibration was set at the stem node of Dendrobieae and Malaxideae with 38.68 Ma (HDP: 30.8–46.6). An additional calibration based on the fossil *Dendrobium winikaphyllum* (Conran et al., 2009) was applied to the stem node of the Australasian *Dendrobium* clade (*D. macropus*, *D. cunninghamii*, and *D. muricatum*), using a uniform distribution with an infinite maximum age and the minimum age constrained to 20.4 Ma, based on the minimum age estimated for the strata containing the fossil (Lindqvist and Lee, 2009; Mildenhall et al., 2014). Ten independent BEAST analyses were run for 30 million MCMC generations, with trees sampled every 1×10^4 generations. To assess convergence of independent runs and determine burn in fractions, log files were assessed in Tracer v.1.5.0 (Rambaut and Drummond, 2007), ensuring the effective sample size of all parameters was at least above 170. Trees from each of the ten runs were combined in LogCombiner (from the BEAST package) with a sampling

frequency of 6000, excluding a cumulative burn in fraction of 19% (run 1 20%, run 2 20%, run 3 17%, run 4 37%, run 5 23%, run 6 18%, run 7 18%, run 8 18%, run 9 8%, run 10 13%). A maximum clade credibility tree was generated in TreeAnnotator (BEAST package) with median node heights.

2.2.5. Ancestral area analysis

Species ranges were determined based on distributions recorded in the world checklist for selected plant families (WCSP, 2018). Six geographic regions were delineated in the ancestral area estimation, largely based on the floristic continents defined in the world geographic scheme for recording plant distributions (WGSRPD) (Brummitt, 2001). The geographic areas defined for the analysis were: 1) Africa, 2) temperate Asia, 3) tropical Asia, 4) Papuasias, 5) Australasia and 6) Pacific. Africa, temperate Asia, Australasia and Pacific correspond to the floristic continents defined in the WGSRPD. The region Papuasias including New Guinea, the Solomon Islands and the Bismarck archipelago, is distinguished from its floristic continent of tropical Asia based on the relatively recent geological history of the Papuasias region (Hall et al., 2011). Ancestral ranges were reconstructed in RASP v 4.0 (Yu et al., 2015) based on 100 randomly chosen maximum clade credibility trees from the BEAST analysis of the plastid alignment, pruned of the outgroups to Dendrobieae. Ancestral ranges were estimated based on 1) a statistical dispersal, vicariance approach S-DIVA (Yu et al., 2010), and 2) a statistical dispersal extinction cladogenesis model (S-DEC) (Beaulieu et al., 2013), replicating the key assumptions outlined in the non-parametric dispersal vicariance approach (Harris and Xiang, 2009; Nylander et al., 2008; Ronquist, 2001, 1997; Ronquist and Sanmartín, 2011) and the dispersal extinction cladogenesis model (DEC) (Ree and Smith, 2008), and also take into account uncertainty in the tree topology. No constraints were applied to dispersal direction or the maximum number of ranges allowed at ancestral nodes. To determine the model of best fit, model testing was carried out in the program RASP using the package BioGeoBEARS (Matzke, 2013a) and the resulting likelihood values of the DIVALIKE and DEC models were compared based on the AIC model weights (Akaike, 1974).

2.3. Results

2.3.1. Sectional relationships based on nuclear ITS sequences

2.3.1.1. Relationships among the major clades

Maximum likelihood and Bayesian phylogenetic reconstructions based on the nuclear ITS region reconstructed Malaxideae (BS 99, PP 1) and Dendrobieae (BS 77, PP 1) in a sister group relationship (BS 76, PP 0.89) (Fig. 2.1, Appendix 2.2). Within Dendrobieae, all *Bulbophyllum* taxa formed a highly supported clade (BS 94, PP 1), resolved in a sister group relationship to Dendrobiinae (BS 61, PP 0.98). Within *Bulbophyllum*, both ML (Fig. 2.1) and Bayesian (Appendix 2.2) analyses resolved three major clades: 1) an Australasian clade, 2) an Afrotropical/Neotropical clade and 3) an Asian clade. The Afrotropical/Neotropical clade (BS 85, PP 0.99) was composed of a Madagascan clade (BS 71, PP 1), resolved in a sister group relationship to a highly supported clade continental African/Neotropical clade (BS 97, PP 1). The continental African/Neotropical clade was split into a Neotropical clade (BS 71, PP 0.94) and a continental African clade (BS 97, PP 1). The Australasian clade included all species sampled from section *Adelopetalum* and sect. *Minutissima* species from Australia and New Zealand. However, the Australasian clade remained unsupported on both ML and Bayesian reconstructions (BS <50, PP < 0.50). Within the Australasian clade several highly supported clades were resolved, however, relationships between these groups were either unresolved or received low support. The Asian clade was composed of predominantly Asian taxa but also included taxa from Australia and the south west Pacific region. However, the Asian clade was also unsupported or poorly supported in the ML and Bayesian analyses (BS <50, PP 0.77).

2.3.1.2. Relationships within the Asian clade

Within the Asian clade, clades a-i were resolved with high support (BS >90 or PP >0.95), while five species were not resolved in a supported relationship with any other taxa, *B. roseopictum* (sect. *Sunipia*), *B. reptans* (sect. *Repantia*), *B. dayanum* (sect. *Acrochaene*), *B. ovalifolium* (sect. *Macrocaulia*) and *B. leopardinae* (sect. *Leopardinae*). Higher level relationships among these groups remained largely unclear due to lack of support. Clade a (BS 72, PP 0.98) included sect. *Coelochilus*, sect. *Fruticicola*, sect. *Macrocaulia*, sect. *Macrouris*, sect. *Pelma*, sect. *Saurocephalum*, sect. *Scyphosepalum*, sect. *Sphaeracron*, sect. *Hoplandra*, sect. *Macrobulbon*, sect. *Micromonanthae*, sect. *Oxysepala*, sect. *Polyblepharon*, sect. *Polymeres*, sect. *Epicranthes*, sect. *Hyalosema*, sect. *Peltopus*, sect. *Papulipetalum*, sect. *Brachypus* and sect. *Codonosiphon*. Clade b (BS 62, PP 0.98) was composed of sections *Trias* and *Biflorae*. Clade c (BS 100, PP 1) included section *Cirrhopetaloides*. Clade d (BS 100, PP 1) was composed of

sections *Cirrhopetalum* and *Ehippium*. Clade e (BS 98, PP 1) included sections *Macrostylida*, *Cirrhopetalum* and *Brachyantha*. Clade f (BS 66, PP 1) was composed of sections *Cirrhopetalum*, *Plumata*, *Desmonanthes*, *Rhytionanthos* and *Sestochilus*. Clade g (BS 100, PP 1) included sections *Lemniscata* and *Tripudianthes*. Clade h (BS 100, PP 1) was composed of section *Racemosae*. Clade i (BS <50, PP 0.99) included sections *Brachystachyae*, *Hoplandra*, *Hirtula*, *Stachysanthes*, *Beccariana*, *Leopardinae*, *Intervallatae*, *Lepidorhiza*, *Sestochilus*, and *Stenochilus*. Sections identified as polyphyletic included *Cirrhopetalum*, *Desmosanthes*, *Rhytionanthos*, *Hoplandra*, *Leopardinae*, *Macrocaulia Polymeres* and *Sestochilus*. Section *Lemniscata* was reconstructed as paraphyletic with section *Tripudianthes*, nested within clade g. Section *Beccariana* was rendered paraphyletic with *B. hamatipes* (sect. *Leopardinae*) nested within clade i2, while section *Stenochilus* was rendered paraphyletic with *B. ericssonii* (sect. *Beccariana*) and *B. patens* (sect. *Sestochilus*) nested within clade i4.

Figure 2.1 Maximum likelihood phylogeny of 145 Bulbophyllum taxa reconstructed from the nuclear ITS region. Bootstrap values >50% are displayed near nodes. Species distributions in Asia and Australasia are coded yellow: temperate Asia; green: tropical Asia; purple: Papuaasia; blue: Australasia; red: South West Pacific.

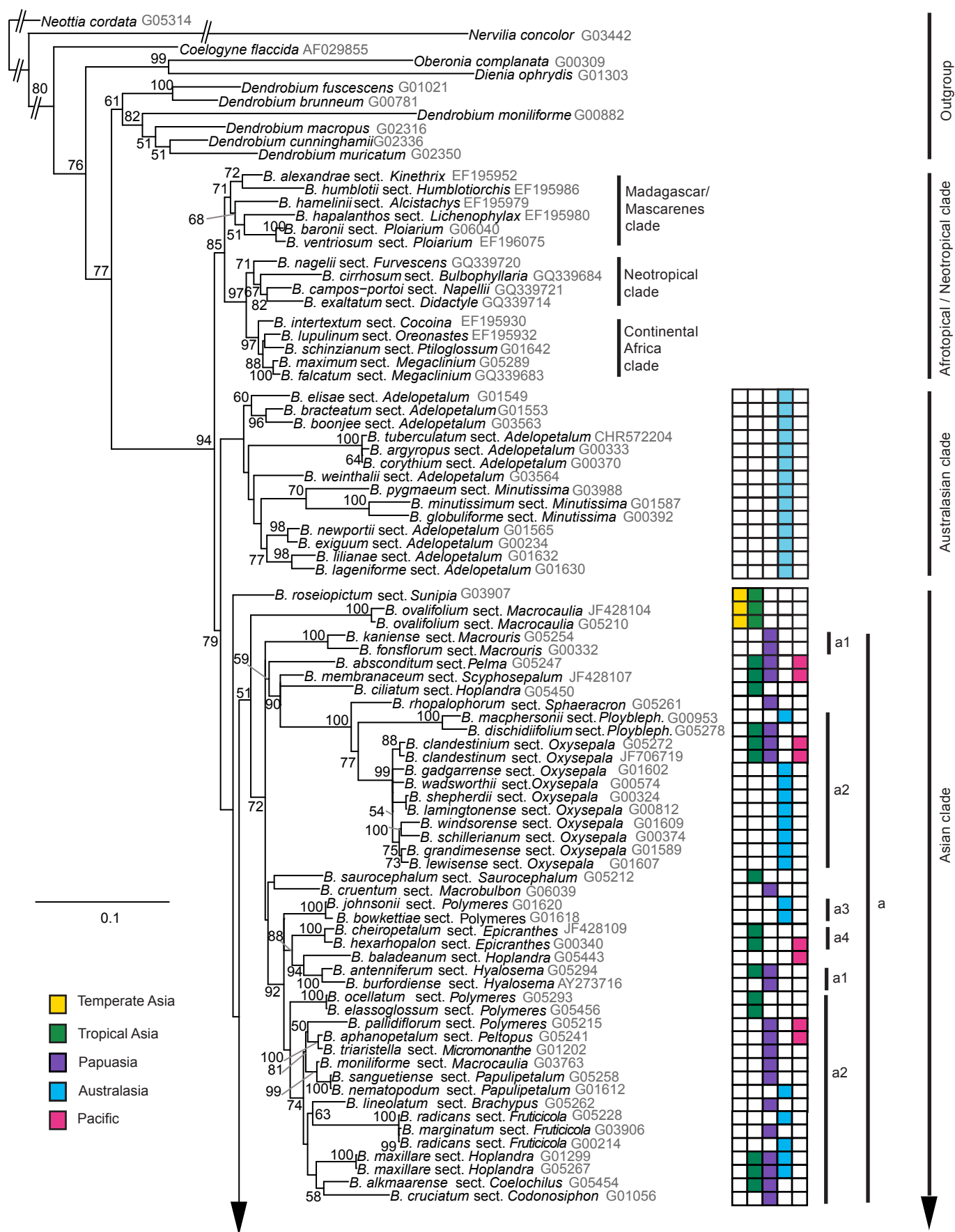


Fig.2.1

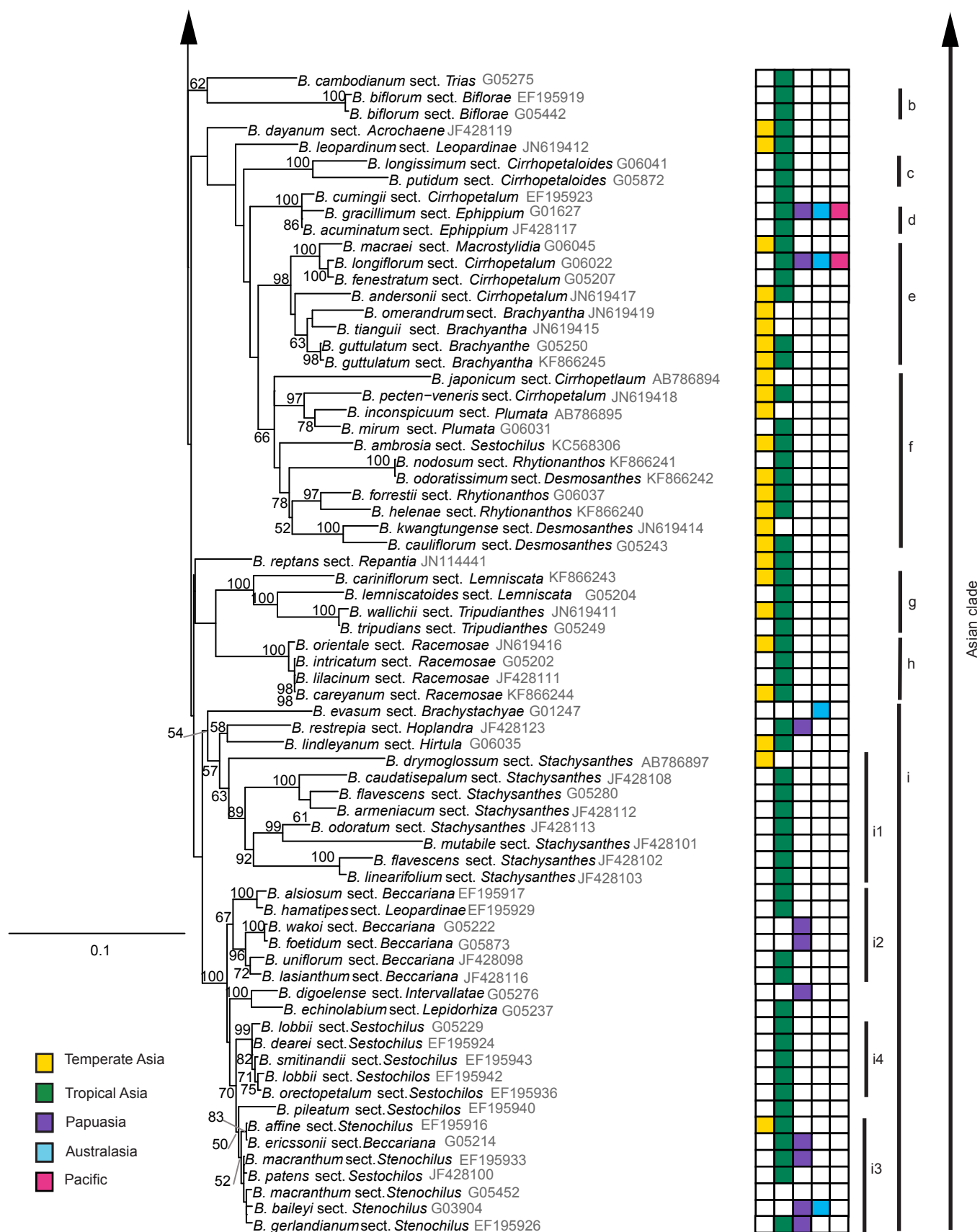


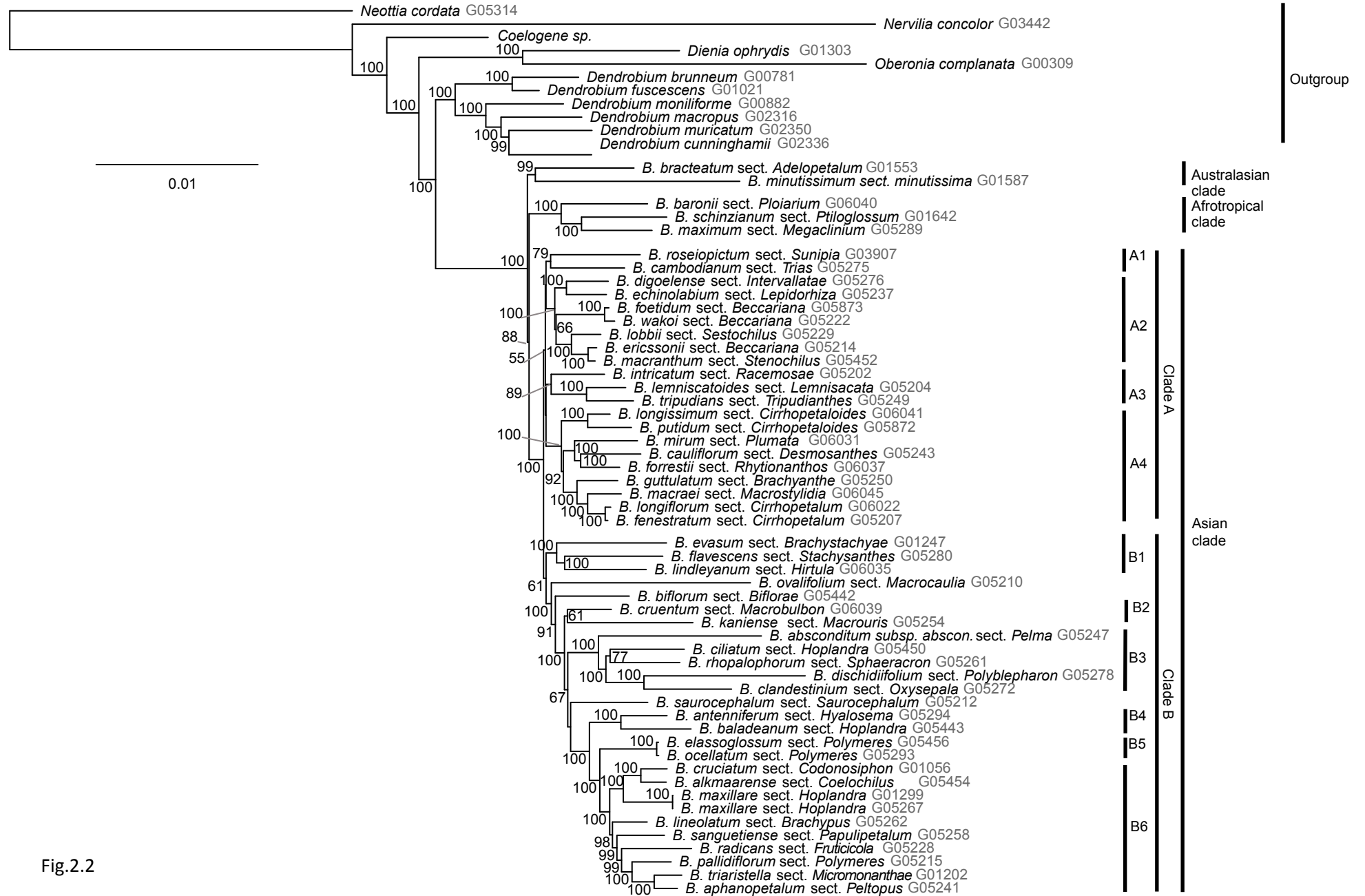
Fig.2.1

2.3.2. Sectional relationships reconstructed from 70 plastid markers

2.3.2.1. *Relationships among the major clades*

The ML and Bayesian phylogenies based on the 70-plastid locus dataset were highly resolved with congruent topology (Fig. 2, Appendix 2.3). Malaxideae and Dendrobieae were reconstructed in a sister group relationship (BS 100, PP 1) and both clades received maximum support. Within Dendrobieae, the sister group relationship between a highly supported Dendrobieae (BS 100, PP 1) and a highly supported Bulbophyllinae (BS 100, PP 1) received maximum support. Within *Bulbophyllum*, a basal split was resolved between the Australasian clade and the remainder of the genus. The highly supported Australasian clade (99/1) included Australian species *B. minutissimum* (sect. *Minutissima*) and *B. bracteatum* (sect. *Adelopetalum*). The remainder of the genus formed a moderately supported clade (BS 88, PP 0.98) that split into an Afrotropical clade and an Asian clade, which both received maximum support. Within the Afrotropical clade the Madagascan species (sect. *Ploiarium*, *B. baronii*) were reconstructed as the sister group to a highly supported clade (BS 100, PP 1) including two species from continental Africa (sect. *Megaclinium*: *B. maximum* and sect. *Ptiloglossum*: *B. schinzianum*). The Asian clade was mainly composed of taxa occurring in temperate and tropical Asia, including Papuasias. However, this lineage also included taxa with distributions extending to, or endemic to the Afrotropics (sect. *Cirrhopetalum*: *B. longiflorum*) Australasia (sect. *Fruticicola*: *B. radicans*; sect. *Brachystachyae*: *B. evasum*; sect. *Hoplandra*: *B. maxillare*; sect. *Cirrhopetalum*: *B. longiflorum*) and the Pacific (sect. *Peltopus*: *B. aphanopetalum*; sect. *Polymeres*: *B. pallidiflorum*; sect. *Hoplandra*: *B. baladeanum*; sect. *Oxysepala*: *B. clandestinum*; sect. *Pelma*: *B. absconditum* subsp. *absconditum*; sect. *Cirrhopetalum*: *B. longiflorum*).

Figure 2.2 Maximum likelihood phylogeny of 53 *Bulbophyllum* species reconstructed from 70 plastid coding regions. Bootstrap values >50% are displayed near nodes.



2.3.2.2. Relationships within the Asian clade

The Asian clade displayed a basal dichotomy between clade A and clade B which both received low bootstrap support and maximum posterior probabilities. Within clade A (BS 55, PP 1) four lineages were resolved with moderate to high support, clade A1–A4. Clade A1 (BS 79, PP 1) included sect. *Trias* (*B. cambodianum*) and sect. *Sunipia* (*B. roseopictum*). Clade A2 (BS 100, PP 1) comprised three highly supported clades which each received maximum support. However, relationships between them were poorly supported. The first clade included sect. *Lepidorhiza* (*B. echinolabium*) and sect. *Intervallatae* (*B. digoelense*), the second clade harboured sect. *Beccariana* (*B. foetidum*, *B. wakoi*) and the third clade comprised sect. *Sestochilus* (*B. lobbii*), sect. *Stenochilus* (*B. macranthum*) and sect. *Beccariana* (*B. ericssonii*). Clade A3 (BS 100, PP 1) included sect. *Racemosae* (*B. intricatum*), sect. *Tripudianthes* (*B. tripudians*) and sect. *Lemniscata* (*B. lemniscatoides*). Clade A4 comprised three highly supported clades, and each received maximum support. The clade including sect. *Cirrhopetaloides* (*B. putidum*, *B. longissimum*) was reconstructed in a sister group to a highly supported group (BS 92, PP 1), comprising one clade including sect. *Plumata* (*B. mirum*), sect. *Rhytionanthos* (*B. forrestii*) and sect. *Desmonanthes* (*B. cauliflorum*) and another harbouring sect. *Brachyantha* (*B. guttulatum*), sect. *Macrostylidia* (*B. macraei*), and sect. *Cirrhopetalum* (*B. longiflorum*, *B. fenestratum*). Relationships among clades A1–A4 were resolved with low support (BS < 50 or PP < 0.7).

Clade B (BS 61, PP 1) was divided into two highly supported lineages, clade B1 and B2. Clade B1 (BS 100, PP 1) comprised sect. *Brachystachyae* (*B. evasum*), sect. *Hirtula* (*B. lindleyanum*) and sect. *Stachysanthes* (*B. flavescens*). *B. ovalifolium* (sect. *Macrocaulia*) was resolved in a sister group relationship (BS 100, PP 1) to a clade including *B. biflorum* (sect. *Biflorae*), *B. saurocephalum* (sect. *Saurocephalum*) and clades B2–B6 (BS 91, PP 1). Clade B2 (BS 61, PP 0.89) included *B. kaniense* (sect. *Macrouris*) and *B. cruentum* (sect. *Macrocaulia*). Clade B3–B6 each reached maximum support. Clade B3 comprised sect. *Pelma* (*B. absconditum* subsp. *absconditum*) and a highly supported clade including sect. *Oxysepala* (*B. clandestinum*) and sect. *Polyblepharon* (*B. dischidiifolium*), sect. *Sphaeracron* (*B. rhopalophorum*) and sect. *Hoplandra* (*B. ciliatum*). Clade B4, B5 and B6 together formed a lineage which received maximum support. Clade B4 diverged first including sect. *Hoplandra* (*B. baladeanum*) and sect. *Hyalosema* (*B. antenniferum*), while clade B5 and B6 were resolved as a highly supported sister group (BS 100, PP 1). Clade B5 included sect. *Polymeres* (*B. occellatum*, *B. elassoglossum*). Clade B6 was composed of sect. *Codonosiphon* (*B. cruciatum*), sect. *Coelochilus* (*B.*

alkmaarensense), sect. *Brachypus* (*B. lineolatum*), sect. *Papulipetalum* (*B. sanguetiense*), sect. *Polymeres* (*B. pallidiflorum*), sect. *Fruticicola* (*B. radicans*), sect. *Hoplandra* (*B. maxillare*), sect. *Peltopus* (*B. aphanopetalum*), and sect. *Micromonanthae* (*B. triaristella*).

Thus, sections *Beccariana*, *Hoplandra* and *Polymeres* were resolved as polyphyletic.

2.3.3. Divergence dating

Based on divergence time estimation, *Bulbophyllum* diverged from *Dendrobium* in the early Oligocene, ca. 33.1 Ma (95% highest probability density, HPD: 27.5–39.1 Ma) (Fig. 3). Crown diversification of *Bulbophyllum* commenced in the late Oligocene, ca. 23.7 Ma (HPD: 17.8–30 Ma) with the divergence of the Australasian clade from the remainder of the genus.

Divergence between the Afrotropical clade and Asian clade was estimated to late Oligocene, ca. 23.2 Ma (HPD: 17.7–29.7 Ma). Crown divergence within the Asian and Afrotropical clades was estimated to have begun during the early Miocene. Within the Afrotropical clade crown diversification commenced ca. 16.4 Ma (HPD: 10.8–23.0 Ma). Within the Asian clade, divergence between the two major clades A and Clade B was dated to ca. 20.3 Ma (HPD: 15.4–26 Ma). Divergence ages between sections within the Asian clade were reconstructed mainly during the Miocene.

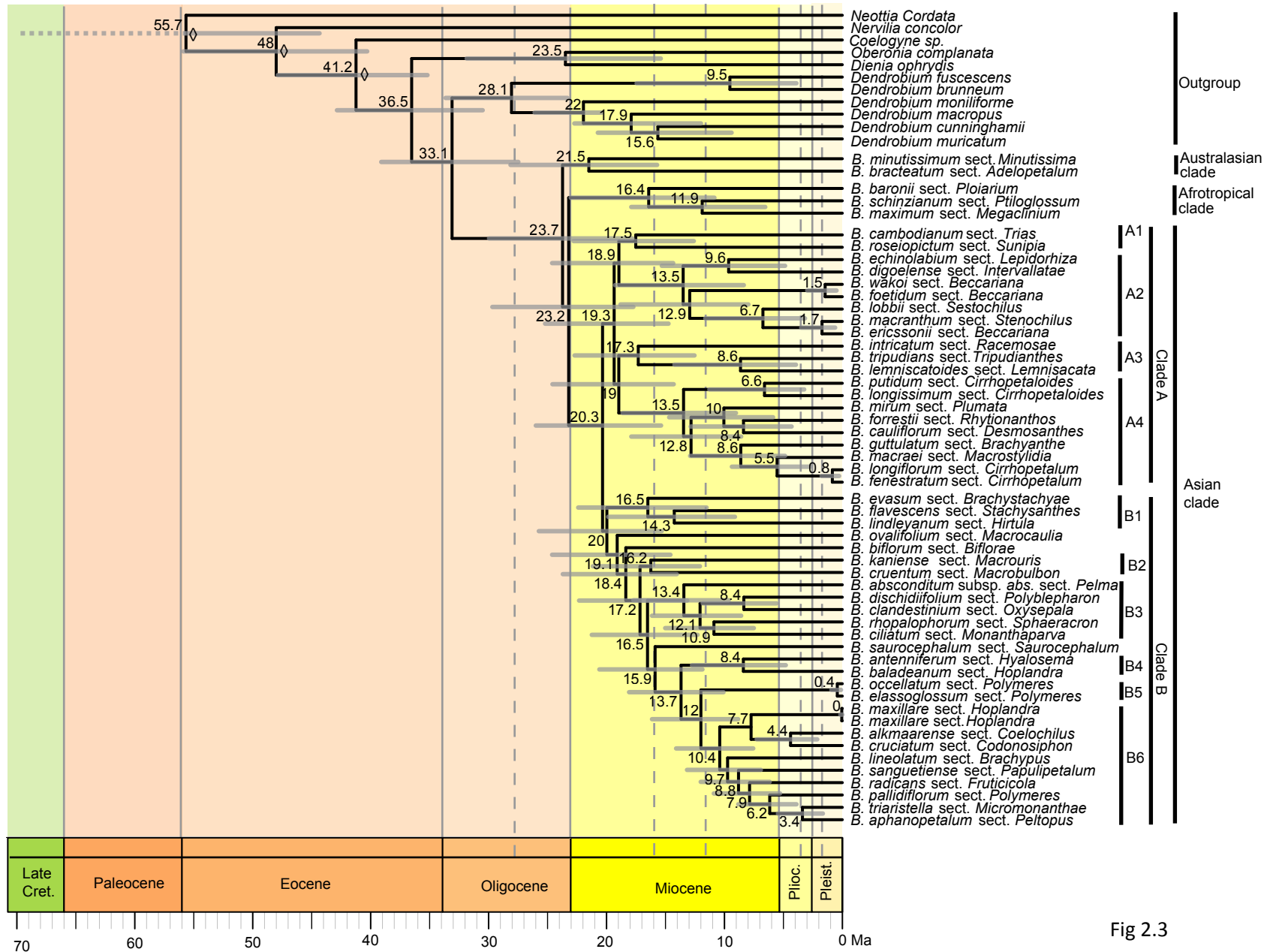


Fig 2.3

Figure 2.3 Maximum clade credibility chronogram for 53 Bulbophyllum species based on 70 plastid coding regions. Divergence dates and 95 % highest posterior density values are indicated adjacent to nodes. Grey bars indicate 95% highest posterior density. The asterisk denotes the node constrained with a fossil calibration point, the diamond shape denotes nodes which were constrained by secondary calibration points.

2.3.4. Ancestral area estimation

The AIC model test identified the dispersal, extinction, cladogenesis model (DEC) as the best fitting model yielding the lowest AIC score (360.2), followed by the dispersal-vicariance model (DIVALIKE) with an AIC score of 376.7 (Appendix 2.4). The results from the ancestral range reconstruction based on the DEC model are presented below. Results from the reconstruction based on the DIVALIKE model are provided in Appendices 2.4 and 2.7.

2.3.4.1. *Spatio-temporal evolution among the major biogeographic lineages*

The ancestral range of *Bulbophyllum* remained unresolved in the ancestral area estimation. The most likely ancestral range inferred for the genus was a widespread distribution across the Afrotropics, tropical Asia and Australasia (relative probability (rel. prob.) 0.97) (Fig. 2.4, Appendices 2.5, 2.6). Australia was reconstructed as the ancestral area of the Australasian clade and was conserved in both representatives of the clade. The most likely ancestral range reconstructed for the Afrotropical/Asian lineage was a widespread distribution in the Afrotropics and Tropical Asia (rel. prob. 0.84). The Afrotropics was reconstructed as the ancestral area of the Afrotropical clade and was conserved for all representatives of the clade. The most likely ancestral area inferred for the Asian lineage was tropical Asia (rel. prob. 0.84). Within the Asian clade, tropical Asia was also reconstructed as the most likely ancestral range for the two main clades, A (rel. prob. 1) and B (rel. prob. 0.76). The alternative ancestral range inferred for clade B was a widespread distribution across tropical Asia and Australasia (rel. prob. 0.24) (Fig. 2.4, Appendices 2.5, 2.6).

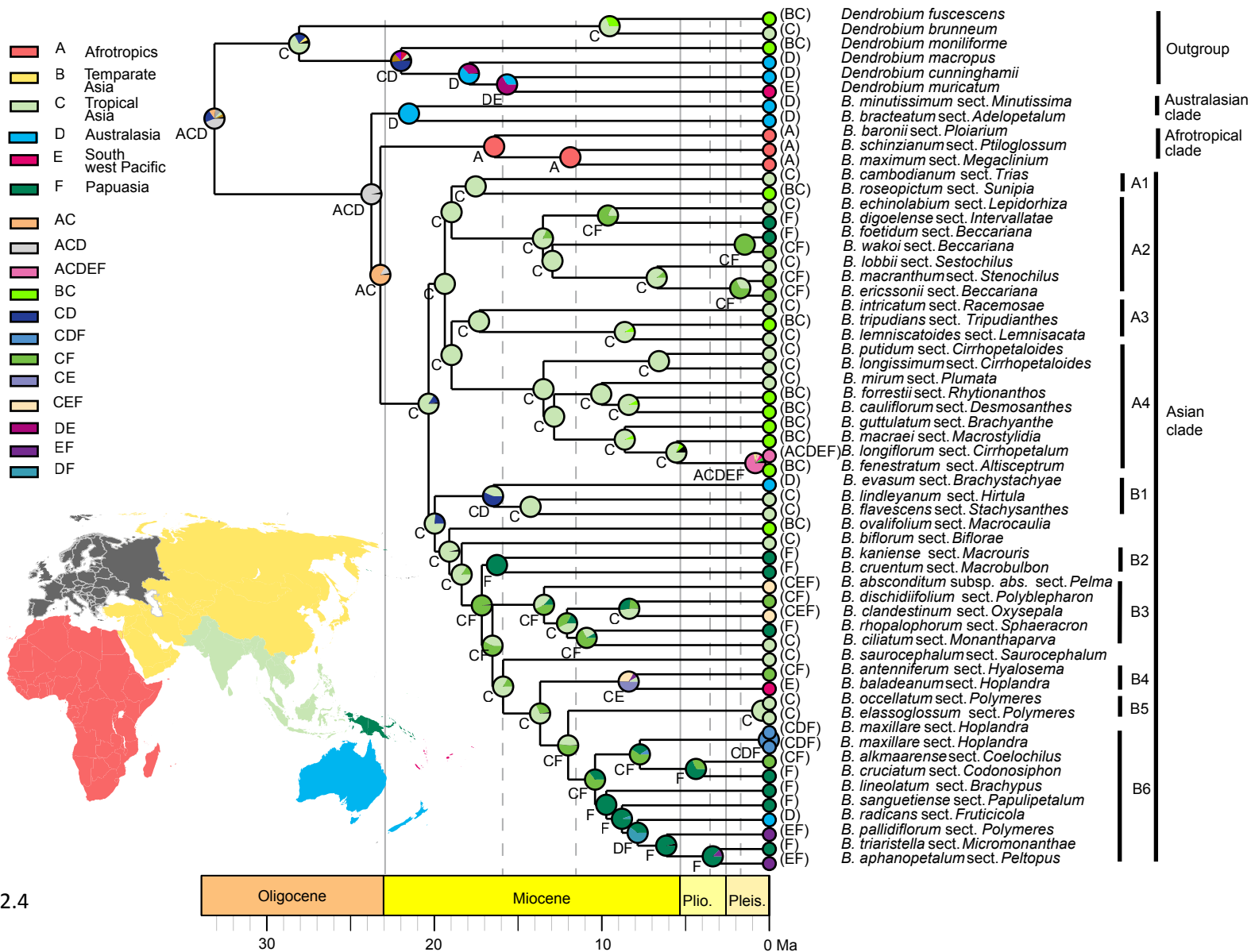


Fig 2.4

Figure 2.4 Ancestral area reconstruction for 53 *Bulbophyllum* species inferred from the DEC model of range evolution.

2.3.4.2. Spatio-temporal evolution within Asian clade A

Within Clade A, diversification mainly took place within the ancestral tropical Asian region. Tropical Asia is reconstructed as the most likely ancestral range for MRCA of the four sub-clades within clade A, A1–A4 (Fig. 2.4, Appendices 2.5, 2.6). Within Clade A, the ancestral range analysis reconstructed at least eight independent range expansions from tropical Asia to temperate Asia within clades A1, A3 and A4 from the early Miocene onwards. At least one range expansion from tropical Asia to temperate Asia was inferred within clade A1, in the lineage that gave rise to sect. *Sunipia* (*B. roseopictum*), from the early Miocene ca. 17.5 Ma. From the late Miocene, range expansion from tropical Asia to temperate Asia was inferred at least once within clade A3, in the lineage giving rise to *B. tripudians* (sect. *Tripudianthes*) after ca. 8.6 Ma, and at least four times within clade A4 in the lineages giving rise to 1) *B. guttulatum* (sect. *Brachyantha*) after ca. 8.6 Ma, 2) *B. cauliflorum* (sect. *Desmonanthes*) after ca. 8.4, 3) *B. forrestii* (sect. *Rhytionanthos*) after ca. 8.4 and 4) *B. macraei* (sect. *Macrostylidia*) after ca. 5.5 Ma.

The most likely ancestral range reconstructed for the MRCA of *B. longiflorum* and *B. fenestratum* was a widespread distribution throughout the Afrotropics, tropical Asia, Papuasia, the Pacific and Australasia (rel. prob. 0.72), inferring widespread dispersal between the late Miocene and late Pleistocene ca. 5.5–0.8 Ma. In this scenario the descendant lineage giving rise to *B. longiflorum* inherited the widespread ancestral range, while the descendant lineage giving rise to *B. fenestratum* underwent a range contraction back to a shared distribution across temperate and tropical Asia. Alternative ancestral ranges reconstructed for the MRCA of *B. longiflorum* and *B. fenestratum* each received relative probabilities below 0.06. Within clade A2 the ancestral range analysis reconstructed range expansion from tropical Asia to Asia. The most likely ancestral range reconstructed for the MRCA of the A2 is tropical Asia (rel. prob. 0.81). Within clade A2, at least three range expansions were inferred from tropical Asia to a shared range including Papuasia from the mid-Miocene onwards. The first instance of range expansion was inferred in the lineage giving rise to the MRCA of *B. digoelense* (sect. *Intervallatae*) and *B. echinolabium* (sect. *Lepidorhiza*) between the mid to late Miocene, ca. 13.5–9.6 Ma (rel. prob. 0.83), with vicariance inferred between tropical Asia and Papuasia in the descendent lineages subsequently. Alternatively, the ancestral tropical Asian range was conserved in the MRCA of *B. digoelense* and *B. echinolabium* (rel. prob. 0.17) and in *B.*

echinolabium, with dispersal inferred from tropical Asia to Papuasias in the lineage giving rise to *B. digoelense* from the late Miocene ca. 9.6 Ma. The second instance of range expansion to Papuasias was inferred in the lineage giving rise to the MRCA of *B. foetidum* and *B. wakai* (sect. *Beccariana*) (rel. prob. 1), between the mid-Miocene and early Pleistocene ca. 12.9–1.5 Ma. The widespread distribution was conserved in the lineage giving rise to *B. wakai*, while range contraction to Papuasias was inferred in the lineage giving rise to *B. foetidum*. The third instance of range expansion to Papuasias was inferred in the lineage giving rise to the MRCA of *B. ericssonii* (sect. *Beccariana*) and *B. macranthum* (sect. *Stenochilus*) (rel. prob. 0.68), between the late Miocene and early Pleistocene ca. 6.7–1.7 Ma, with both descendent lineages inheriting the widespread range. Alternatively, the ancestral tropical Asian range was conserved in the MRCA of *B. ericssonii* and *B. macranthum* (rel. prob. 0.32), with range expansion inferred in both the descendant lineages from the early Pleistocene ca. 1.7 Ma. The ancestral range analysis also reconstructed an alternative scenario inferring range expansion from tropical Asia to Papuasias in the MRCA of Clade A2 (*B. digoelense*, *B. echinolabium*, *B. foetidum*, *B. wakai*, *B. lobbii*, *B. ericssonii* and *B. macranthum*) between the early Miocene and mid-Miocene ca. 18.9–13.5 Ma (rel. prob. 0.19) (Fig. 2.4, Appendices 2.5, 2.6).

2.3.4.3. Spatio-temporal evolution within Asian clade B

Within clade B, the ancestral range of the MRCA of clade B1 remains unresolved. A shared range across tropical Asia and Australasia was reconstructed as the most likely ancestral range (rel. prob. 0.57), inferring range expansion from tropical Asia to Australasia during the early Miocene between ca. 20–16.5 Ma (Fig. 2.4, Appendices 2.5, 2.6). In this scenario, the shared distribution is maintained in *B. evasum* (sect. *Brachystachyae*), while range contraction to tropical Asia is inferred in the MRCA of *B. lindleyanum* (sect. *Hirtula*) and *B. flavescens* (sect. *Stachysanthes*). Alternatively, the tropical Asian ancestral range is conserved in the MRCA of *B. evasum*, *B. lindleyanum* and *B. flavescens* (rel. prob. 0.43), and range expansion to Australasia was inferred subsequently in the lineage giving rise to *B. evasum*, from the late Miocene ca. 16.5 Ma. At least one range expansion from tropical Asia to temperate Asia was inferred within clade B in the lineage giving rise to *B. ovalifolium* (sect. *Macrocaulia*) from the early Miocene ca. 19.1 Ma. Range expansion from tropical Asia to a shared range that also included Papuasias was inferred in the MRCA of clades B2–B6 in the early Miocene ca. 18.4–17.2 Ma (rel. prob. 0.98). Subsequently, range contraction to Papuasias is inferred in the lineage giving rise to the MRCA of clade B2, while two alternative scenarios are presented for the lineage giving rise

to MRCA of clades B3-B6: 1) the shared range across tropical Asia and Papuasias was conserved in the MRCA of clades B3-B6 (rel. prob. 0.58), or 2) the lineage giving rise to the MRCA of clades B3-B6 underwent a range contraction to tropical Asia (rel. prob. 0.42).

Range evolution within clade B3 during the mid to late Miocene remained largely unresolved. Alternative ranges reconstructed include 1) tropical Asia, 2) Papuasias or 3) a widespread distribution across tropical Asia and Papuasias. At least two dispersal events to the Pacific region were inferred in the lineages giving rise to 1) *B. absconditum* subsp. *absconditum* (sect. *Pelma*) from the mid-Miocene ca. 13.4 Ma and 2) *B. clandestinum* (sect. *Oxysepala*) from the late Miocene ca. 8.4 Ma. Range evolution in the lineage giving rise to the MRCA of clade B4 also remained unresolved. The most likely ancestral range reconstructed for the MRCA of clade B4 is a shared distribution across tropical Asia and the Pacific (rel. prob. 0.5).

Alternatively, a widespread distribution across tropical Asia, Papuasias and the Pacific was reconstructed (rel. prob. 0.33), while other scenarios received relative probabilities below 0.01. The ancestral range of the MRCA of clades B5 and B6 was also unresolved. Two alternative ancestral ranges were presented with similar likelihoods: 1) a shared distribution between tropical Asia and Papuasias (rel. prob. 0.52) and 2) tropical Asia (rel. prob. 0.47), while a third scenario of a Papuasian ancestral range received a relative probability less than 0.01.

From the late Miocene diversification occurs predominantly within clade B6. Two alternative ancestral ranges were reconstructed for the MRCA of clade B6: 1) a shared distribution across tropical Asia and Papuasias (rel. prob. 0.64) and 2) Papuasias (rel. prob. 0.36). Diversification among lineages within clade B6 was inferred to have taken place largely within Papuasias during the late Miocene to late Pliocene. At least two range expansions events were inferred to Australasia from the late Miocene: 1) from a shared distribution including tropical Asia and Papuasias to a widespread distribution across tropical Asia, Papuasias and Australasia in the lineage giving rise to *B. maxillare* (sect. *Hoplandra*) from ca. 7.7 Ma., and 2) from Papuasias to a shared distribution across Papuasias and Australasia in the lineage giving rise to the MRCA of *B. radicans* (sect. *Fruticicola*), *B. pallidiflorum* (sect. *Polymeres*), *B. triaristella* (sect. *Micromonanthae*) and *B. aphanopetalum* (sect. *Peltopus*) ca. 8.8–7.9 Ma. Within clade B6, at least two range expansions from Papuasias to a widespread distribution including the Pacific were inferred from Papuasias to the Pacific region in the lineages that gave rise to 1) *B. pallidiflorum* (sect. *Polymeres*) from the late Miocene ca. 6.2 Ma and 2) *B. aphanopetalum* (sect. *Peltopus*) from the late Pliocene ca. 3.4 Ma.

2.4. Discussion

2.4.1. Infrageneric relationships within *Bulbophyllum*

2.4.1.1. Higher level relationships

This study has clarified higher level infrageneric relationships within *Bulbophyllum*, identifying three major clades within the genus: an Australasian clade, an Asian clade and an Afrotropical/Neotropical clade, each resolved with high support in phylogenetic reconstructions based on a 70 plastid cds dataset (Fig. 2.2, Appendix 2.3). Phylogenetic reconstruction based on three nuclear ribosomal regions also resolved an Australasian clade, an Asian clade and an Afrotropical/Neotropical clade (Fig. 2.1, Appendix 2.2). The Australasian clade was composed of sect. *Adelopetalum*, from Australia, New Zealand and New Caledonia, and sect. *Minutissima* species from Australia and New Zealand (Fig. 2.1, Fig. 2.2, Appendix 2.2, Appendix 2.3). The clade is referred to here as the Australasian clade, based on its distribution predominantly found within the Australasian floristic continent including Australia and New Zealand (Brummitt, 2001). The Asian clade was comprised predominantly of Asian taxa but also included taxa from Papuasia, Australia and New Caledonia, while the Afrotropical/Neotropical clade included all the Afrotropical and Neotropical taxa (Fig. 2.1, Fig. 2.2, Appendices 2.1, 2.2). Reconstructions based on the 70 gene plastid alignment resolved the Australasian clade as an early diverging lineage in a sister group position to the remainder of the genus (Fig. 2.1). The latter group included the Afrotropical clade and the Asian clade. Overall, the reconstruction based on the three nuclear ribosomal regions was poorly supported and relationships between the three major clades remained unsupported (Fig. 2.2.). The ITS is a rapidly evolving region and the chance of saturation whereby numerous substitutions occur at the same position, masking the phylogenetic signal, increases over time (Alvarez and Wendel, 2003). Consequently, the phylogenetic utility of the region for resolving higher level relationships can be limited (Alvarez and Wendel, 2003). The major lineages are also separated by very short branch lengths indicating these groups are differentiated based on a very small number of mutations. Further studies to increase sampling from the nuclear genome are required with the aim to improve support and confidence in the relationships resolved.

The results support previous molecular phylogenetic analyses based on 1–7 nuclear and plastid markers that identified major Afrotropical/Neotropical and Asian clades (Fischer et al., 2007; Smidt et al., 2011; Gamisch et al., 2015; 2019). The results presented here further clarify relationships among the major lineages by identifying an Australasian clade. The Australasian

clade has not been identified in previous molecular phylogenetic studies due to a broadly defined Asian-Pacific region and limited sampling from the Australasian clade including only three representatives (Gamisch et al., 2015; 2019). For example, many studies did not include representatives of the Australasian clade (Graveendeel et al., 2004; Fisher et al., 2007; Smidt et al., 2011). Analyses based on 1–7 nuclear and plastid markers resolved a basal dichotomy within the genus between an Afrotropical/Neotropical clade and an Asian/Australasian clade (Graveendeel et al., 2004; Fisher et al., 2007; Smidt et al., 2011; Pridgeon et al., 2014). However, relationships between the two major clades were resolved with low support and thus higher-level evolutionary relationships within the genus remained unclear. A recent analysis based on the ITS region reconstructed major clades within the genus with moderate to high support but like previous studies did not differentiate between the Asian and Australasian regions, and instead recognised a broad Asian-Pacific region (Gamisch and Comes, 2019). In contrast to previous studies of *Bulbophyllum* that recognised a broad Asian-Pacific region, this study differentiated the Asian and Australasian floristic continents as distinct biogeographic regions and identified an Australasian lineage formed by sect. *Adelopetalum* and sect. *Minutissima pro parte*.

2.4.1.2. Intersectional relationships

Taxonomic treatments of Asian and Australasian *Bulbophyllum* have largely been informed by morphology (Reichenbach, 1861; Bentham and Hooker, 1883; Pfitzer, 1889; Schlechter, 1913; Holttum, 1953; Seidenfaden, 1973; 1979; Dressler, 1993; Garay et al., 1994; Szlachetko and Margonska, 2001; Clements and Jones, 2002; Chen and Vermeulen, 2009; Vermeulen and O’Byrne, 2011; Pridgeon et al., 2014; Vermeulen et al., 2014; Chase et al. 2015) and are yet to be validated with a robust phylogenetic framework. The 70 plastid loci phylogeny provided a resolved and largely well supported phylogenetic framework that clarified broad relationships between sections from the Asian and Australasian region (Fig. 2.2).

Sampling for the phylogenetic analyses was aimed at resolving phylogenetic relationships between major evolutionary lineages and hence assessment of the monophyly of *Bulbophyllum* sections was limited. Nevertheless, of 28 sections for which two or more representatives were sampled, eight were identified as polyphyletic: sections *Cirrhopetalum*, *Desmosanthes*, *Rhytionanthos*, *Hoplandra*, *Leopardinae*, *Macrocaulia*, *Polymeres* and *Sestochilus* (Fig. 2.1, 2.2). Three additional sections were identified as paraphyletic: sect. *Lemniscata* with sect. *Tripudianthes* nested within, sect. *Beccariana* with *B. hamatipes* (sect. *Leopardinae*) nested within, and sect. *Stenochilus* with *B. ericssonii* (sect. *Beccariana*) and *B.*

patens (sect. *Sestochilus*) nested within (Fig. 2.1). Previous phylogenetic analyses have also identified sect. *Polymeres*, sect. *Beccariana*, sect. *Cirrhopetalum*, sect. *Desmonanthes*, sect. *Leopardinae* sect. *Drymoda* as polyphyletic (Pridgeon et al. 2014, Hu, 2018). Evidence from phylogenetic reconstructions in the present study and previous research shows further revision of the sectional classification of *Bulbophyllum* is required to accurately reflect evolutionary relationships within the genus.

2.4.2. Spatio-temporal evolution

2.4.2.1. Early spatio-temporal evolution of *Bulbophyllum*

Molecular dating analyses inferred that *Bulbophyllum* emerged in the early Oligocene ca. 33.1 Ma, and Australasian, Asian and Afrotropical ancestral lineages emerged in the early evolution of the genus (Fig. 2.3, Fig. 2.4). The ancestral area reconstruction placed the origin of the Australasian lineage in Australasia in the late Oligocene, ca. 23.7 Ma. The analyses showed the Afrotropical and Asian lineages emerged in the late Oligocene, ca. 23.2 Ma, with the ancestral range of the Afrotropical lineage placed in the Afrotropics, while the Asian lineage was shown to have emerged in tropical Asia (Fig. 2.3, Fig. 2.4). Previous divergence dating studies of *Bulbophyllum* estimated slightly younger divergence ages, placing the origin of the genus in the late Oligocene, ca. 25.8 Ma, and both the Asian clade and the Afrotropical/Neotropical clade, in the mid-Miocene (Gamisch and Comes, 2019). The divergence dates reconstructed based on the 70 plastid *cds* dataset predate results from previous studies, however the previous estimates fell within the upper confidence margins. Therefore, these results largely support previous estimates.

The present-day distribution of *Bulbophyllum* in Australia, New Caledonia, Africa, Madagascar, India and the Neotropics suggests a Gondwanan origin of the genus. However, this hypothesis is clearly rejected by divergence dating analyses. The break-up of Gondwana occurred during the Cretaceous (McLoughlin, 2001). Divergence dating analyses carried out in previous studies have placed the origin of the Epidendroideae orchid subfamily in the Palaeocene (Chomicki et al., 2015; Givnish et al., 2015) and *Bulbophyllum* in the late Oligocene (Givnish et al., 2015, Gamisch and Comes, 2019). These studies show that *Bulbophyllum* emerged at a time when the land masses of Australia, New Caledonia, Africa, Madagascar, India and South America had largely assumed their present-day positions. Therefore, the divergence of the Australasian, Afrotropical and Neotropical lineages within *Bulbophyllum* is not consistent with a vicariance hypothesis associated with the break-up of Gondwana. The divergence dates between the

Australasian, the Asian and the Afrotropical/Neotropical lineages inferred in the divergence dating and ancestral range reconstructions (Fig. 2.3, Fig. 2.4) implicate transoceanic long distance dispersal (LDD) in the evolution of major biogeographic lineages within the genus. The results presented in this study support previous studies that highlight the important role of LDD in the spatio-temporal evolution of *Bulbophyllum* and more broadly across the evolutionary history of Orchidaceae (Smidt et al., 2011; Givnish et al., 2016; Gamisch et al., 2019).

Following the break-up of Gondwana in the Palaeocene, the Australasian plate drifted north during the Eocene and Oligocene, eventually colliding with the Asian plate in the late Oligocene ca. 23 Ma (Hall et al. 2013). The collision of the two landmasses initiated major geological uplift in the region of Papuasias at the northern edge of the Australasian plate approximately 12 Ma. Within *Bulbophyllum*, divergence between the tropical Asian and Australasian ancestral lineages indicates dispersal between these two regions has taken place during the late Oligocene ca. 23 Ma (Fig. 2.4), coinciding with the collision of the Asian and Australasian plates (Hall et al. 2011). Long distance dispersal is inferred between Asia and Australasia across the deep-water channels that separated the Asian and Australasian plates at the time (Hall et al. 2011). The deep-water channels that separate Asia and Australasia are considered a strong biogeographic barrier for animals, however, phylogenetic studies show that many plant lineages have transgressed these barriers (Sniderman and Jordan, 2011, Richardson et al., 2012; Crayn et al., 2015; Yap et al., 2018). The late Oligocene disjunction inferred between the Asian and Australian *Bulbophyllum* lineages represents one of the earliest disjunctions inferred between these regions (Sniderman and Jordan, 2011; Crayn et al., 2015).

Two alternative scenarios may be proposed for the inferred dispersal between the Asian/Australasia region and the Afrotropics (Fig. 2.4). The first is trans-oceanic LDD across the Indian ocean, possibly via Madagascar. This may have been from tropical Asia to the Afrotropics, or from Australasia to the Afrotropics, at a time when the Australasian plate was positioned lower in the southern hemisphere, mostly below the tropic of Capricorn. Multiple transoceanic LDD events have been inferred within the Orchidaceae, including from south east Asia to Africa, across the Indian Ocean, giving rise to the Angraecinae and Aerangidae (Givnish et al., 2016). The minute wind dispersed seeds that characterise the family provide an efficient dispersal vector even across large ocean barriers. Transoceanic dispersal has also previously been inferred within *Bulbophyllum*, from continental Africa to South America, giving rise the

Neotropical *Bulbophyllum* lineage (Smidt et al., 2011). Alternately, dispersal between the Asian/Australasia region to the Afrotropics may have occurred via the northern rim of the Indian Ocean during climatic maxima when continuous mega-thermal forests provided a migration pathway across the boreotropical route (Tiffany, 1985; Sanmartin et al., 2001; McElwain, 2018). A migration along the boreotropical route has been proposed for several rainforest lineages (Zhou et al., 2012; Joyce et al., 2018). Previous studies of *Bulbophyllum* have dismissed this scenario for range shifts between the Asian and Afrotropical regions because the estimated divergence between the Asian/Australasian and Afrotropical lineages occurred subsequent to the Eocene climatic optimum (Gamisch et al., 2019). However, a forested migration pathway may have also been present along the boreotropical route during the mid-Miocene climatic optimum (17–15Ma), coinciding with the lower estimates for divergence between the Asian and Afrotropical *Bulbophyllum* lineages (Zachos, 2001; 2008).

The geographic origins of the genus remain unclear. The ancestral range analyses reconstructed a broad shared geographic range for the MRCA of the genus between the Afrotropics, Asia and Australasia (Fig. 2.4). Hence, the direction of dispersal between the Asian, Afrotropical and Australasian regions in the early evolution of the genus also remains unclear. A broad analysis of the spatio-temporal evolution of the Orchidaceae reconstructed the ancestral range for *Bulbophyllum* and the tribe Dendrobieae in southeast Asia (Givnish et al., 2016). However, the study included a single representative for each genus, assigned with a geographic range representing the distribution of the genus. Previous ancestral range reconstructions within *Bulbophyllum* utilising Bayesian methods have placed the origin of the genus in the Asian-Pacific region and inferred range expansion from the Asian-Pacific region to the Afrotropics and then to the Neotropics via LDD (Gamisch and Comes 2019). To accurately reconstruct the finer scale spatio-temporal evolution within the Dendrobieae increased sampling of distinct biogeographic lineages within genera is required from Dendrobieae and the closely related tribes Malaxideae and Podochileae. Resolution of relationships among the major biogeographic lineages within *Bulbophyllum* will also require increased sampling of molecular markers from the nuclear genome.

2.4.2.2. *Spatio-temporal evolution of the Asian clade*

Within the Asian clade of *Bulbophyllum* numerous range expansions and range shifts were inferred from the ancestral range of the clade in tropical Asia to temperate Asia, Papuasias, Australasia and the South Pacific regions (Fig. 2.4). Range expansions into temperate Asia,

predominantly into China, may have occurred via LDD or overland migration. Range expansion and range shifts from tropical Asia to Papuasias, Australasia and New Caledonia transgress deep water biogeographic barriers. The southeast Asian landmasses are separated from Papuasias by deep water straits, and from Australia and New Caledonia by the Arafura, Torres Strait and Coral seas. Therefore, LDD is inferred to explain range expansion and range shifts reconstructed within the genus from tropical Asia to Papuasias, Australia or New Caledonia. Within the Asian lineage, at least seven independent dispersal events were inferred from tropical Asia to Papuasias from the early Miocene (Fig. 2.4). These ages are consistent with the period of floristic exchange inferred between the two regions in 49 flowering plant groups between 33 Ma to 1 Ma based on dated molecular phylogenies (Crayn et al., 2015). Range expansion from tropical Asia to Papuasias gave rise to the Papuasian clade B6, comprising predominantly Papuasian sections *Brachypus*, *Codonosiphon*, *Coelochilus*, *Fruticicola*, *Hoplandra*, *Papulipetalum*, *Peltopus*, *Polymeres* and *Micromonanthae*. The ancestral Papuasian range is largely conserved for internal nodes within clade B6, providing evidence for *in situ* diversification within Papuasias (Fig. 2.4). The divergence of clade B6 and subsequent diversification within the clade was reconstructed to the late Miocene from ca. 10.4 Ma, coinciding with the major uplift of the Central Range of New Guinea. The significant mountain building that occurred in Papuasias during this time is identified as a likely driver of divergence within this lineage, facilitating allopatric speciation. A study testing the impacts of various traits on net diversification within Orchidaceae showed invasion of mountain ranges in the Andes and New Guinea highlands had the largest positive effect on net species diversification within lineages (Givnish et al. 2015). The authors concluded the high peaks, deep valleys and diversity of communities along altitudinal gradients present barriers to gene flow facilitating allopatric speciation. The ancestral range reconstruction within *Bulbophyllum* supports this hypothesis, providing evidence of diversification within Papuasias coinciding with a period of major mountain building.

Range expansions from Papuasias to Australia and New Caledonia inferred in clade B6 demonstrate Papuasias has provided a stepping stone for the movement of *Bulbophyllum* from tropical Asia to the Australasian and Pacific regions (Fig. 2.4). At least two range expansions from Papuasias to New Caledonia were inferred in the lineages that gave rise to 1) *B. pallidiflorum* (sect. *Polymeres*) and 2) *B. aphanopetalum* (sect. *Peltopus*). At least two other independent dispersal events to New Caledonia were inferred within the Asian clade B, in the lineages giving rise to 1) *B. baladeanum* (sect. *Hoplandra*) and 2) *B. absconditum* subsp. *absconditum* (sect. *Pelma*) and dispersal to Pacific islands (Fiji, Marianas and the federated

states of Micronesia) in the lineage giving rise to *B. clandestinum* (*Oxysepala*). However, it is unclear if these dispersals were via New Guinea or directly from tropical Asia. At least one dispersal event from tropical Asia to the Pacific region was also inferred within the Asian clade A in the lineage giving rise to the widespread species *B. longiflorum* (sect. *Cirrhopetalum*). New Caledonia has a long geological history of isolation in the Coral Sea following its separation from Gondwana in the late Cretaceous (Neall and Trewick, 2008). The island harbours a unique biota including many endemic taxa (Lowry et al., 2004). Geological evidence indicates the island was at least partially submerged during the Palaeocene and Eocene prompting hypotheses the island's biota has arrived via LDD since the Oligocene (Pelletier, 2006). However, the occurrence of ancestral lineages contradicts the notion that the island has undergone complete inundation since breaking away from Gondwana, while alternative hypotheses proposing ancestral lineages have persisted on other landmasses in the region throughout the Cenozoic have also been proposed (Ladiges and Cantril, 2007). Within *Bulbophyllum*, LDD to New Caledonia is not inferred until the Neogene, from the mid-Miocene onwards, ca 13.4 Ma.

From the Papuan clade B6, at least two independent dispersal events were inferred to Australia with 1) range expansion in the lineage that gave rise to *B. maxillare* and 2) range shift in the lineage that gave rise to *B. radicans* (Fig. 2.4). Divergence of the Australian species *B. radicans* in the late Miocene (ca. 7.9 Ma) may have been the result of vicariance or LDD. New Guinea and Australia together form the Australasian continental shelf and the two regions have been joined by land bridges during periods of low sea levels associated with glacial cycles. The ancestor of *B. radicans* may have migrated from New Guinea to Australia via these land bridges if continued tracts of rainforests were present. Palynological records indicate the Neogene was a period of aridification on the Australian continent transforming the Australian landscape from one dominated by rainforest to one dominated by open vegetation communities (Hill et al., 2017). Pollen records from the early to mid-Miocene indicate that rainforest remained a major component of the Australian vegetation, however aridification was well underway with woodland and heath communities expanding across the continent (Hill et al., 2017). Although pollen records are absent from the late Miocene period, their absence indicates drier conditions, and records from the Pliocene show that by this time open communities had expanded across the northern Australia presenting a dry biogeographic barrier between New Guinea and the Australian rainforests that were contracting to the mesic eastern coast where they occur today (Hill et al. 2017). This region of open vegetation

communities would have presented a significant biogeographic barrier to *Bulbophyllum* species which are epiphytes adapted to mesic habitats, with most species endemic to wet forest communities. Thus, vicariance should be considered as a possible driver of divergence within lineages where a range shift was inferred between New Guinea and Australia. The divergence of *B. radicans* in the late Miocene coincides with this period of aridification. Presently, *B. radicans* occurs in rainforest of the Wet Tropics region on Australia's east coast (Jones, 2006). Range expansion from Papuasias in the lineage that gave rise to *B. radicans* may have occurred across an exposed land bridge between New Guinea and Australia when rainforest vegetation was relatively intact across northern Australia, with subsequent speciation caused by vicariance with the emergence of the dry biogeographic barrier across northern Australia. Alternatively, the divergence of *B. radicans* was the result of LDD and subsequent allopatric speciation.

2.4.2.3. Evolutionary origins of *Bulbophyllum* in Australasia

Bulbophyllum is represented in the floristic continent of Australasia with 35 species (Brummitt, 2001; Clements and Jones, 2002; WCSP, 2018). The highest diversity is found on the Australian continent where 33 species occur, predominantly within montane forest communities, while New Zealand harbours two endemic species. So far, the spatio-temporal evolution of Asian/Australasian *Bulbophyllum* is poorly understood. Previous taxonomic treatments recognised several morphological groups (either at sectional or genus level), however, relationships among these groups had remained unclear (Clements and Jones, 2002). Previous studies of *Bulbophyllum* have included up to eight Australasian species (Gamisch and Comes, 2019). Twelve *Bulbophyllum* sections are represented in Australasia, namely sections *Adelopetalum* (Fitzg.) J.J. Verm. (1993), *Brachystachyae* Benth. & Hook.f. (1883), *Cirrhopetalum* (Lindl.) Rchb.f. (1861), *Ephippium* Schltr. (1913), *Fruticicola* Schltr. (1913), *Hoplandra* J.J. Verm. (2008), *Minutissima* Pfitz. (1889), *Polymeres* Ridl. (1896), *Oxysepala* (Wight) Rchb.f. (1861), *Papulipetalum* Schltr. (1913), *Polyblepharon* Schltr. (1913), *Stenochilus* J.J.Sm. (1914). Section *Adelopetalum* represents a predominantly Australian section (nine species) with two species also known from New Caledonia and one species from New Zealand (Pridgeon et al., 2014). Sections *Brachystachyae*, *Cirrhopetalum*, *Ephippium*, *Fruticicola*, *Hoplandra*, *Minutissima*, *Polymeres*, *Oxysepala*, *Papulipetalum*, *Polyblepharon* and *Stenochilus* represent predominantly Asian groups that include one to eight Australasian species (Pridgeon et al., 2014). Phylogenetic analysis of the nuclear ITS region reconstructed the Australasian species from sect. *Adelopetalum* and sect. *Minutissima* within the Australasian clade, while the Australasian

species from other sections were found in different phylogenetic positions across the Asian clade (Fig. 2.1). These results indicate that the Australasian *Bulbophyllum* species do not have a single evolutionary origin with a subsequent radiation within the Australasian region, but rather have different evolutionary origins. The results provide evidence that multiple dispersals have occurred from tropical Asia and Papuasia into Australasia. Reconstructions based on 70 plastid coding regions show sect. *Adelopetalum* and Australasian species of sect. *Minutissima* comprise a highly supported early diverging Australasian lineage that emerged in Australasia in the early Miocene (Fig. 2.4). The ancestral range of *Bulbophyllum* remains unclear and therefore it remains unclear if this early diverging Australasian lineage descended from an Australasian ancestor for *Bulbophyllum* or colonised Australasia via LDD in the early evolution of the genus. The other sections represented in Australasia, sections *Brachystachyae*, *Cirrhopetalum*, *Ephippium*, *Fruticicola*, *Hoplandra*, *Polymeres*, *Oxysepala*, *Papulipetalum*, *Polyblepharon* and *Stenochilus*) are nested within the highly supported Asian clade, which emerged in tropical Asia from the late Oligocene to the early Miocene (Fig. 2.4). The placement of these sections within different highly supported clades within the Asian clade provides evidence for multiple independent dispersal events from tropical Asia into Australasia (Fig. 2.4). Phylogenetic reconstructions based on the ITS marker including 97% of Australasian species resolved nine lineages of Australasian taxa within the Asian clade (Fig. 2.4). Thus at least nine independent dispersal events were inferred from the Asian lineage into Australasia from the mid-Miocene onwards. Dispersal may have been direct, from tropical Asia into Australasia across the Arafuran Sea, or via Papuasia as a 'stepping stone' for migration into Australasia. Dispersal from Papuasia may have occurred via 1) LDD across the Arafuran, Torres Strait and Coral Seas or 2) a terrestrial route during periods when a land bridge existed between New Guinea and Australia.

Seven out of twelve *Bulbophyllum* sections found in Australia are represented by a single extant Australian species (sect. *Stenochilus*: *B. baileyi*, sect. *Cirrhopetalum*: *B. longiflorum*, sect. *Ephippium*: *B. gracillimum*, sect. *Brachystachyae*: *B. evasum* and sect. *Papulipetalum*: *B. nematopodum*, sect. *Fruticicola*: *B. radicans*, sect. *Hoplandra*: *B. maxillare*) (Fig. 2.1). The remaining sections include a group of closely related Australasian species, indicating diversification has occurred within the Australasian region (Fig. 2.1). The earliest diverging Australasian lineage represents the largest group comprising sect. *Adelopetalum* and Australasian species of sect. *Minutissima* (Fig. 2.1). *In situ* diversification in Australia was also inferred in sect. *Polymeres* (*B. johnsonii* and *B. bowkettiae*), sect. *Polyblepharon* (*B.*

macphersonii and *B. sladeanum*) and sect. *Oxysepala* (*B. gadgarrense*, *B. wadsworthii*, *B. lamingtonensis*, *B. shepherdii*, *B. schillerianum*, *B. windsorensis*, *B. grandimesiense* and *B. lewisense*) (Fig. 2.1). Within these Australasian radiations, phylogenetic relationships between species were largely unsupported in phylogenetic reconstructions from the single nuclear ITS region, and therefore the spatio-temporal evolution of these groups in the Australasian region remains unclear.

Papuasias harbours the centre of diversity of *Bulbophyllum* species, where 658 species are recognised (WCSP, 2018). The wet montane and forest communities of New Guinea provide an ideal environment to support the epiphytic and lithophytic habit that is dependent on high moisture availability. Relative to the significant diversity found in Papuasias, Australasia harbours a low diversity of *Bulbophyllum* species. Based on the species-area relationship, the high diversity of species found in New Guinea relative to Australia can be explained by the considerably larger area of wet forest communities in New Guinea. Perhumid forests are the dominant vegetation type on the island of New Guinea and therefore the island has a larger capacity for mesic adapted species. Australia, however, presents a predominantly arid environment where wet forest communities are restricted to isolated fragments along the east coast (Hill et al., 2017). Cool temperate forests of Gondwanan origin once spread across the Australian continent, however, changes in the climate during the Neogene transformed the Australian landscape from one dominated by rainforest to one dominated by open vegetation communities (Hill et al., 2017) not suitable for the mesic adapted *Bulbophyllum* species. The ancestral area analysis placed the earliest origin of *Bulbophyllum* in Australasia during the late Oligocene ca. 23.9 Ma with the divergence of the Australasian clade (Fig. 2.4). During the late Oligocene to the early Miocene rainforests remained a major component of the Australian vegetation (Hill et al., 2017). During the mid – late Miocene to Pliocene, rainforests contracted to the moist east coast regions where they occur today (Hill et al., 2017). A mass extinction of rainforest taxa occurred during this time (Byrne et al., 2011), and the paucity of *Bulbophyllum* species in Australia might be due to high rates of extinction during this period.

Further studies including comprehensive sampling are required to reconstruct the number, chronology and migration route for dispersals from Asia into Australasia and to assess the time in which these lineages have had to diversify. Sections represented in Australasia by a single species or few species may represent recent dispersal events that have had little time to diversify subsequently. The ability of new immigrants to colonise and persist in Australia's established wet forest communities should also be considered. In contrast to the perhumid equatorial forests of south east Asia and New Guinea, Australian mesic forests receive lower

annual precipitation and have a seasonal weather pattern including a dry season, that is periodically accentuated by the southern oscillation index. Australia's tropical forests also experience high natural disturbance in the form of cyclones (Webb, 1958; Turton and Siegenthaler, 2004). This dynamic system presents both opportunities for migrants in the form of increased niche diversity and turnover and also significant challenges which may have limited the establishment and diversification of *Bulbophyllum* species in Australia. The epiphytic habit of *Bulbophyllum* provides unique opportunities to exploit niches throughout the canopy (Gravendeel et al., 2004). However, the seasonally dry climate of Australia's mesic forests presents a critical challenge for epiphytes and many *Bulbophyllum* species are largely restricted to montane forests that receive year-round moisture from cloud stripping (Jones, 2006).

2.4.3. Conclusions

Genome skimming has produced a highly informative 70 plastid cds dataset that has clarified infrageneric relationships within *Bulbophyllum* and provided a well resolved phylogenetic framework identifying broad sectional relationships within *Bulbophyllum*. The phylogenetic reconstruction based on the 70 plastid cds dataset resolved three major lineages with high support. Firstly, an Australasian lineage was identified within *Bulbophyllum* comprising section *Adelopetalum* and Australasian species of section *Minutissima*. Secondly, an Afrotropical clade was resolved, supporting major lineages resolved in previous studies. Finally, an Asian lineage was resolved, providing evidence for a monophyletic Asian clade. Reconstruction of the spatio-temporal evolution of the genus reveals disjunctions between Asia, Australasia and the Afrotropics between the late Oligocene and the early Miocene, rejecting a vicariance explanation for divergence among the major biogeographic lineages with the break-up of Gondwana. The late Oligocene disjunction between Asia and Australasia is among the earliest disjunctions recorded for plants between the two regions. Paraphyletic sections identified among the Asian and Australasian taxa in the present study are in line with previous analyses which identified taxonomic issues in sectional classification of the genus highlighting the need for further infrageneric revision within *Bulbophyllum*. Within the early diverging Australian clade, relationships were largely unsupported. Further analysis is required to resolve evolutionary relationships in this group and provide insights into its spatio-temporal evolution in Australasia.

2.5. Chapter summary

- To reconstruct broad-level infrageneric relationships, 148 *Bulbophyllum* species were sampled, representing 63% of Asian and Australasian sections (42 sections).
- Genome skimming with high throughput sequencing was carried out for 92 samples and 70 plastid coding regions and the ribosomal regions (ITS1, 5.8s, ITS2) were used in subsequent phylogenomic analysis, with the inclusion of 67 ITS accessions from GenBank.
- Maximum Likelihood and Bayesian analyses based on 70 plastid coding regions resolved three highly supported clades within the genus: 1) an Asian clade, 2) an Australasian clade and 3) an Afrotropical/Neotropical clade.
- Eleven sections were identified as polyphyletic (sections *Cirrhopetalum*, *Desmosanthes*, *Rhytionanthos*, *Hoplandra*, *Leopardinae*, *Macrocaulia*, *Polymeres* and *Sestochilus*) or paraphyletic (sections *Lemniscata*, *Beccariana*, *Stenochilus*).
- Molecular divergence time analyses placed the origin of *Bulbophyllum* in the early Oligocene, ca. 33.3 Ma. Crown diversification was estimated to have commenced in the late Oligocene, ca. 24 Ma and a rapid divergence between the three major lineages was inferred during the late Oligocene 24–23.4 Ma.
- Ancestral area reconstructions based on maximum likelihood using two models (DEC, DIVA) placed the origins of the Australasian clade in Australasia, the Afrotropical/Neotropical clade in the Afrotropics and the Asian clade in tropical Asia. Within the Asian clade, divergence among sections took place mainly throughout the Miocene and Pliocene.
- The divergence dates between the Australasian, the Asian and the Afrotropical/Neotropical biogeographic lineages within *Bulbophyllum* implicate transoceanic long distance dispersal in the evolution of major biogeographic lineages within the genus.
- Multiple independent long-distance dispersals were inferred from tropical Asia to temperate Asia, Australasia and the South Pacific and within the Tropical Asian region dispersal events were inferred westward to Papuasias.
- The divergence of the predominantly Papuasian clade B6 and subsequent diversification within the clade was reconstructed to the late Miocene. This coincided with the major uplift of the Central Range of New Guinea, which is identified as a likely driver of divergence within this lineage.

Chapter three: The spatio-temporal and morphological trait evolution of the Australasian clade of *Bulbophyllum* (sections *Adelopetalum* and *Minutissima*)

*This chapter is modified from the manuscript “Evolutionary relationships and spatio-temporal evolution of the hyperdiverse orchid genus *Bulbophyllum*: phylogenomic insights from the Australasian lineage” by Simpson, L., Clements, M.A., Crayn, D.M., Paule, J., Nargar, K. which is in review for publication in the journal *Molecular Phylogenetics and Evolution*.

3.1. Introduction

Phylogenetic analyses of 70 plastid coding regions and three nuclear ribosomal ITS markers identified an early diverging Australasian clade within *Bulbophyllum* comprising sections *Adelopetalum* and *Minutissima* (Chapter two, Fig. 2.1, 2.2).

Bulbophyllum section *Adelopetalum* (Fitzg.) J.J. Verm. is the only section within *Bulbophyllum* with a centre of diversity in Australia (Vermeulen, 1993; Brummitt, 2001; Pridgeon et al., 2014,). Eleven other sections include Australian species (namely sections *Brachystachyae* Benth. & Hook.f. (1883), *Cirrhopetalum* (Lindl.) Rchb.f. (1861), *Ehippium* Schltr. (1913), *Fruticicola* Schltr. (1913), *Hoplandra* J.J. Verm. (2008), *Minutissima* Pfitz. (1889), *Polymeres* Ridl. (1896), *Oxysepala* (Wight) Rchb.f (1861), *Papulipetalum* Schltr. (1913), *Polyblepharon* Schltr. (1913), *Stenochilus* J.J.Sm. (1914)), however the centre of diversity of each of these sections lies within the floristic continent of tropical Asia (Brummitt, 2001). Section *Adelopetalum* comprises 12 tropical to temperate epi-lithophytic species. Nine of these species occur in eastern Australia in the montane forest communities of the Great Dividing Range, two species are endemic to the montane forests of New Caledonia (*B. corythium*, *B. lingulatum*), and one is endemic to the lowland coastal forests of New Zealand (*B. tuberculatum*).

Section *Adelopetalum* was circumscribed (Vermeulen, 1993) based on morphological affinities recognised among ten species from Australia and New Caledonia previously assigned to *Bulbophyllum* sections *Desmosanthes*, *Racemosae* and *Sestochilus* (Dockrill, 1969; 1992). Subsequent treatments recognised an additional two species, *B. weinthalii* and *B. exiguum* within the section (Clements and Jones, 2002). Section *Adelopetalum* is characterised by thin creeping rhizomes that adpress to the host, anchored by filamentous roots, and small, crowded to widely spaced pseudobulbs that give rise to a small, single flat leaf at the apex. The inflorescence is single to few-flowered, with small white, cream or yellow flowers, sometimes with red or purple patterns. The petals are smaller than the sepals but similar in shape, with the base of the lateral sepals fused to

the column foot. The fleshy three-lobed labellum is firmly hinged to the apex of the column foot. A cladistic analysis of sect. *Adelopetalum* based on morphological characters resolved two main clades, differentiated by characters such as the prominence and shape of the lower margin of the stelidia (Vermeulen, 1993). However, the phylogenetic utility of morphological traits is yet to be tested in a molecular phylogenetic framework.

Bulbophyllum section *Minutissima* comprises 23 epiphytic species distributed across Thailand, Sumatra, Java, New Guinea, Australia, New Caledonia and New Zealand (Pridgeon et al., 2014). This section is characterised by plants an epi-lithophytic habit with creeping or straggling rhizomes and pseudobulbs that are distinct or minute compared to the size of the plant, bearing one (usually persistent) leaf and a single flowered inflorescence. The flowers are resupinate with the basal node of the pedicel above the attachment of the floral bract. Sepals are free and more or less equally lobed with one to three veins, petals that are usually single veined and two–four pollinia (Pridgeon et al., 2014). The type species of Section *Minutissima*, *B. minutissimum*, has also been associated with sect. *Macrocaulia* species, such as *B. moniliforme*, based on the morphological affinity of their minute pseudobulbs (Seidenfaden, 1979). However, morphological studies have identified unique floral and vegetative characteristics in the Australian sect. *Minutissima* species, for example an apical cavity in the pseudobulb that contains internal stomata (Jones and Clements, 2001).

In previous phylogenetic analyses based on three nuclear ribosomal regions (ITS1, 5.8s, ITS2), which included near-complete sampling of sect. *Adelopetalum* and representatives sampling from section *Minutissima*, a number of relationships were not resolved with strong bootstrap support (Chapter two, Fig. 2.1). Phylogenetic reconstructions based on 70 plastid cds included only representative sampling of the type species from sect. *Adelopetalum* and sect. *Minutissima* (Chapter two, Fig. 2.2). Sampling from sect. *Adelopetalum* and sect. *Minutissima* was also limited in previous molecular phylogenetic studies (Gamisch et al., 2015; Gamisch and Comes, 2019), which included at most four representatives. Consequently, phylogenetic relationships among sections *Adelopetalum* and *Minutissima* remain poorly understood.

The aims of this phylogenomic study were to reconstruct:

1. interspecific relationships within sections *Adelopetalum* and *Minutissima*;
2. patterns of spatio-temporal evolution within the Australasian clade; and
3. morphological trait evolution within the Australasian clade.

3.2. Methods

3.2.1. Sampling

In total, 64 samples representing 45 species were included in the study. For *Bulbophyllum* sect. *Adelopetalum* 28 samples were included, representing all 12 species recognised for the section. For *Bulbophyllum* sect. *Minutissima* eight samples representing five species comprising all four Australasian species and one (of ca. 19) tropical Asian species were included. For the reconstruction of broad-level relationships in *Bulbophyllum*, representatives of the Asian, continental African, Madagascan, and southern American clades were included based on previous phylogenetic studies (Fischer et al., 2007; Smidt et al., 2011; Vermeulen et al., 2014). The outgroup comprised representatives of subtribe Dendrobiinae and tribes Malaxideae, Arethuseae, Nervilleae, and Neottieae based on previous molecular phylogenetic studies (Górniak et al., 2010; Givnish et al., 2015). Details of the plant material studied, and voucher information are provided in Table 3.1 and a complete list of markers included from each sample is provided in Appendix 3.1.

Table 3.1 Details of plant material used in this study. New accessions generated for this study are indicated in bold. Accessions included in divergence age estimations are indicated with an asterisk.

Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>B. argyropus</i> (Endl.) Rchb.f.	<i>Adelopetalum</i> (Fitzg.) J.J. Verm. (1993)	CANB: Orchid Research Group 5613	CNS_G00333	—	x
<i>B. argyropus</i>	<i>Adelopetalum</i>	CANB: Orchid Research Group 7052	CNS_G03566*	x	—
<i>B. baronii</i> Ridl.	<i>Ploiarium</i> Schltr. (1925)	CANB: Orchid Research Group 6242	CNS_G06040*	x	—
<i>B. boonjee</i> B. Gray & D.L. Jones	<i>Adelopetalum</i>	CANB: Jones, D.L. 4226	CNS_G03563	—	x
<i>B. boonjee</i>	<i>Adelopetalum</i>	CANB: Gray, B. 9761	CNS_G07175*	x	—
<i>B. bracteatum</i> (Fitzg.) F.M. Bailey	<i>Adelopetalum</i>	CANB: Orchid Research Group 5159	CNS_G00383	—	x
<i>B. bracteatum</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 497	CNS_G01553*	x	—
<i>B. ciluliae</i> Bianch. & J.A.N. Bat.	<i>Xiphizusa</i> Rchb.f. (1852)	HUEFS: Smidt, E.C. & al. 805	ITS:GQ339698*	—	x
<i>B. corythium</i> N. Hallé	<i>Adelopetalum</i>	CANB: Clements, M.A. 11238	CNS_G00370*	—	x

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Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>B. pallidiflorum</i> Schltr.	<i>Polymeres</i> J.J.Verm. & O'Byrne (2008)	CANB: Clements, M.A. 11200	CNS_G05215*	x	—
<i>B. elisae</i> (F. Muell.) Benth.	<i>Adelopetalum</i>	CNS: Simpson, L. 498	CNS_G01548	—	x
<i>B. elisae</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 499	CNS_G01549	—	x
<i>B. elisae</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 500	CNS_G01550*	—	x
<i>B. evasum</i> T.E. Hunt & Rupp	<i>Brachystachyae</i> Benth. & Hook.f. (1883)	CANB: Gray, B. 8744	CNS_G01247*	x	—
<i>B. exiguum</i> F. Muell.	<i>Adelopetalum</i>	CNS: Schulte, K. 121	CNS_G00234	—	x
<i>B. exiguum</i>	<i>Adelopetalum</i>	CANB: 667983	CNS_G00322	—	x
<i>B. exiguum</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 494	CNS_G01558*	—	x
<i>B. globuliforme</i> Nicholls	<i>Minutissima</i> Pfitz. (1889)	CANB: McCraith, G. 86	CNS_G00392	—	x
<i>B. globuliforme</i>	<i>Minutissima</i>	CNS: Simpson, L. 501	CNS_G01584*	—	x
<i>B. hoehnei</i> E.C. Smidt & Borba	<i>Xiphizusa</i>	HUEFS: E.C. Smidt & al. 700	ITS: GQ339700*	—	x
<i>B. humblotii</i> Rolfe	<i>Lupulina</i> Pfitz. (1889)	WU: FS1008	ITS: EF195986* <i>ycf1</i> : KJ558543*	—	x
<i>B. intricatum</i> Seidenf.	<i>Racemosae</i> Benth. & Hook.f. (1883)	CANB: McCraith, G. 83	CNS_G05202*	x	—

Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>B. keekee</i> N. Hallé	<i>Minutissima</i>	CANB: Clements, M.A 5675	CNS_G07329*	x	—
<i>B. lageniforme</i> F.M. Bailey	<i>Adelopetalum</i>	CNS: Simpson, L. 180A	CNS_G04503	—	x
<i>B. lageniforme</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 167B	CNS_G03707*	x	—
<i>B. lageniforme</i>	<i>Adelopetalum</i>	CNS: Schulte, K. 95	CNS_G01630	—	x
<i>B. lilianae</i> Rendle	<i>Adelopetalum</i>	CNS: Schulte, K. 87	CNS_G01632	—	x
<i>B. lilianae</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 496	CNS_G01561	—	x
<i>B. lilianae</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 337E	CNS_G07754*	x	—
<i>B. lingulatum</i> Rendle	<i>Adelopetalum</i>	CANB: Clements, M.A. 7959	CNS_G06046*	x	—
<i>B. lobbii</i> Lindl.	<i>Sestochilus</i> (Breda) Benth & Hook.f. (1883)	CANB: Orchid Research Group 5166	CNS_G05229*	x	—
<i>B. longissimum</i> (Ridl.) J.J.Sm.	<i>Cirrhopetaloides</i> Garay, Hamer & Siegerist (1994)	CANB: Orchid Research Group 5525	CNS_G06041*	x	—
<i>B. maxillare</i> (Lindl.) Rchb.f.	<i>Hoplandra</i> J.J. Verm. (2008)	CANB: Jones, D.L. 3587	CNS_G05267*	x	—
<i>B. maximum</i> (Lindl.) Rchb.f.	<i>Megaclinium</i> (Lindl.) Summerh (1921)	CANB: Orchid Research Group 6250	CNS_G05289*	x	—
<i>B. minutissimum</i> (F. Muell.) F. Muell.	<i>Minutissima</i>	CNS: Simpson, L. 502	CNS_G01585	—	x

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Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>B. minutissimum</i>	<i>Minutissima</i>	CNS: Simpson, L. 503	CNS_G01587*	x	—
<i>B. moniliforme</i> E.C. Parish & Rchb.f.	<i>Macrocaulia</i> (Blume) Aver. (1994)	CANB: Clements, M.A. 9605	CNS_G03763*	x	—
<i>B. mucronatum</i> (Blume) Lindl.	<i>Minutissima</i>	CANB: Clements, M.A. 12379	CNS_G05440*	x	—
<i>B. newportii</i> (F.M. Bailey) Rolfe	<i>Adelopetalum</i>	CNS: Simpson, L. 492	CNS_G01562	—	x
<i>B. newportii</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 495	CNS_G01565	—	x
<i>B. newportii</i>	<i>Adelopetalum</i>	CNS: Simpson, L. 155E	CNS_G03754*	x	—
<i>B. cambodianum</i> (Christenson) J.J. Verm., Schuit. & de Vogel	<i>Trias</i> (Lindl.) J.J. Verm., Schuit. & de Vogel (2014)	CANB: Orchid Research Group 3077	CNS_G05275*	x	—
<i>B. ocellatum</i> Cootes & M.A. Clem.	<i>Polymeres</i> (Blume) J.J. Verm. & O'Byrne (2008)	CANB: Clements, M.A. 11603	CNS_G05293*	x	—
<i>B. pervillei</i> Rolfe ex Scott-Elliot	<i>Lupulina</i>	WU: FS818	ITS: EF196049* <i>ycf1</i> : KJ558551*	—	x
<i>B. pygmaeum</i> (Sm.) Lindl.	<i>Minutissima</i>	CANB: Molloy, B.P.J. 062/98	CNS_G04599*	x	—
<i>B. pygmaeum</i>	<i>Minutissima</i>	CANB: Molloy, B.P.J. 134/99	CNS_G03988	—	x
<i>B. rhopalophorum</i> Schltr.	<i>Sphaeracron</i> Schltr. (1913)	CBG: 9013094	CNS_G05261*	x	—

Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>B. schinzianum</i> Kraenzl.	<i>Ptiloglossum</i> Lindl. (1862)	CANB: Clements, M.A. 11554	CNS_G01642*	x	—
<i>B. tuberculatum</i> Colenso	<i>Adelopetalum</i>	CANB: Molloy, B.P.J. 112/99	CNS_G03993	—	x
<i>B. tuberculatum</i>	<i>Adelopetalum</i>	CHR: 572204	CHR572204*	—	x
<i>B. weinthalii</i> ssp. <i>weinthalii</i> R.S. Rogers	<i>Adelopetalum</i>	CNS: Simpson, L. 493	CNS_G01624	—	x
<i>B. weinthalii</i> ssp. <i>striatum</i> D.L. Jones	<i>Adelopetalum</i>	CANB: Orchid Research Group 7186	CNS_G03564	—	x
<i>B. weinthalii</i> ssp. <i>weinthalii</i>	<i>Adelopetalum</i>	BRI: Mathieson, M.T. 730	CNS_G06024*	x	—
<i>Coelogyne flaccida</i> Lindl.		WIS: v0289172	n/a	—	x
<i>Dendrobium brunneum</i> Schuit. & Peter B. Adams		CANB: Orchid Research Group 5485	CNS_G00781*	x	—
<i>Dendrobium cunninghamii</i> Lindl.		CANB: Molloy, B.P.J. 061/98	CNS_G02336*	x	—
<i>Dendrobium fuscescens</i> (Griff.) Summerh.		CANB: Orchid Research Group 6920	CNS_G01021*	x	—

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Species name	Section	Voucher details	DNA / GenBank Accession number	Sequencing method	
				Illumina	Sanger
<i>Dendrobium macropus</i> (Endl.) Rchb.f.		CANB: Ziesing, P.D. 345	CNS_G02316*	x	—
<i>Dendrobium moniliforme</i> (L.) Sw.		CANB: Clements, M.A. 8262	CNS_G00882*	x	—
<i>Dendrobium muricatum</i> Finet		CANB: Clements, M.A. 3093	CNS_G02350*	x	—
<i>Dienia ophrydis</i> (J. Koenig) Seidenf.		CBG: 9306410	CNS_G01303*	x	—
<i>Neottia cordata</i> (L.) Rich.		CANB: Clements, M.A. 9865	CNS_G05314*	x	—
<i>Nervilia concolor</i> (Blume) Schltr.		CANB: Roberts, L. ORG 3787	CNS_G03442*	x	—
<i>Oberonia complanata</i> (A. Cunn.) M.A. Clem. & D.L. Jones		CNS: 135322	CNS_G00309*	x	—

3.2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted as described previously (Chapter two, section 2.2.2).

3.2.2.1. Illumina sequencing

To clarify broad-level phylogenetic relationships in *Bulbophyllum*, shotgun high-throughput sequencing was carried out for 25 *Bulbophyllum* species to recover plastid protein-coding regions (cds) and high copy nuclear ribosomal regions (Table 3.1, Appendix 3.1). Libraries were constructed and sequenced as described previously (Chapter two, section 2.2.2.2).

3.2.2.2. Sanger sequencing

To examine interspecific relationships, sequences of three nuclear ribosomal regions (ITS1, 5.8s, ITS2) and two plastid cds (*matK*, *ycf1*) generated for 24 additional samples were included for *Bulbophyllum* sections *Adelopetalum* and *Minutissima* (Appendix 3.1). Amplification and sequencing of the nuclear ribosomal ITS region was carried out as described previously (Chapter two, section 2.2.2.2). Amplifications for *matK* were carried out with the primers 19F and, 1326R (Cuénoud et al., 2002), and for *ycf1* with primers 3720F, intR, intF, 5500R (Neubig et al., 2008). PCR reactions were carried out in 20 µL volumes with 4 µL 5x High-Fidelity Buffer, 0.8 µL each of forward and reverse primer (10µM), 0.4µL dNTPs (10 mM), 0.6µL DMSO, 0.25 µL iProof High-Fidelity DNA polymerase (5U/µL), (Thermo Fisher Scientific, Waltham, USA), 11.15 µL H₂O, and 2 µL gDNA (ca. 5 ng/µL). For *matK*, an initial denaturation step was carried out at 98 °C for 45 sec followed by 35 cycles of 98 °C denaturation for 10 sec, 60 °C annealing for 45 sec and 72 °C extension/elongation for 45 sec, with a final extension at 72 °C for 10 min. For *ycf1*, PRC reactions were carried out with the same conditions as for *matK*, except for the annealing temperature which was set to 63 °C. PCR products were cleaned and sequenced as described previously (Chapter two, section 2.2.2.2).

3.2.2.3. Assembly and alignment

Sequences were assembled and edited in Geneious R10 (Kearse et al., 2012). Illumina sequences were assembled to a reference plastid cds set extracted from *Dendrobium catenatum* (GenBank accession number KJ862886) and for *ycf68* from *Anoectochilus roxburghii* (KP776980). To build a reference for the nuclear ribosomal ITS-ETS region, Illumina reads of *B. boonjee* (CNS_G07175) were first mapped to the ITS-ETS region of *Corallorhiza trifida* (JVF2676a). To extend the region assembled, the *B. boonjee* Illumina reads were then mapped to the *B. boonjee* consensus sequence generated in the initial step, yielding a *B. boonjee* reference including seven nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). Illumina sequences for all other samples were assembled against the resulting *B.*

boonjee ETS-ITS reference. Assemblies were carried out with the highest quality threshold and a minimum coverage of ten reads. The quality of the assemblies was checked and edited manually where required. For the Sanger sequences, bidirectional reads were assembled in Geneious and edited manually. Additional sequences were sourced from GenBank for *Bulbophyllum* sect. *Pantoblepharon*, sect. *Lupulina*, and sect. *Xiphizusa* (Fischer et al., 2007; Smidt et al., 2011; Gamisch et al. 2015), and from DRYAD (<https://doi.org/10.5061/dryad.n9r58>) for *Coelogyne flaccida* (Givnish et al., 2015) (Table 1., Appendix 3.1). DNA sequences were combined into supermatrices for the nuclear ribosomal ITS-ETS region and each 70 plastid cds. Alignments were carried out for each region separately using MAFFT v.7.222 (Katoh et al., 2005, 2002) with the default settings, and then concatenated into matrices. Two matrices were created: 1) a nuclear supermatrix including seven ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) comprising 64 accessions (alignment length: 6241 bp, number of variable sites: 1267 (20%)), and 2) a plastid supermatrix including 70 plastid coding regions, including 62 accessions (alignment length: 61719 bp, no. of variable sites: 15194 (25%)). For divergence time estimations, sampling was reduced to one representative per species (indicated by an asterisk in Table 3.1), and two alignments were analysed: 1) a plastid supermatrix including 70 plastid coding regions, comprising 43 accessions (alignment length: 61719bp, no. of variable sites: 15194 (25%)), and 2) a nuclear alignment including three ribosomal markers (ITS1, 5.8s, ITS2) including 45 accessions (alignment length: 747bp, no. of variable sites: 547 (73.2%)).

3.2.3. Phylogenetic analysis

Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference. Reconstructions were carried out based on a GTR gamma substitution model identified among the top performing models in tests carried out in MrModeltest2 v.2.3 (Nylander, 2004) on preliminary plastid and nuclear datasets (Appendix 2.1). ML analyses were carried out in the program RAxML v. 8.2.4 (Stamatakis, 2014) applying the rapid hill climbing algorithm with a GTR gamma nucleotide substitution model. Bootstrap analyses were conducted with 1,000 replicates under the same settings.

Bayesian analyses were conducted in MrBayes 3.2.5 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) applying a GTR gamma model. Three independent MCMC runs with three heated chains each were carried out for 5×10^6 generations sampling trees every 1×10^4 generations. Convergence of independent runs was confirmed by the average standard deviation of split frequencies dropping below 0.01. Bayesian all compatible

consensus trees with posterior probability values of nodal support were constructed excluding the first 1.25×10^6 generations as burn-in.

3.2.4. Divergence time estimation

Divergence times were estimated applying a GTR gamma substitution model (Tavare, 1986) as implemented in BEAST2 v. 2.4.8 (Bouckaert et al., 2014). We tested two clock models 1) strict clock (Zuckerkandl and Pauling, 1965) and 2) relaxed lognormal clock (Drummond et al., 2006) and two models of speciation and extinction: 1) Yule and 2) birth, death (Gernhard et al., 2008; Yule, 1925). To determine the model of best fit, marginal likelihoods were estimated in Tracer v1.6, using a posterior simulation-based analog of Akaike's information criterion through Markov Chain Monte Carlo (AICM), with 1000 bootstrap replicates (Beale et al., 2012). Three secondary calibration points and one fossil were used for calibration as described previously (Chapter two, section 2.2.4). To assess convergence of independent runs and determine burn in fractions, log files were assessed in Tracer v.1.5.0 (Rambaut and Drummond, 2007), ensuring the effective sample size of all parameters was above 200. From the analyses based on the 70 plastid cds dataset, nine runs reached convergence and were combined in LogCombiner (from the BEAST package) with a sampling frequency of 8000, excluding a cumulative burn in fraction of 16%. A maximum clade credibility tree was generated in TreeAnnotator (BEAST package) with median node heights. From the analyses based on the ITS nuclear ribosomal dataset, all runs reached convergence and were combined in LogCombiner with a sampling frequency of 10000, excluding a 10% burn in fraction (3×10^6 generations for each run).

3.2.5. Ancestral range analysis

The distribution of species of *Bulbophyllum* sect. *Adelopetalum* and closely related species from sect. *Minutissima* was assessed from 881 distribution records downloaded from the Atlas of Living Australia (www.ala.org.au). Six geographic regions were coded based on major disjunctions: A) Australian east coast, B) Norfolk Island, C) Lord Howe Island, D) New Caledonia, and E) New Zealand. Ten species occur on Australia's east coast (*B. boonjee*, *B. bracteatum*, *B. elisae*, *B. exiguum*, *B. globuliforme*, *B. lageniforme*, *B. lilianae*, *B. minutissimum*, *B. newportii* and *B. weinthalii*). One species is found along Australia's east coast, on Norfolk Island, and Lord Howe Island (*B. argyropus*). Two species occur in New Zealand (*B. pygmaeum* and *B. tuberculatum*) and one species in New Caledonia (*B. corythium*). Ancestral ranges were estimated from 100 randomly selected maximum clade credibility trees obtained from the BEAST analysis of the plastid supermatrix, pruned of the outgroups to sect.

Adelopetalum and its closest relatives from sect. *Minutissima*, in RASP v. 4.0 (Yu et al., 2015). Ancestral ranges were estimated based on 1) the statistical dispersal-vicariance approach S-DIVA (Yu et al. 2010), and 2) the statistical dispersal-extinction cladogenesis model S-DEC (Beaulieu et al., 2013)., replicating the key assumptions outlined in the non-parametric dispersal-vicariance approach (Harris and Xiang, 2009; Nylander et al., 2008; Ronquist, 1997; 2001; Ronquist and Sanmartín, 2011) and the dispersal-extinction cladogenesis model (DEC) (Ree and Smith, 2008) respectively, and also take into account uncertainty in tree topology. No constraints were applied to dispersal direction or the maximum number of ranges allowed at ancestral nodes. To determine the model of best fit, model testing was carried out in the program RASP using the package BIOGEOBEARS (Matzke, 2013a) and the resulting likelihood values for the DIVALIKE and DEC models were compared using the AIC score (Akaike, 1974).

3.2.6. Ancestral trait reconstruction

To reconstruct ancestral trait evolution, eighteen morphological characters were coded for each species of sect. *Adelopetalum* and its closest relatives from sect. *Minutissima*. Characters were: 1) nodes between pseudobulbs, 2) pseudobulb shape, 3) pseudobulb surface, 4) pseudobulb ribs, 5) pseudobulb sheath, 6) leaf surface, 7) leaf margin, 8) leaf petiole, 9) photosynthetic pit at the leaf base, 10) number of flowers per inflorescence, 11) peduncle surface, 12) ovary surface, 13) tepal surface, 14) number of veins in petals, 15) labellum shape, 16) wings along lower margin of the stelidia, 17) shape of wings along lower margin of the stelidia, and 18) attachment of the labellum. Details of character states and character matrix are provided in the Appendix 3.11. The ancestral character analyses were based on the chronogram produced in BEAST, pruned of outgroups. Character states at ancestral nodes were estimated in Mesquite v. 3.04 (Maddison and Maddison, 2015) using maximum likelihood as optimization criterion.

3.2.7. Genome size estimation

To estimate absolute genome sizes (2C-values, Greilhuber et al., 2005) within sect. *Adelopetalum*, flow cytometric measurements were conducted for eight *Bulbophyllum* species with one to four individuals per species, including a total of 18 samples. The measurements were carried out with fresh leaves using a CyFlow space (Partec, Münster, Germany) fitted with 30 mW green solid-state laser (532 nm). Leaves of the analysed sample and internal standard (*Pisum sativum* cv. "Ctirad"; (2C=9.09 pg) Doležel et al., 1998) were co-chopped using a razor blade in a plastic Petri-dish containing 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto, 1990). The suspension was filtered through Partec CellTrics® 30µm in order to remove tissue debris and incubated for at least 5 min at room temperature. Isolated

nuclei in filtered suspension were stained with 1 ml of Otto II buffer (0.4 M $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$) containing propidium iodide (PI) and RNase IIA (both at final concentrations of $50 \mu\text{g} \cdot \text{ml}^{-1}$). Fluorescence intensities of at least 5000 particles (nuclei) per measurement were recorded and nuclei counts for both the samples and the internal standard were approximately the same. Three to four replicate measurements of each sample were carried out on different days to minimize potential random instrumental error. Fluorescence histograms were visualized using the FloMax v2.4d software (Partec, Münster, Germany). Only histograms with coefficients of variation $< 4 \%$ for the G_0/G_1 peak of the sample were considered. 2C-values were inferred on the basis of the sample/standard fluorescence histogram ratio using the formula given by Doležal and Bartoš (2005).

3.3. Results

3.3.1. Phylogenetic relationships

3.3.1.1. Relationships reconstructed from plastid coding regions

The ML and Bayesian phylogenies based on the 70 loci plastid supermatrix were well resolved and supported and congruent with each other (Fig. 1 and Appendix 3.2). The monophyly of tribe Dendrobieae and its sister group relationship with Malaxideae received maximum support (BS 100, PP 1). The monophyly of *Dendrobium s.l.* and *Bulbophyllum* and their sister relationship received maximum support. Within *Bulbophyllum*, all species of sect.

Adelopetalum and sect. *Minutissima* species from Australia (*B. minutissimum*, *B. globuliforme*), New Zealand (*B. pygmaeum*) and New Caledonia (*B. keekee*), formed a clade that received moderate to high support (BS 95, PP 1). The clade is predominantly distributed within the Australasian floristic continent, including Australia and New Zealand (Brummitt, 2001) and is therefore termed here the Australasian clade. The Australasian clade was resolved in sister group relationship to a moderately supported clade including the remainder of the genus (BS 83, PP 1). The latter group split into two highly supported clades: an Afrotropical clade (BS 100, PP 1) and an Asian clade (BS 100, PP 1), which was composed of species from Asia plus one species, *B. evasum*, (sect. *Brachystachyae*), from Australasia.

Within the Australasian clade six main lineages were resolved with high support: 1) minutissimum clade, 2) *B. pygmaeum*, 3) bracteatum clade, 4) *B. weinthalii*, 5) argyropus clade, and 6) newportii clade. The minutissimum clade (BS 100, PP 1), comprising *B. minutissimum*, *B. globuliforme*, and *B. keekee* (sect. *Minutissima*), was resolved in a sister group relationship to the remainder of the Australasian clade. The five remaining lineages together formed the highly supported *Adelopetalum* clade (BS 95, PP 1) which comprised all species of sect. *Adelopetalum* plus *B. pygmaeum* (sect. *Minutissima*). *Bulbophyllum pygmaeum* formed a weakly to moderately supported sister group relationship (BS <50, PP 0.98) with a highly supported bracteatum clade (BS 91, PP 1), comprising *B. bracteatum*, *B. boonjee*, and *B. elisae*. Within the bracteatum clade, *B. elisae* was sister to a highly supported clade (BS 100, PP 1) unifying *B. bracteatum* and *B. boonjee* (Fig. 1). *Bulbophyllum weinthalii* (sect. *Adelopetalum*) formed a highly supported sister group relationship (BS 99, PP 1) with a highly supported argyropus clade (BS 100, PP 1), including *B. argyropus*, *B. corythium*, and *B. tuberculatum* (sect. *Adelopetalum*).

Figure 3.1 Maximum likelihood phylogenetic reconstruction of 51 Bulbophyllum samples based on the supermatrix of 70 plastid coding regions. Bootstrap values and posterior probabilities are given adjacent to nodes.

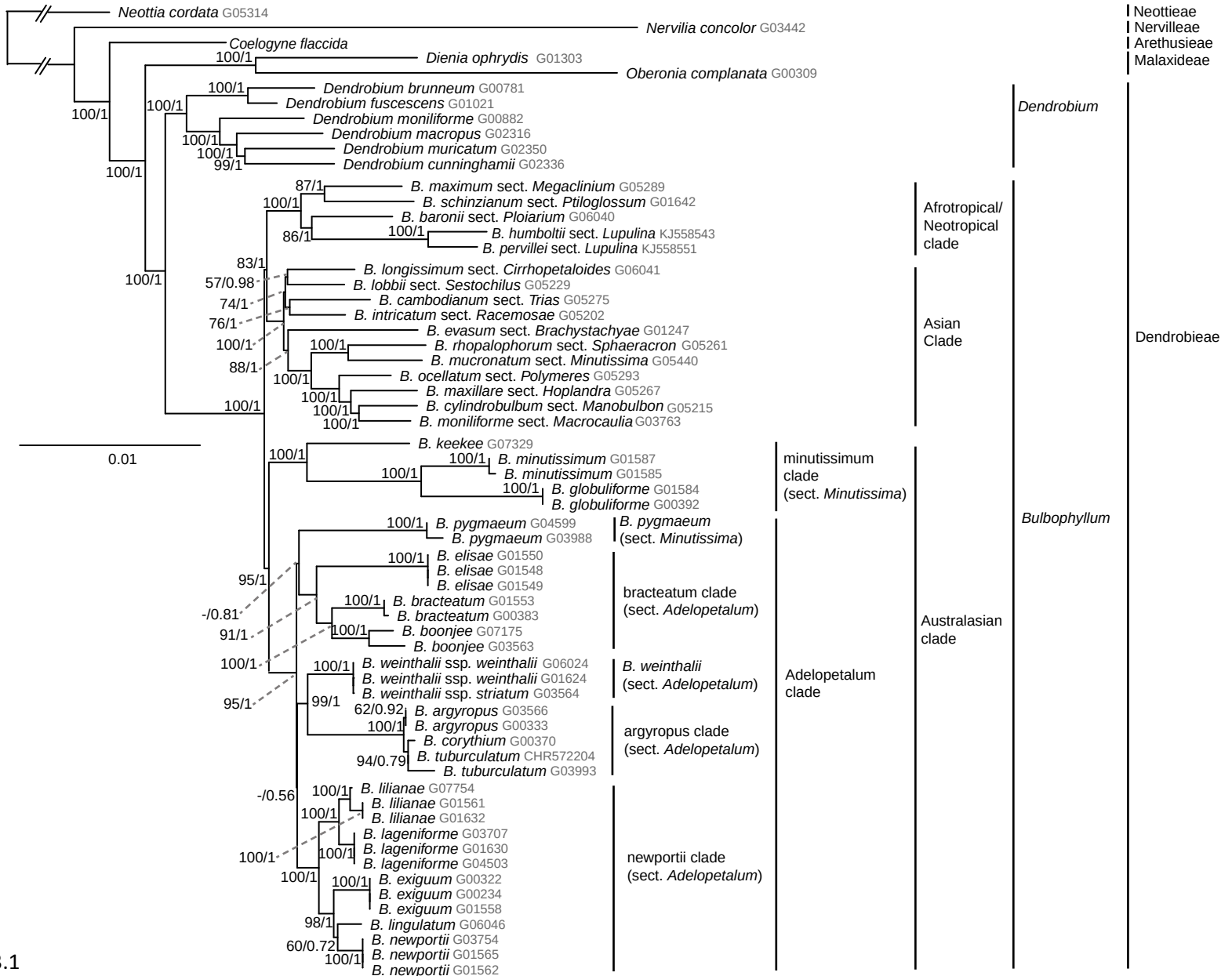


Fig. 3.1

The highly supported newportii clade (BS 100, PP 1) included *B. lageniforme*, *B. lilianae*, *B. lingulatum*, *B. exiguum*, and *B. newportii*, which split into a dichotomy between two highly supported clades. The first clade (BS 100, PP 1) included *B. lageniforme* and *B. lilianae* and the second clade (BS 98, PP 1) was composed of *B. exiguum* and a weakly to highly supported group (BS 98, PP 0.71) with *B. lingulatum* and *B. newportii*. Interspecific relationships within the Australasian clade were largely well resolved, however relationships among the main lineages within the Adelopetalum clade remained partly unclear due to low support values. Each species formed a highly supported clade, except within the argyropus clade among the closely related species *B. argyropus*, *B. corythium*, and *B. tuberculatum*. *Bulbophyllum argyropus* samples were unified in a poorly to moderately supported group (BS 62, PP 0.92). *Bulbophyllum corythium* and *B. tuberculatum* were part of a highly supported clade (BS 94, PP 1), however, relationships within this clade were unresolved. Section *Minutissima* was found to be polyphyletic, with the Asian species, *B. mucronatum*, found within the Asian clade, while the Australasian species, including the sectional type species *B. minutissimum*, were included in the Australasian clade. Within the Australasian clade, *B. pygmaeum* (sect. *Minutissima*) was nested within the highly supported Adelopetalum clade, rendering sect. *Adelopetalum* paraphyletic.

3.3.1.2. Relationships reconstructed from nuclear ribosomal markers

ML and Bayesian tree reconstructions based on seven nuclear ribosomal markers (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) showed several moderately to highly supported clades. These corresponded to highly supported clades in the 70 loci plastid phylogenies including *Bulbophyllum* (BS 93, PP 1), Afrotropical/Neotropical clade (BS 85, PP 0.98), Asian clade (BS 92, PP 1), Australasian clade (BS 94, PP 1), bracteatum clade (BS 82, PP 0.95), argyropus clade (BS 100, PP 1), minutissimum clade (BS 85, PP 1), newportii clade (BS 65, PP 1) (Fig. 2 and Appendix 3.3). However, some relationships reconstructed among these groups were incongruent with the topology reconstructed from the plastid dataset.

Figure 3.2 Maximum likelihood phylogenetic reconstruction of 53 Bulbophyllum samples based on seven nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). Bootstrap values and posterior probabilities are given adjacent to nodes.

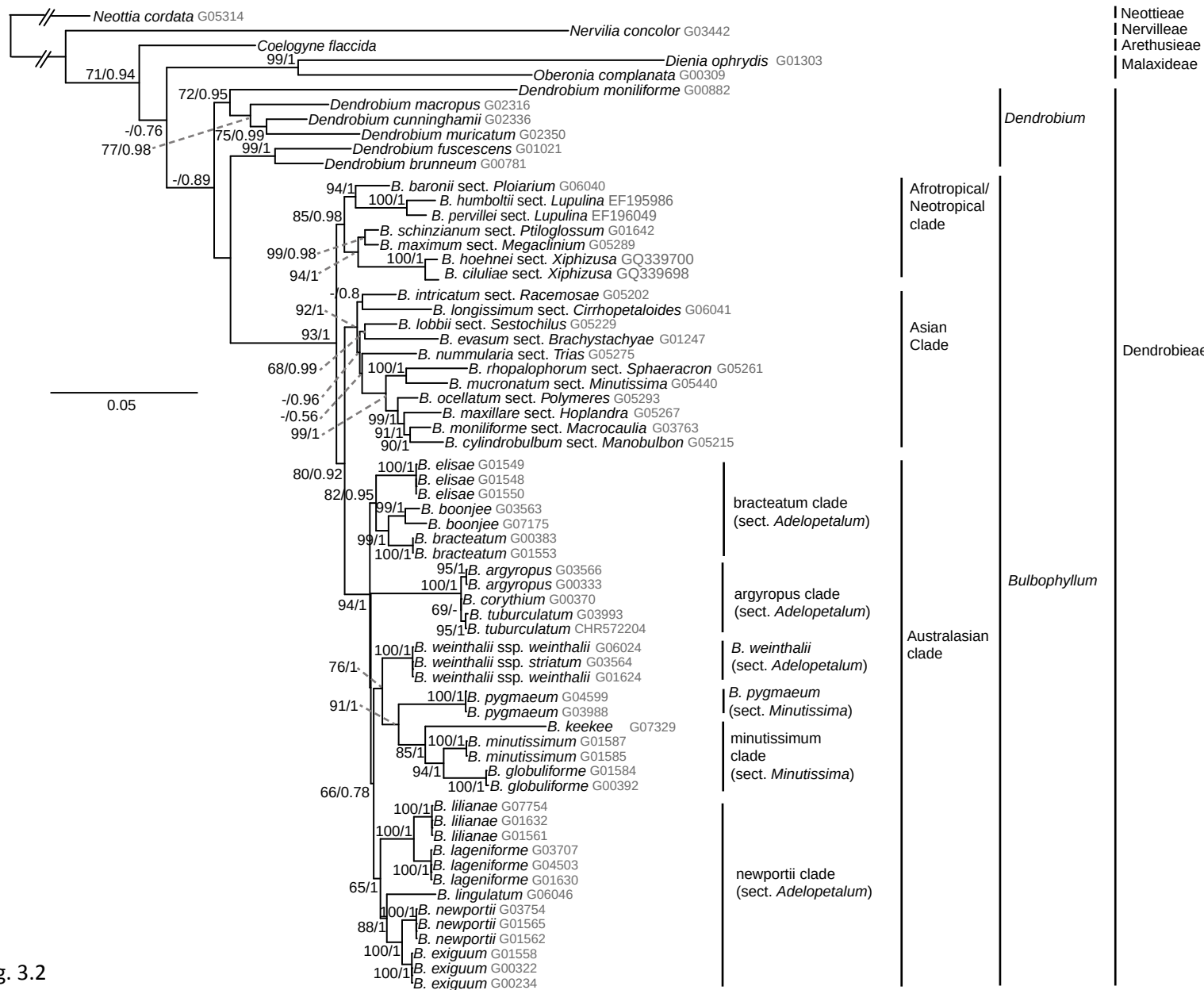


Fig. 3.2

One incongruence between the plastid and nuclear phylogenies concerned the relationships among the Australasian, Asian and Afrotropical/Neotropical lineages within the genus. Relationships based on the plastid phylogeny reconstructed the Afrotropical and Asian lineages in a moderate to highly supported group (BS 83, PP 1), which was resolved in a sister group relationship to the highly supported Australasian clade (BS 95, PP 1). However, in reconstructions based on the nuclear ribosomal regions, the Asian and Australasian clade were resolved in a moderately supported relationship (BS 80, PP 0.92), and together formed a sister group relationship with the moderately supported Afrotropical/Neotropical clade (BS 85, PP 0.98).

Within the Australasian clade, the phylogenetic position of *B. pygmaeum* and *B. weinthalii* was incongruent between the plastid and nuclear phylogenies. In phylogenetic reconstructions based on the plastid dataset, *B. pygmaeum* was nested within the highly supported Adelopetalum clade (BS 95, PP 1). However, relationships reconstructed from the nuclear dataset resolved *B. pygmaeum* together with the minutissimum clade, forming a highly supported group (BS 91, PP 1) including all Australasian species of sect. *Minutissima*. In phylogenetic reconstructions based on the plastid dataset, *B. weinthalii* formed a highly supported sister group relationship with the argyropus clade. However, relationships reconstructed from the nuclear dataset resolved *B. weinthalii* in a highly supported sister group relationship to a clade comprising the Australasian sect. *Minutissima* species (*B. pygmaeum* + the minutissimum clade). Similar to relationships resolved in the plastid phylogeny, reconstructions based on the nuclear dataset showed sect. *Minutissima* as polyphyletic, with the Asian species nested within the Asian clade, while the Australasian species were reconstructed within the Australasian clade.

3.3.2. Divergence time estimation

The divergence dating analysis based on a relaxed log normal clock and birth death prior on speciation and extinction was identified as the model of best fit based on marginal likelihoods estimated for alternative models (Appendix 3.4). The divergence dating reconstructions based on the relaxed log normal clock and birth death prior estimated similar divergence ages for major clades in analyses based on 70 plastid cds or three nuclear ribosomal markers (Fig. 3.3, Appendix 3.5).

Figure 3.3 Maximum clade credibility chronogram for Bulbophyllum based on 70 plastid coding regions. Black circles indicate highly supported nodes (PP > 0.95). Divergence dates and 95 % highest posterior density values are indicated adjacent to nodes. Grey bars indicate 95% highest posterior density. The asterisk denotes the node constrained with a fossil calibration point, the diamond denotes nodes which were constrained by secondary calibration points.

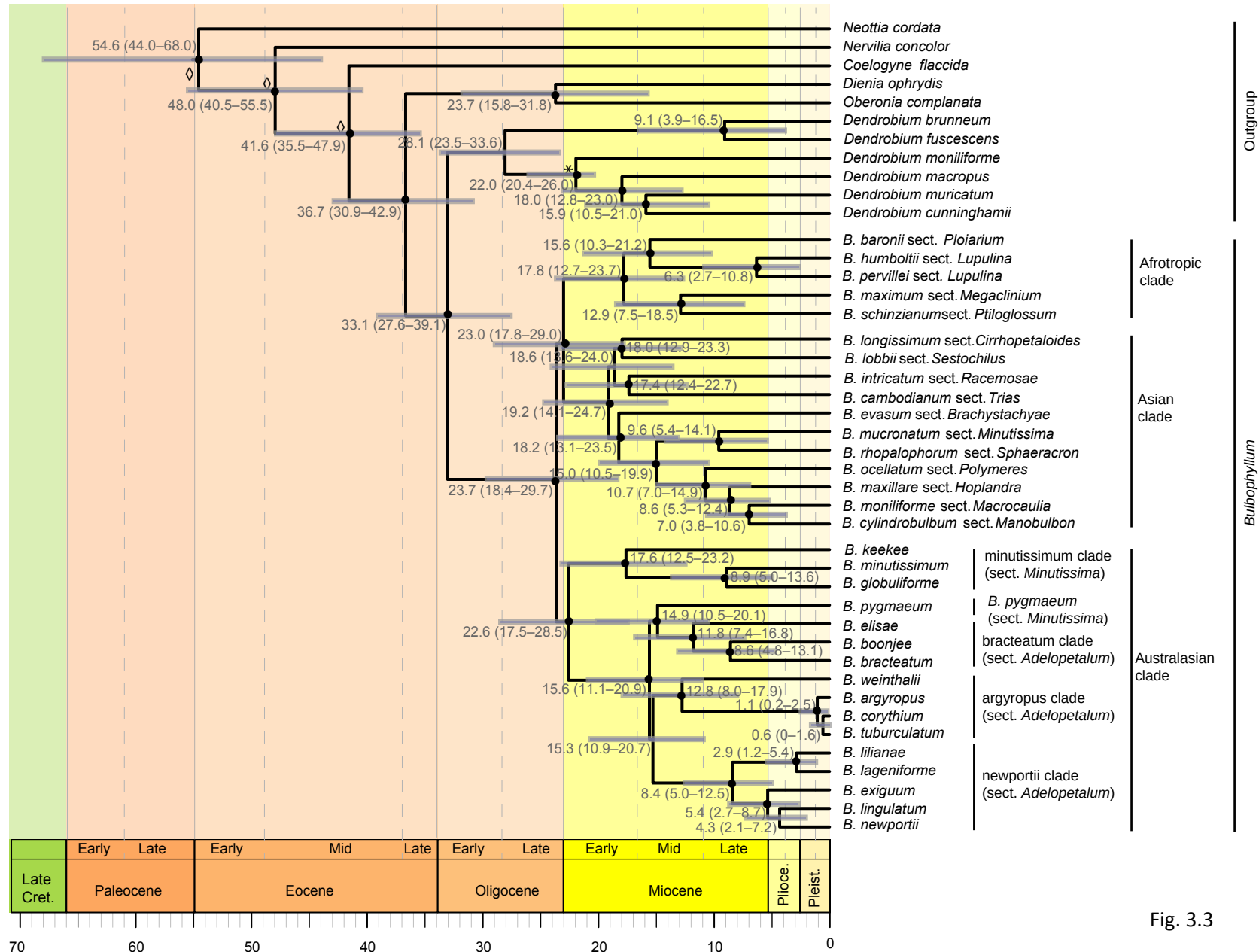


Fig. 3.3

The divergence dating analysis based on the plastid dataset was well resolved and highly supported (Fig. 3.3). Backbone relationships reconstructed in the nuclear dataset were less well supported (Appendix 3.5). The 95% HPD intervals were generally larger in the analysis based on the nuclear dataset and usually were included within the 95% HPD ranges estimated from the analysis based on the plastid dataset. In the following results from the divergence time estimations based on 70 plastid cds are presented.

According to the divergence time estimation based on 70 plastid cds (Fig. 3.3), *Bulbophyllum* diverged from *Dendrobium* in the early Oligocene, ca. 33.1 Ma (95% highest posterior probability density, HPD: 27.6–39.1). The crown age of *Bulbophyllum* was dated to the late Oligocene, ca. 23.7 Ma (HPD: 18.4–29.7), with the divergence between the Australasian clade from the remainder of the genus. Divergence between the Asian clade and the Afrotropical/Neotropical clade was estimated to have taken place during the early Miocene, ca. 23.0 Ma (HPD: 17.8–29.0). The crown age of the Australasian clade was estimated to the early Miocene, ca. 22.6 Ma (HPD: 17.5–28.5), with the split of the minutissimum clade from the Adelopetalum clade, comprising the remainder of the Australasian clade. The crown age of the Adelopetalum clade was dated to the mid-Miocene, ca. 15.6 Ma (HPD: 11.1–20.9). The stem age for 1) the bracteatum clade was dated to ca. 14.9 Ma (HPD: 10.5–20.1); 2) the lineage giving rise to *B. weinthalii* to ca. 12.8 (8.0–17.9); 3) the argyropus clade to ca. 12.8 (8.0–17.9); and the newportii clade to ca. 15.3 Ma (HPD: 10.9–20.7), in the mid-Miocene.

Within the minutissimum clade, crown divergence commenced in the early Miocene ca. 17.6 Ma (node g., HPD 12.5–23.2) with the divergence of *B. keekee* from the ancestor of the lineage that gave rise to *B. minutissimum* and *B. globuliforme*. Divergence between *B. minutissimum* and *B. globuliforme* was estimated to have occurred in the late Miocene, ca. 8.9 Ma (HPD: 5.0–13.6). Diversification within the bracteatum clade commenced during the mid-Miocene, with the split of the lineage that gave rise to *B. elisae* ca. 11.8 Ma (HPD: 7.4–16.8). The stem ages of lineages giving rise to 1) *B. bracteatum* and 2) *B. boonjee* were estimated to late Miocene ca. 8.6 Ma (HPD: 4.8–13.1). The crown age of the argyropus clade, comprising the closely related species *B. argyropus*, *B. corythium*, and *B. tuberculatum*, was dated to the late Pleistocene, ca. 1.1 Ma (HPD: 0.2–2.5). Divergence within the newportii clade commenced in the late Miocene, ca. 8.4 Ma (HPD: 5.0–12.5). *Bulbophyllum exiguum* was estimated to have diverged from the lineage including *B. lingulatum* and *B. newportii* at the start of the Pliocene, ca. 5.4 Ma (HPD: 2.7–8.7), with divergence between the latter two species estimated during the early Pliocene ca. 4.3 Ma (HPD: 2.1–7.2). Divergence between *B. lageniforme* and *B. lilianae* was dated to the late Pliocene, ca. 2.9 Ma (HPD: 1.2–5.4) (Fig. 3.3).

3.3.3. Ancestral range reconstruction

The ancestral ranges estimated based on the DIVALIKE or DEC model yielded largely similar results (Fig. 3.4, Appendix 3.9). The most likely ancestral range reconstructed was consistent for respective nodes in each analysis, with some variation in the relative probabilities assigned to alternative ancestral range scenarios. The AIC model selection identified the DIVALIKE as the best fit yielding the lowest AIC score of 64.8, compared to the DEC with an AIC score of 73.4 (Appendix 3.6). Here results are reported from the analysis based on the dispersal-vicariance model.

Ancestral range estimation placed the most probable origin of the Australasian clade in mainland Australia (rel. prob. 0.62). The second most probable ancestral range reconstructed was a widespread distribution including Australia and New Caledonia (rel. prob. 0.24), whereas all other alternative range scenarios received low relative probabilities (<0.05) (Fig. 3.4). The analysis inferred that mainland Australia has been largely maintained as the ancestral range within the Australasian clade. At least three independent range expansions and one range shift were inferred eastwards to other Australasian landmasses: to Norfolk Island and Lord Howe Island, to New Zealand, and to New Caledonia (Fig. 3.4). A range expansion from Australia to New Caledonia was inferred in lineage that gave rise to the MRCA of the minutissimum clade (rel. prob. 0.70), during the early Miocene, between ca. 22.6–17.6 Ma. Subsequent lineage divergence led to a solely New Caledonian distribution for the lineage that gave rise to *B. keekee* between the early and late Miocene, ca. 17.6–8.9 Ma, whereas Australia was the ancestral range inferred for the MRCA of *B. minutissimum* and *B. globuliforme*. Alternatively, the Australian ancestral range was maintained in the MRCA of the minutissimum clade (rel. prob. 0.19), as well as the MRCA of *B. minutissimum* and *B. globuliforme*, and a range shift to New Caledonia occurred in the lineage giving rise to *B. keekee* from the early Miocene ca. 17.6 Ma onwards.

A range shift from Australia to New Zealand was inferred in the lineage giving rise to *B. pygmaeum* from the mid-Miocene (ca. 14.9 Ma) onwards, while the ancestral Australian range was conserved in the MRCA of the bracteatum clade (rel. prob. 100%). Alternatively, range expansion from Australia to New Zealand was inferred for the MRCA of *B. pygmaeum* and the bracteatum clade (rel. prob. 38.1%) in the mid-Miocene, with subsequent lineage divergence in the mid-Miocene, ca. 14.9–11.8 Ma resulting in a distribution of *B. pygmaeum* in New Zealand and the MRCA of the bracteatum clade in Australia. Range expansion was

reconstructed in the lineage that gave rise to the MRCA of the argyropus clade, from Australia to a widespread distribution including Australia, New Caledonia, New Zealand, and Lord Howe and Norfolk Island from the mid-Miocene ca. 12.8 Ma to the Pleistocene, ca. 1.1 Ma (rel. prob. 0.72). Alternative ranges presented for the MRCA of the argyropus clade included 1) Australia, New Caledonia, New Zealand and Norfolk Island (0.10), or 2) Australia, New Caledonia, New Zealand and Lord Howe Island (0.10), while all other ranges received relative probabilities below 0.02. Range reduction was inferred subsequently from the late Pleistocene, ca. 1.1 Ma, onwards, to: 1) Australia, Lord Howe and Norfolk Island in the lineage giving rise to *B. argyropus*, 2) New Caledonia in the lineage that gave rise to *B. corythium* and 3) New Zealand in the lineage that gave rise to *B. tuberculatum*. Within the newportii clade, the ancestral area analysis reconstructed a range expansion from Australia to New Caledonia in the lineage that gave rise to the MRCA of *B. newportii* and *B. lingulatum*, during the early Pliocene between ca. 5.4 to 4.3 Ma, with range contraction occurring subsequently, to Australia in the lineage that gave rise to *B. newportii*, and to New Caledonia in the lineage that gave rise to *B. lingulatum*. Alternatively, the ancestral Australian range was maintained in the MRCA of *B. newportii* and *B. lingulatum* (rel. prob. 0.16), and a range shift from Australia to New Caledonia was inferred in the lineage that gave rise to *B. lingulatum* from the early Pliocene, ca. 5.4 Ma, onwards.

Figure 3.4 Historical biogeography of the Australasian clade. A) ancestral area reconstruction based on a dispersal-vicariance like model; b) geographic regions delineated in the biogeographic analysis; c) legend of colour-coded geographic regions and shared ancestral areas. Values adjacent to nodes indicate bootstrap support.

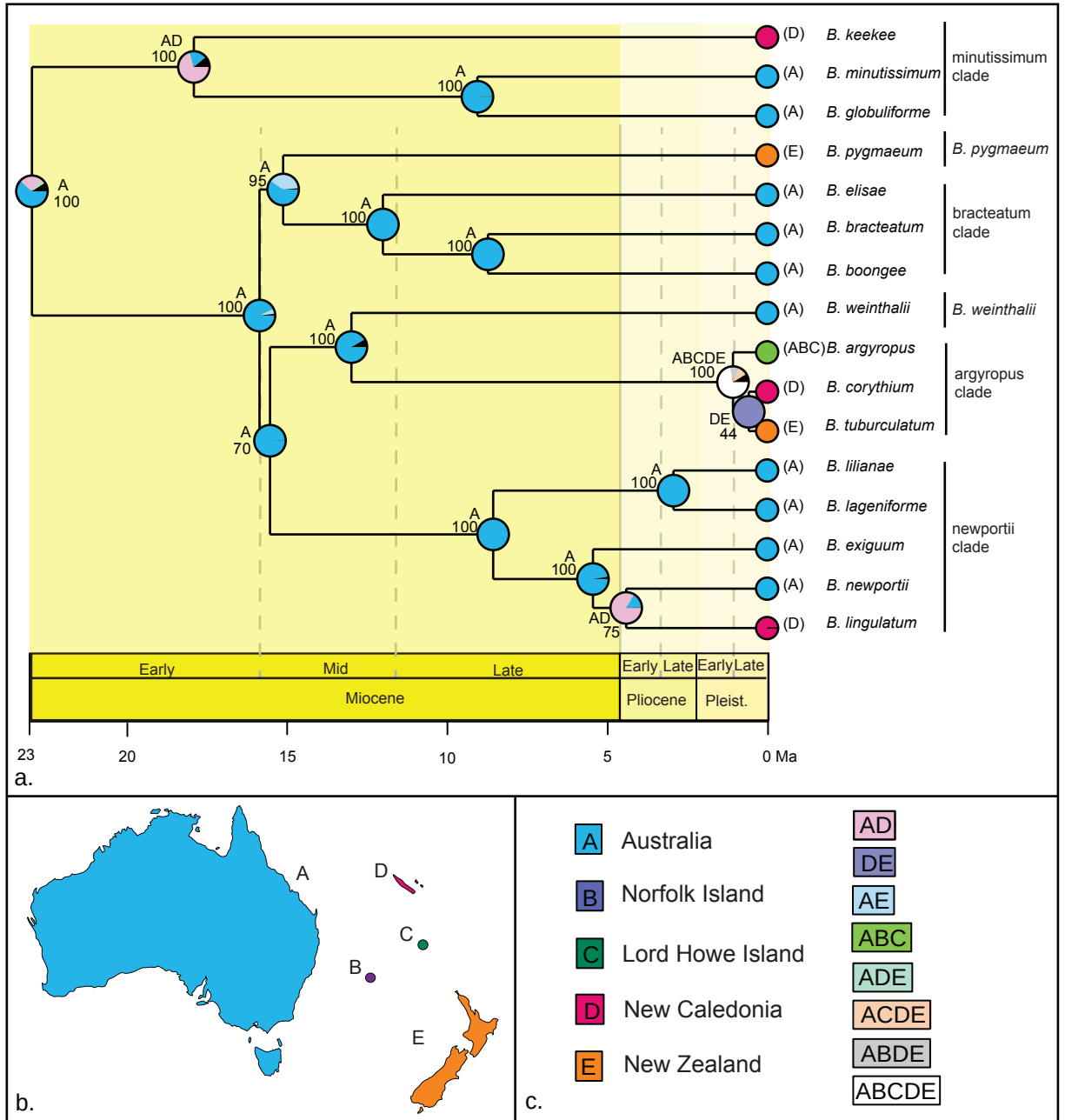


Fig. 3.4

3.3.4. Ancestral character reconstruction

Photosynthetic pit at the base of the leaf: a photosynthetic pit at the base of the leaf was inferred to have evolved once in the most recent common ancestor (MRCA) of sect.

Minutissima species from Australia, B. minutissimum and B. globuliforme, while the absence of a photosynthetic pit was reconstructed as the ancestral state for the Adelopetalum clade and the most likely ancestral state for the MRCA of the minutissimum clade and the Australasian clade (Fig. 5a).

Leaf petiolation: character state reconstructions inferred that a sessile leaf evolved once in the MRCA of sect. *Minutissima species from Australia, B. minutissimum and B. globuliforme*, while a petiolate leaf was inferred as the ancestral state for the Adelopetalum clade and the most likely ancestral state for the MRCA of the minutissimum clade and the Australasian clade (Fig. 5b).

Nodes between pseudobulbs: one or more nodes between pseudobulbs was inferred as the ancestral state for the Adelopetalum clade and the most likely ancestral state for the minutissimum clade and the Australasian clade (Fig. 5c). The absence of nodes between the pseudobulbs was inferred to have evolved once in the MRCA of sect. *Minutissima species from Australia, B. minutissimum and B. globuliforme* (Fig. 5c).

Peduncle surface: a glabrous peduncle surface was reconstructed as the most likely ancestral state for the Australasian clade, which was maintained in the bracteatum, argyropus and newportii clades, the MRCA of the minutissimum clade and in *B. keekee* (Fig. 5d). A single origin for a verrucate peduncle surface was inferred in the MRCA of sect. *Minutissima species from Australia, B. minutissimum and B. globuliforme*, and a single origin for a hirsute peduncle surface was inferred in *B. pygmaeum* (Fig. 5d).

Tepal surface: a glabrous tepal surface was reconstructed as the most likely ancestral state for the Australasian clade and was retained in the bracteatum, argyropus, and newportii clades, the MRCA of the minutissimum clade, and in *B. keekee* (Fig. 5e). A hirsute tepal surface was inferred to have evolved at least two times independently: 1) in the MRCA of sect. *Minutissima species from Australia, B. minutissimum and B. globuliforme* and 2) in *B. pygmaeum* (Fig. 5e).

Figure 3.5 Maximum likelihood reconstructions of the most likely ancestral character states in the Australasian clade. A) Photosynthetic pit at base of leaf, b) leaf petiolation, c) nodes between pseudobulbs, d) peduncle surface, e) tepal surface, f) labellum.

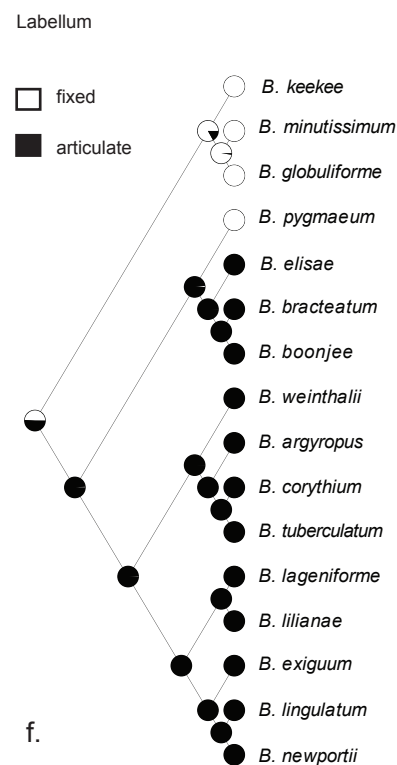
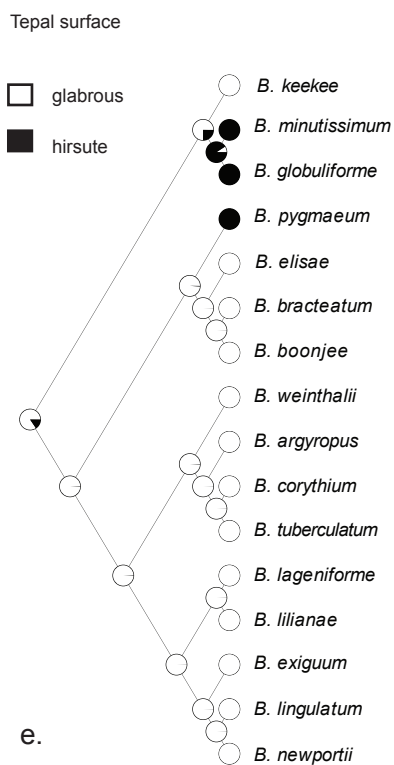
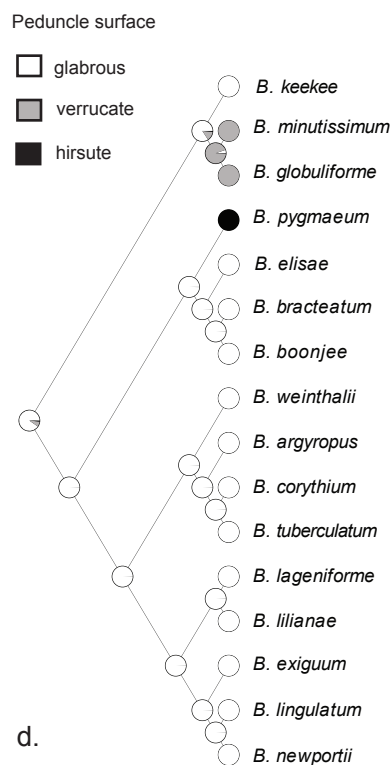
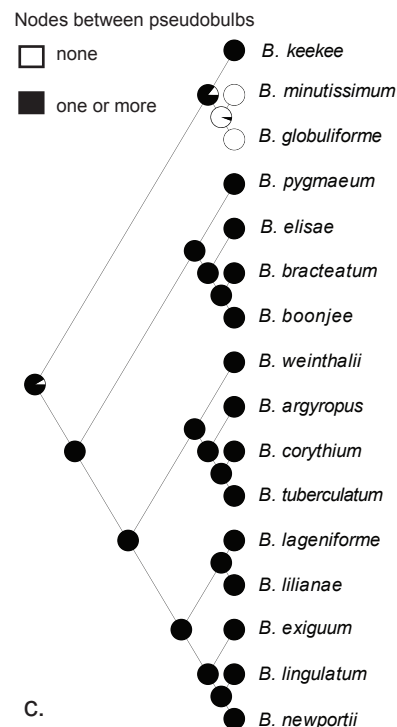
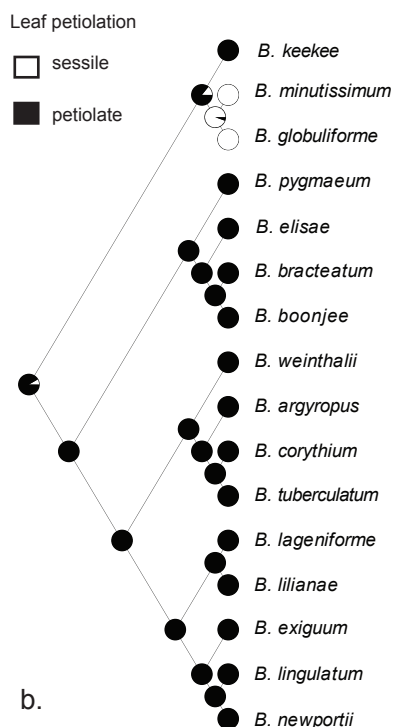
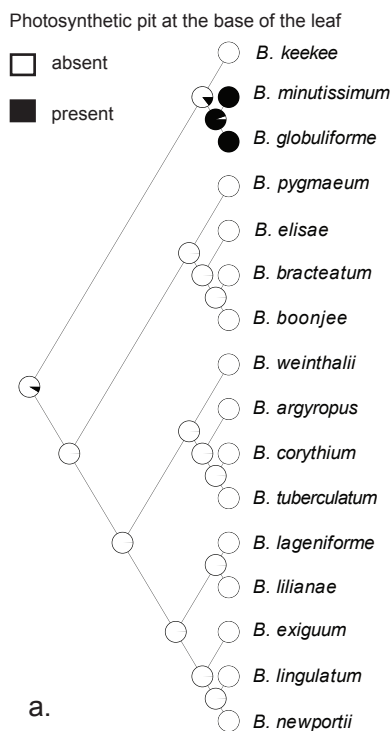


Fig 3.5

Labellum: the ancestral character state for the Australian clade was ambiguous, while a fixed labellum was reconstructed as the most likely ancestral state for the Adelopetalum clade and this was largely maintained within the Adelopetalum clade. Two independent origins for a fixed labellum were inferred: 1) in the minutissimum clade and 2) in *B. pygmaeum* (Fig. 5f).

Leaf surface: a glabrous leaf surface was reconstructed as the ancestral state for the Australasian clade and this state was retained within the clade with the exception of *B. pygmaeum*, which evolved a rugose leaf surface (Fig. 6a).

Leaf margin: an attenuate leaf margin was reconstructed as the ancestral state for the Australasian clade and was conserved within the clade with the exception of *B. pygmaeum*, which evolved a crenulate leaf margin (Fig. 6b).

Pseudobulb sheath: a membranous pseudobulb sheath was inferred as the ancestral state for the Australasian clade and this state was retained in the minutissimum, bracteatum and newportii clades (Fig. 6c). A scarious sheath was reconstructed as the most likely ancestral state for the MRCA of the clade including *B. weinthalii* and the argyropus clade (Fig. 6c).

Wings along the lower margin of the stelidia: inconspicuous wings on the lower margin of the stelidia were inferred as the ancestral state for the Australasian clade and this state is largely conserved within the clade (Fig. 6d). Prominent wings on the lower margin of the stelidia were inferred to have evolved once, in the MRCA of the newportii clade (Fig. 6d).

Shape of wings on the lower margin of the stelidia: the ancestral state for the shape of wings on the lower margin of the stelidia in the newportii clade was ambiguous (Fig. 6e). Deltoid wings were inferred as the most likely ancestral state in the MRCA of *B. lageniforme* and *B. lilianae* while toothed stelidia wings were inferred as the most likely ancestral state for the MRCA of *B. lingulatum*, *B. newportii*, and *B. exiguum* (Fig. 6e).

Pseudobulb ribs: inconspicuous ribs were reconstructed as the ancestral state for the Australasian clade which was largely conserved within the clade (Fig. 6f). Prominent ribs were inferred to have evolved once, in the newportii clade (Fig. 6f).

Figure 3.6 Maximum likelihood reconstructions of the most likely ancestral character states in the Australasian clade, continued. A) Leaf surface, b) leaf margin, c) pseudobulb sheath, d) wings along lower margin of stelidia, e) shape of wings along lower margin of stelidia, f) pseudobulb ribs.

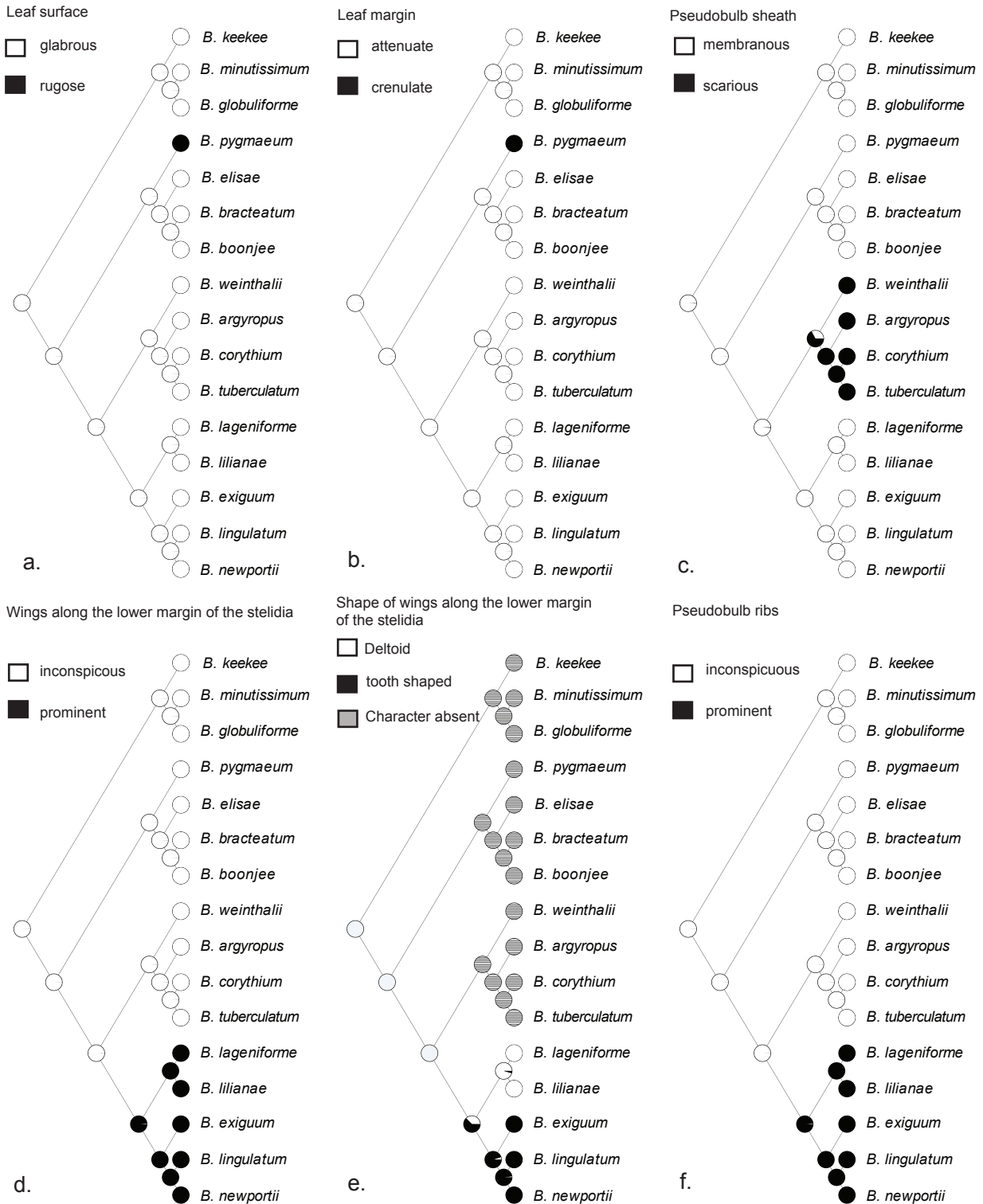


Fig 3.6

Pseudobulb shape: the ancestral state of the pseudobulb shape for the Australasian clade was ambiguous (Fig. 7a). Ellipsoid pseudobulbs were reconstructed as the most likely ancestral state for the Adelopetalum clade and the MRCA of 1) the bracteatum clade and *B. pygmaeum* and 2) the argyropus clade and was maintained within the bracteatum and argyropus clades. An ovoid pseudobulb was inferred as the most likely ancestral state for the newportii clade. The character reconstruction showed two origins for the globose pseudobulbs in 1) the minutissimum clade and 2) in *B. pygmaeum* (Fig. 7a).

Labellum shape: the ancestral state of the labellum shape for the Australasian clade was ambiguous (Fig. 7b). A trilobed labellum with prominent side lobes was reconstructed as the most likely ancestral state for the Adelopetalum clade which was retained in the bracteatum and argyropus clades. An obscurely trilobed labellum was inferred to have evolved once, in the newportii clade and two origins of an entire labellum were inferred, 1) in the minutissimum clade and 2) in *B. pygmaeum* (Fig. 7b).

Number of veins per petal: ancestral character reconstruction showed the number of veins per petal as a highly homoplastic character (Fig. 7c).

Pseudobulb surface: the ancestral state of the pseudobulb surface for the Australasian clade was ambiguous (Fig. 7d). The most likely ancestral state for the Australasian clade was a crenulate surface, which is maintained in *B. keekee*, *B. pygmaeum*, and the bracteatum clade. A rugose pseudobulb surface was inferred as the most likely ancestral state for the MRCA of the argyropus clade and *B. pygmaeum* and was maintained within that group. Two likely origins of a smooth and slightly wrinkled pseudobulb surface were inferred in 1) the MRCA of sect. *Minutissima* species from Australia, *B. minutissimum* and *B. globuliforme* and 2) the newportii clade, where it was conserved with the exception of *B. lageniforme*, in which a warty pseudobulb evolved. The ancestral character state of the pseudobulb surface for the Australasian clade remained ambiguous (Fig. 7d).

Figure 3.7 Maximum likelihood reconstructions of the most likely ancestral character states in the Australasian clade, continued. A) Pseudobulb shape, b) labellum shape, c) number of veins per petal, d) pseudobulb surface, e) number of flowers per inflorescence, f) ovary surface.

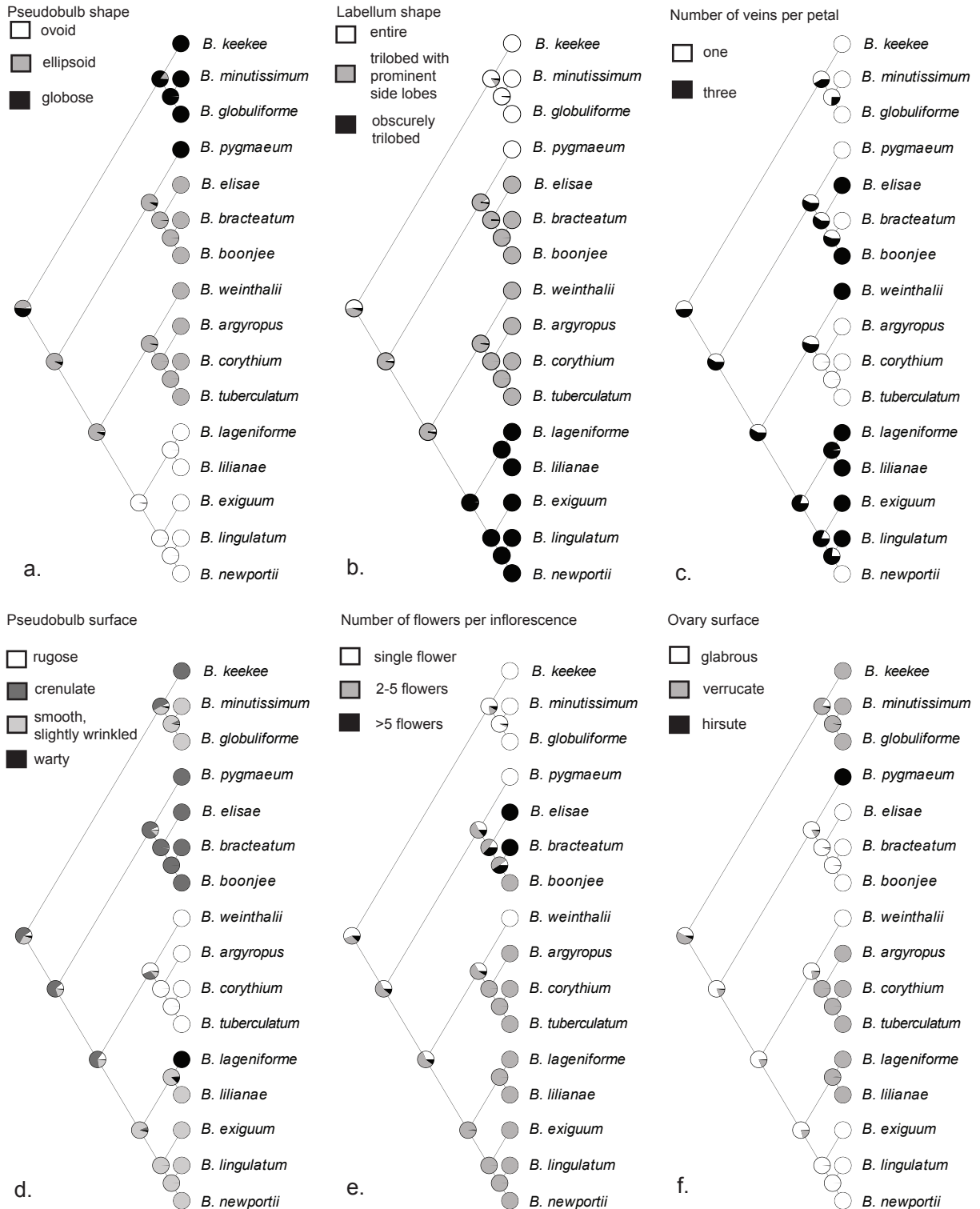


Fig 3.7

Number of flowers per inflorescence: the ancestral state for the number of flowers per inflorescence in the Australasian clade remained ambiguous (Fig. 7e). Two to five flowers were inferred as the ancestral character state for the MRCA of 1) the argyropus clade and 2) the newportii clade. For the MRCA of the bracteatum clade the ancestral state remained ambiguous, with *B. boonjee* characterised by 2-5 flowers while *B. bracteatum* and *B. elisae* each have more than 5 flowers per inflorescence. A single flower per inflorescence was inferred to have evolved at least three times independently in: 1) the MRCA of the minutissimum clade, 2) *B. pygmaeum*, and 3) *B. weinthalii* (Fig. 7e).

Ovary surface: the ancestral state for the ovary surface in the Australasian clade remained ambiguous (Fig. 7f). A glabrous ovary surface was reconstructed as the most likely ancestral state in the MRCA of the Adelopetalum clade and this state was maintained in the bracteatum newportii clades. A verrucate ovary surface was inferred to have evolved at least three times independently: 1) in the MRCA of the minutissimum clade, 2) in the MRCA of the argyropus clade and 3) in the MRCA of *B. lageniforme* and *B. lilianae*. A hirsute ovary surface was inferred to have evolved at least once, in *B. pygmaeum* (Fig. 7f).

3.3.5. Genome size estimates

The variation in fluorescence intensity recorded for sample replicates measured on different days was below 2% (with the exception of *B. newportii* (LS155A), Appendix 3.12). Genome size (2C-values) was largely conserved within sect. *Adelopetalum* and ranged between 2.51 pg and 3.19 pg (mean 2.74 pg) (Fig. 8, Appendix 3.12). The coefficients of variation for the G₀/G₁ peak of all analysed samples ranged from 1.00 to 3.92 (mean 3.12). Within species, low variation in genome size was recorded in all eight species with standard deviation observed within species between 0 and 0.064 pg (Fig. 8, Appendix 3.12).

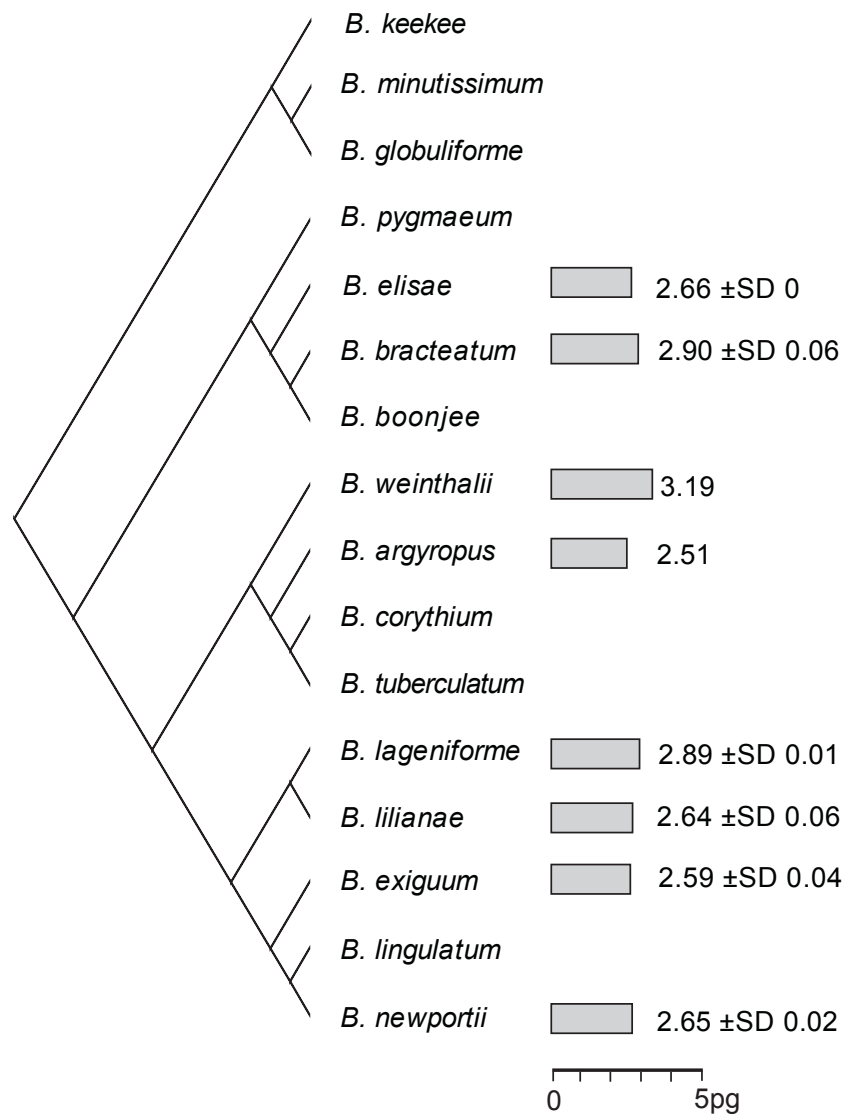


Figure 3.8. Genome sizes in *Bulbophyllum* sect. *Adelopetalum*. 2C value [pg] were estimated from flow cytometric measurements. Grey bars indicate mean genome size with mean genome size and standard deviation for the species reported adjacent.

3.4. Discussion

3.4.1. Phylogenetic relationships

All species from section *Adelopetalum* and section *Minutissima* species from Australia, New Zealand and New Caledonia were reconstructed within a highly supported clade in analyses based on 70 plastid loci (BS 95, PP 1) and seven nuclear ribosomal regions (BS 94, PP 1) (Fig. 3.1, Fig. 3.2). The Afrotropical/Neotropical clade (BS 85–100, PP 0.98–1) and Asian clade (BS 92–100, PP 1) were each reconstructed with moderate to high support in analyses based on plastid and nuclear datasets (Fig. 3.1, Fig. 3.2). The Australasian, Asian and Afrotropical/Neotropical clades reconstructed are consistent with the major clades resolved in phylogenetic reconstructions based on 70 plastic cds and three nuclear ribosomal regions (ITS1, 5.8s, ITS2) presented in Chapter two (Fig. 2.1, Fig. 2.2). In reconstructions based on three nuclear ribosomal regions presented in Chapter two (Fig. 2.1, Appendix 2.1), the Asian and Australasian clades remained poorly supported (BB <50, PP <0.50). With increased sampling of the nuclear ribosomal ITS cistron to include seven nuclear ribosomal markers (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) the nuclear phylogeny now provides support for the monophyly of the Australasian (BS 94, PP 1) and Asian clades (BS 85, PP 0.99) (Fig. 3.2). Based on results from phylogenetic analyses of 70 plastid cds and five nuclear ribosomal markers (Fig. 3.1, Fig. 3.2, Appendices 3.2, 3.3), relationships among the Afrotropical/Neotropical, Asian, and Australasian lineages remained unclear due to incongruence between the plastid or nuclear phylogenies. Reconstructions based on 70 plastid coding regions recovered the Australasian clade in a sister group relationship to a moderately to highly supported clade including the Afrotropical/Neotropical and Asian lineages (BS 83, PP1) (Fig. 3.1, Appendix 3.2). Reconstructions based on the nuclear ribosomal regions recovered the Afrotropical/Neotropical lineage in a sister group relationship to a moderately supported clade comprising the Australasian and Asian lineages (BS 80, PP 92) (Fig. 3.2, Appendix 3.3). Increased sampling from the nuclear genome is required to further clarify broad-level infrageneric relationships within the genus.

This study revealed that sect. *Minutissima* is polyphyletic when treated in the broader sense including Asian species such as *B. mucronatum* (sect. *Minutissima*). The phylogenetic analyses reconstructed the Australasian species of sect. *Minutissima*, including the type species *B. minutissimum*, in the highly supported Australasian clade (BS 94–95, PP 1), but placed the Asian sect. *Minutissima* species, *B. mucronatum*, within the highly supported Asian clade (BS 92–100, PP 1). The section assignment of *B. mucronatum* requires assessment. The species has

been previously recognised within sect. *Hybochilus* Schltr. (1912), however the phylogenetic relationship among taxa within this group is yet to be tested with molecular data. Previous molecular studies have not revealed the polyphyly of sect. *Minutissima* due to limited sampling including only representatives of the section from the Asian region (Pridgeon et al., 2014; Gamisch et al., 2015, Gamisch and Comes, 2019). Taxonomic treatments have also identified morphological affinities among *B. minutissimum* and sect. *Macrocaulia* (Seidenfaden, 1979). For example, the minute pseudobulbs that characterise *B. minutissimum* also characterise several species of sect. *Macrocaulia* such as *B. moniliforme*, a similarity that has resulted in these species being frequently confounded. However, the phylogenetic analysis does not support a close relationship between *B. moniliforme* (sect. *Macrocaulia*) and *B. minutissimum* (sect. *Minutissima*) but instead reconstructed *B. moniliforme* nested within the highly supported Asian clade. The results support previous morphological studies differentiating Australian species of sect. *Minutissima* (Jones and Clements, 2001) and indicate minute pseudobulbs are a trait that has evolved more than once independently within the genus.

Within the Australasian clade, analyses based on the 70 plastid loci (Fig. 3.1, Appendix 3.2) showed a dichotomous split between the highly supported minutissimum (BS 100, PP 1) and Adelopetalum (BS 95, PP 1) clades. Species were reconstructed in each of these clades according to their sectional placement with the exception of New Zealand endemic *B. pygmaeum* (sect. *Minutissima*), which was nested within the Adelopetalum clade. However, supported incongruence was detected in the placement of *B. pygmaeum* (sect. *Minutissima*) and *B. weinthalii* (sect. *Adelopetalum*) between plastid and nuclear phylogenies (Fig. 3.1, Fig. 3.2, Appendices 3.2, 3.3). In reconstructions based on seven nuclear ribosomal regions, all Australasian species of sect. *Minutissima*, including *B. pygmaeum*, were reconstructed in the highly supported minutissimum clade (BS 91, PP 1). This clade was resolved in a sister group relationship to *B. weinthalii* (sect. *Adelopetalum*) (BS 76, PP1) (Fig. 3.2, Appendix 3.3). Relationships between *B. weinthalii* + the minutissimum clade and the three highly supported lineages from sect. *Adelopetalum* (bracteatum, argyropus and newportii clades) remained unclear due to poor nodal support. Discordant relationships among *B. weinthalii*, *B. pygmaeum*, minutissimum, newportii, bracteatum and argyropus clades, reconstructed in analyses based on either the plastid or nuclear datasets may be the result of past hybridisation between the lineages giving rise to the minutissima and Adelopetalum clades and suggests *B. weinthalii* and *B. pygmaeum* may each be of hybrid origin. Today, *B. pygmaeum* (sect. *Minutissima*) occurs in lowland rainforests of New Zealand, in sympatry with *B. tuberculatum*

(sect. *Adelopetalum*), while *B. weinthalii* (sect. *Adelopetalum*) occurs in sympatry with *B. globuliforme* (sect. *Minutissima*) in *Araucaria* forests in Australia.

The results revealing the polyphyly of sect. *Minutissima*, with species reconstructed within both Asian and Australasian clades, warrant further revision of the sectional classification of *Bulbophyllum*. The type for the section, *B. minutissimum*, was placed within the highly supported minutissimum clade within the Australasian lineage. Further studies including additional Asian species assigned to sect. *Minutissima* and representative sampling of related Asian sections is required to confirm the systematic placement of these species. The high support for the *Adelopetalum* clade based on the plastid alignment (BS 95, PP 1) provides evidence for the monophyly of section *Adelopetalum* (Fitzg.) J.J. Verm. (1993), however the group is rendered paraphyletic by *B. pygmaeum* (sect. *Minutissima*) and discordance between the plastid and nuclear phylogenies was also found. To clarify the relationships between section *Adelopetalum* and the Australasian section *Minutissima* group, further studies based on increased number of loci from the nuclear genome are required.

3.4.2. Spatio-temporal evolution

Analyses based on both plastid and nuclear datasets placed the divergence of *Bulbophyllum* from *Dendrobium* in the early Oligocene (Fig. 3.3, Appendix 3.5). A rapid divergence among the Australasian, the Asian, and the Afrotropical/Neotropical clades was estimated between the late Oligocene and early Miocene, during the early evolution of *Bulbophyllum* (Fig. 3.3, Appendix 3.5). The divergence time estimations for the major Asian, Afrotropical/Neotropical and Australasian clades were consistent with findings from the broad-level phylogenetic analysis of *Bulbophyllum* (Chapter two, Fig. 2.3). Divergence dating analyses and ancestral area estimation placed the origin of the Australasian clade on the Australian continent in the late Oligocene to early Miocene. It also showed diversification within the Australasian clade has largely occurred on the Australian continent and that the ancestral area was retained in the most recent common ancestors of the six major lineages within the Australasian clade (Fig. 3.3, Fig. 3.4). Previous phylogenetic analyses of *Bulbophyllum* have shown a strong biogeographic signal among lineages within the genus with major clades largely confined to biogeographic regions such as Madagascar, continental Africa, South America and Asia (Gravendeel et al., 2004; Fischer et al., 2007; Smidt et al., 2011; Gamisch et al., 2015; 2019). Ancestral range reconstructions show that the Australasian clade is also geographically confined (Fig. 3.4). While long distance dispersal (LDD) was inferred as an important process in the evolution of major lineages within *Bulbophyllum* in Chapter two, the conserved distribution of ancestral

ranges in the Australasian clade indicates that LDD with successful recruitment and persistence has been relatively rare in this clade. Similarly, studies of diverse Neotropical orchids found that, despite evidence for recurrent migration across major biogeographic barriers, *in situ* speciation within regions was the dominant biogeographical process (Perez-Escobar and Chomicki et al., 2017). A broad scale analysis of the spatio-temporal evolution of the Orchidaceae revealed the consequential role LDD dispersal has played in the diversification of the Orchidaceae, inferring the products of LDD events have given rise to 97% of orchid species (Givnish et al., 2016). However, the rate of LDD inferred across the evolution of the family was relatively low at 0.8 events Ma^{-1} (Givnish et al., 2016). Although the minute wind-dispersed seeds of orchids have a high dispersal potential, successful establishment in a new area may be limited by a number of factors, such as the presence of mycorrhizal partners necessary for germination and development, a suitable host or substrate and microclimatic conditions, and the availability of pollinators (Arditti and Ghani, 2000; Jersáková and Malinová, 2007; McCormick et al., 2012). These results are consistent with previous studies that have identified *in situ* diversification as the dominant biogeographic process, despite evidence for LDD. This suggests that the complex requirements for successful establishment, rather than dispersal limitations, play an important role in constraining the geographic distribution of orchids (Givnish et al., 2016; Perez-Escobar and Chomicki et al., 2017).

Within the Australasian clade, interspecific relationships were largely well resolved (Fig. 3.1, 3.2). Diversification within the clade occurred mainly during the Miocene and Pliocene when extensive changes to the distribution of forest vegetation were occurring on the Australian continent in response to climatic changes (Martin, 2006). During the early Miocene, Australian vegetation diversified in response to aridification of the Australian continent and the abrupt shift to a cool dry climate during the mid-Miocene resulted in considerable fragmentation of rainforest habitats (Martin, 2006). The Australasian clade comprises epiphytic species that occur in mesic forest habitats and thus diversification and fragmentation of these habitats were likely drivers of allopatric lineage divergence within the clade. Sister group relationships were identified between two species pairs, which occupy disjunct distributions in Australia's northern wet tropical rainforests, and the south-eastern rainforests (*B. boonjee*/*B. bracteatum* and *B. newportii*/*B. exiguum*). These relationships support the hypothesis that the diversification and fragmentation of forest habitats in Australia has been an important driver of lineage divergence in Australia's mesic biome (Byrne et al., 2011, Simpson et al., 2018).

Within the Australasian clade, at least three independent range expansions and one range shift were inferred from continental Australia across the Coral and Tasman Seas to Lord Howe and Norfolk Island, New Zealand, and New Caledonia between the early Miocene and the early Pleistocene (Fig. 4). Each landmass has a long history of isolation from Australia, indicating colonisation of these islands by *Bulbophyllum* species from the Australasian lineage has been via LDD. Once forming part of Gondwana, New Zealand and New Caledonia were separated from Australia during the late Cretaceous, ca. 80 Ma (New Zealand) and the early Palaeocene ca. 65 Ma (New Caledonia) (Matthews et al., 2016). Lord Howe Island and Norfolk Island were formed from volcanic activity during the late Miocene ca. 7 Ma and late Pliocene ca. 2.3 Ma, respectively (Jones and McDougal, 1973; McDougal et al., 1981).

Two independent LDD events were inferred from the Australian continent to New Caledonia during the Neogene, subsequent to a period of inundation over much of the island from the late Cretaceous to the early Eocene (McLoughlin, 2001). The colonisation of New Caledonia by Australasian *Bulbophyllum* coincides with the emergence of suitable habitat on the island. *Araucaria* was identified as an early coloniser of the island, based on divergence of the predominantly New Caledonian *Araucaria* clade from *A. cunninghamii* endemic to Australia and New Guinea in the early Miocene, ca. 21.6 Ma (Pillon, 2012). The earliest colonisation of *Bulbophyllum* on the island was estimated to the early Miocene between 22.6–17.6 Ma, in the MRCA of the minutissimum clade including species that are predominantly found within *Araucaria* forests in New Caledonia (*B. keekee*) and Australia (*B. globuliforme*) (Jones and Clements, 2001). The estimated period of dispersal inferred for the MRCA of *B. lingulatum* and *B. newportii*, both predominantly found in rainforest, coincides with the emergence of rainforest habitat on the island in the late Miocene (Pillon, 2012). The results further highlight the importance of suitable habitat as a precondition to successful establishment and suggest that range expansion may be more limited by availability of the complex biotic and abiotic requirements of orchids, rather than dispersal (Perez-Escobar and Chomicki et al., 2017).

Dispersal from continental Australia to New Zealand was inferred for the lineage that gave rise to *B. pygmaeum* sometime from the mid-Miocene onwards. Dispersal from the Australian continent eastward was also inferred for the MRCA of the argyropus clade, composed of the closely related species *B. argyropus*, *B. corythium*, and *B. tuberculatum*. The results indicate that the ancestor of this group diverged from the lineage that gave rise to *B. weinthalii*, on the Australian east coast in the mid Miocene and subsequent dispersal eastward has facilitated

range expansion to other landmasses in the Pacific Ocean (i.e. Lord Howe Island, Norfolk Island, New Caledonia, and New Zealand). *Bulbophyllum argyropus* occurs on Australia's east coast, Lord Howe Island, and Norfolk Island, *B. corythium* in New Caledonia, and *B. tuberculatum* in New Zealand. Divergence between these species was estimated to have begun relatively recently, during the late Pleistocene, with the disjunct distribution on these islands suggesting allopatric speciation as the likely mode of divergence within the group. The species are morphologically similar and taxonomic treatments recognise one to three species within the group (Halle, 1981; Vermeulen, 1993; Clements and Jones, 2002). Further studies are required to clarify species delimitation and dispersal patterns within this recent diversification utilising population level sampling and molecular sequencing techniques suited to resolving relationships in this species complex, such as reduced genome representation high-throughput sequencing approaches (Peterson et al., 2012).

The pattern of eastward dispersal observed in the Australasian clade is consistent with dispersal patterns from Australia across the Coral and Tasman Seas inferred in other angiosperms, including *Abrotanella*, *Dendrobium*, *Dracophyllum*, *Hebe*, *Korthalsella*, *Leucopogon*, *Northofagus*, *Oreobolus* and *Rytidosperma* (Linder, 1999; Molvray et al., 1999; Lavarack et al., 2000; Swenson et al., 2001; Wagstaff et al., 2002; 2006; 2010; Chacón et al., 2006; Puente-Lelièvre et al., 2013). Eastward dispersal in the southern hemisphere has been inferred significantly more often than in the reverse direction and is likely facilitated by the westerly winds that initiated after the rifting of Australia and South America from Antarctica during the Eocene (Sanmartín et al., 2007). A biogeographic study of Orchidaceae identified Australia as the second most important biogeographic source region in the family globally (Givnish et al., 2016). This study found that within *Bulbophyllum*, Australia has also functioned as a source region and the results showed that other Australasian regions, such as Lord Howe and Norfolk Island, New Caledonia, and New Zealand, were sink regions for *Bulbophyllum* dispersing east from continental Australia.

3.4.3. Morphological trait evolution in the Australasian clade

Ancestral character reconstruction identified phylogenetically informative and homoplasious characters within the Australasian clade (Fig. 5–7). Synapomorphies were identified for the newportii clade, the argyropus clade and the Australian sect. *Minutissima* species, *B. minutissimum* and *B. globuliforme*. Four characters were identified as highly homoplasious in the Australasian clade: 1) the number of veins per petal, 2) pseudobulb surface, 3) number of

flowers per inflorescence and 4) ovary surface (Fig. 7c-f). The results provide an independent test of the phylogenetic utility of morphological traits used in a previous cladistic analysis of sect. *Adelopetalum* that recovered two main clades within the section, one comprising *B. lageniforme*, *B. lilianae*, *B. lingulatum*, and *B. newportii*, and the other uniting *B. argyropus*, *B. bracteatum*, and *B. elisae* (Vermeulen 1993). The phylogenetic and ancestral character reconstructions presented in this study found strong support for the first clade recovered in Vermeulen's cladistic analysis (which corresponds to the *newportii* clade) and for the presence of wings along the lower margin of the stelidia being a synapomorphy for this clade. Additional synapomorphies identified for the *newportii* clade in the ancestral character reconstruction were ovoid shaped pseudobulbs, prominent pseudobulb ribs, and an obscurely trilobed labellum (Fig. 6f, 7a, b). The second group recovered by Vermeulen (1993) included three species reconstructed in the phylogenetic analyses based on molecular data within either the *bracteatum* clade (*B. bracteatum*, *B. elisae*) or the *argyropus* clade (*B. argyropus*). Relationships among these two lineages remained unclear in the phylogenetic analyses due to low support. However, the ancestral trait reconstruction identified four shared character traits between the *bracteatum* clade and the *argyropus* clade (prominently trilobed labellum, inconspicuous wings along the lower margin of the stelidia, and an ellipsoid pseudobulb that lacks prominent ribs), suggesting these two groups may be closely related (Fig. 6d, f, 7a, b). Further studies based on additional nuclear markers are required to clarify relationships between the main lineages of the *Adelopetalum* clade and their character evolution. The sister group relationship between *B. bracteatum* and *B. argyropus* recovered in the cladistic study by Vermeulen (1993) was not supported by the analyses based on molecular data. The ancestral character reconstruction demonstrated that at least one of the character states, number of veins per petal, which was identified as a synapomorphy for the two species in the cladistic study, is homoplasious within the Australasian clade (Fig. 7c).

Ancestral character state reconstructions showed that the Australian *Bulbophyllum* species from sect. *Minutissima*, *B. minutissimum* and *B. globuliforme*, are morphologically distinct from the remaining species of the Australasian clade and can be delineated based on three character states: 1) the presence of a photosynthetic pit at the base of the leaf, 2) a sessile leaf, 3) the absence of nodes between the pseudobulbs (Fig. 5a-c). The results support previous studies that identified these traits as synapomorphies for these two Australian species of sect. *Minutissima* (Jones and Clements, 2001). The evolution of reduced leaves and a photosynthetic pit at the base of the leaf are functional traits that reduce water loss,

indicating low water availability presented a selective pressure on the lineage giving rise to the MRCA ancestor of *B. minutissimum* and *B. globuliforme*. Australasian *Bulbophyllum* species predominantly occur in rainforest, while *B. minutissimum* and *B. globuliforme* occur in habitats such as littoral, mangrove, sclerophyll and *Araucaria* forests, that are characterised by open canopies and lower humidity where minimising moisture loss is likely to be advantageous. Three shared character states were identified between the Australasian sect. *Minutissima* species, *B. minutissimum* and *B. globuliforme* from Australia, *B. keekee* endemic to New Caledonia, and *B. pygmaeum* endemic to New Zealand. Shared character states are globose pseudobulbs and a fixed, entire labellum (Figs. 5f, 7a, b). Relationships reconstructed from the nuclear dataset indicate these character states may represent synapomorphies for the Australasian species of sect. *Minutissima*.

To assess if divergence within sect. *Adelopetalum* was influenced by chromosomal alterations (e.g. polyploidy, aneuploidy, dysploidy), we estimated the genome size of eight representatives of the section. So far, genome size is only poorly documented within the family Orchidaceae, with genome size estimations available for only about 1.4% of the species (Leitch et al., 2009). In spite of the paucity of genome size data, (allo-) polyploidisation was found to lead to large genome sizes in the orchid subfamilies Orchidoideae, Vanillioideae, and Cypripedioideae (Jones et al., 1998; Aagaard et al., 2005; Jersáková et al., 2013) and complex chromosomal rearrangements such as fusions and fissions, loss of telomeric repeats, gain or loss of rDNA sites or inversions were suggested to be responsible for the variable basic chromosome numbers in the subfamily Epidendroideae (Moscone et al., 2007). Genome sizes (2C-values) estimated for *Bulbophyllum* sect. *Adelopetalum* were comparatively small, ranging from 2.51pg/1C to 3.18pg/1C (Fig. 8, Appendix 3.12). Observed low genome size variation does not suggest the presence of polyploidy. However, the small variation and the presence of the outlier 2C-values (*B. argyropus*, *B. weinthalii*) are in line with chromosome numbers reported for the section ($2n=36, 38$; Dawson et al., 2007; Appendix 3.12) and suggest that complex chromosomal evolutionary processes (aneuploidy, dysploidy) played a role in the evolution of the sect. *Adelopetalum*.

Conclusions

This phylogenomic study has provided evidence for a monophyletic Australasian lineage within *Bulbophyllum* and reconstructed evolutionary relationships within this clade. Within the

Australasian clade diversification took place predominantly within Australia's mesic biome along the east coast of the continent, and allopatric speciation was identified as likely driver of lineage diversification. Several eastward LDD events were inferred to neighboring regions in the South Pacific, demonstrating the importance of the Australian continent as source for the orchid floras in the Australasian and Pacific region. The study provided insights into morphological trait evolution within the Australasian clade and identified taxonomically valuable characters as well as several highly homoplasious traits. Results from the phylogenetic analyses revealed sect. *Minutissima* as polyphyletic, demonstrating that sectional classification within *Bulbophyllum* requires further revision.

3.5. Chapter summary

- Evolutionary relationships within the Australasian *Bulbophyllum* clade were reconstructed from a 70 plastid cds supermatrix and a seven nuclear ribosomal supermatrix (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). Sampling included 28 samples from sect. *Adelopetalum*, representing all 12 species recognised for the section and eight samples from sect. *Minutissima*, representing five species comprising all four Australasian species and one (of ca. 19) tropical Asian species.
- Phylogenetic analyses resolved all sect. *Adelopetalum* and Australasian species of sect. *Minutissima* within a highly supported Australasian clade (BS 94-95, PP 1) and reconstructed six well supported clades within the Australasian lineage.
- Section *Minutissima* was identified as polyphyletic with species reconstructed in the Asian and Australasian clades.
- Divergence time estimations, based on a relaxed molecular clock model incorporating fossil and secondary calibration points, placed the origin of the Australasian clade in the late Oligocene to early Miocene, ca. 23–18 Ma, and ancestral range analysis within the Australasian clade based on a dispersal-vicariance model identified the Australian continent as the ancestral area of the Australasian clade.
- Ancestral range analysis showed subsequent diversification mainly took place in east Australia where all six major clades originated during the Miocene. Retraction and fragmentation of eastern Australian rainforests from the early Miocene onwards is seen as a likely driver of lineage divergence within the Australasian clade, facilitating allopatric speciation.
- Several independent long-distance dispersal events from the Australian continent to other Australasian landmasses (Lord Howe Island, Norfolk Island, New Zealand, and New Caledonia) were inferred from the early Miocene onwards, and this eastwards dispersal pattern was found to be in accordance with the predominantly westerly winds of the southern hemisphere.
- Maximum likelihood ancestral character reconstructions of nine floral and nine vegetative characters within the Australasian clade provided insights into evolution of morphological traits and their taxonomic value.

Chapter four: Phylogeography of a mountaintop endemic epiphyte of the Australian Wet Tropics: *Bulbophyllum lageniforme* F.M. Bailey (Orchidaceae)

4.1. Introduction

The Australian Wet Tropics Bioregion harbours a unique assemblage of mesic forest habitats distributed along 450km of coastal ranges on Australia's northeast coast (Nix, 1991). The rich biodiversity of the region, including a high proportion of endemic taxa, is recognised globally and protected under United Nations world heritage status (Metcalf and Ford, 2008; Williams et al., 2009; FEFAC, 2017). The highest diversity of endemic species is found in the upland forests distributed across the ranges and mountaintops, reaching elevations up to 1622m (Williams et al., 2010). These upland communities, together with upland communities in the southeast Queensland Bioregion harbour relic populations of Gondwanan derived rainforest lineages that once covered the Australian continent before the Miocene aridification that caused extensive contraction of Australia's mesic habitats (Martin, 2006; Hill et al., 2011). Today, Australia's Wet Tropics has a monsoonal climate with a distinct winter dry season. The upland forest habitats that occur approximately 800m above sea level provide cool wet climatic conditions throughout the year as they intercept orographic clouds, providing moisture through cloud stripping (McJannet et al., 2007; Wet Tropics Management Authority, 2014). However, rising global temperatures are expected to lift the altitude of the orographic cloud base, critically reducing moisture availability. For this reason, tropical upland communities are considered at high risk from climate change (Still et al., 1999; Foster, 2001; Williams et al., 2012). Studies modelling the distribution of Wet Tropics mountaintop endemics under future climates scenarios have predicted significant declines, with many species qualifying as critically endangered (Hilbert et al. 2001; Hilbert and Williams, 2003; Williams et al., 2003; Shoo et al., 2005a; Shoo et al., 2005b; Williams and Hilbert, 2006; Costion et al., 2015). To mitigate against extinction, *ex situ* conservation action has been recommended (Costion et al., 2015) and programs for the *ex situ* conservation of Wet Tropics mountaintop endemic plants have been initiated (Mountner, 2019).

Evidence from palynological and spatial modelling studies have shown that Wet Tropics rainforests have periodically expanded and contracted in response to past climatic fluctuations, which have been pronounced during the glacial cycles of the late Quaternary (Web and Tracy, 1981; Nix, 1991; Kershaw 1994; Kershaw et al., 2005; Graham et al., 2006; Hilbert et al., 2007; Vanderwal et al., 2009). During arid climates associated with glacial

periods, rainforest habitat retracted into multiple rainforest refugia, and subsequently expanded during wet interglacial periods. Under contemporary climatic conditions, rainforest refugia persist in the northern, central and southern regions of the Wet Tropics, which are separated by major biogeographic disjunctions. The major refugia are found in the central Wet Tropics region, along and adjacent to the central Wet Tropics escarpment, the Atherton Tablelands (Vanderwal et al., 2009). Rainforest refugia in this region include Mt Bartle Frere, Mt Bellenden Ker, the uplands of the Malbon Thompson and Lamb Ranges and the upland rainforests of the Atherton Tablelands, namely Mt Edith, Mt Haig, Mt Fisher and Mt Baldy. Rainforest refugia in the central Wet Tropics region are separated from multiple smaller refugia in the northern Wet Tropics region by a significant band of savannah habitat known as the Black Mountain Corridor (BMC) (Moritz et al., 2000; Crisp et al., 2017;). Rainforest refugia of the northern Wet Tropics include the Carbine Tableland, the Windsor Tableland, Thornton Peak and Mt Finnigan. The southern Wet Tropics region also harbours small areas of upland rainforest along the Paluma Range and Mt Elliot. However, the long-term persistence of rainforest in this region throughout the Quaternary remains uncertain (Graham et al., 2012).

The legacy of habitat fragmentation during restrictive climates may be reflected in the spatial genetic structure of species (Hewitt, 2000; Byrne et al., 2011). Environmental niche modelling for Wet Tropics rainforest endemic animal species suggests habitats were highly fragmented under past restrictive climates. Phylogeographic studies of mesic adapted reptiles, amphibians and invertebrates have identified population genetic structure indicating species have persisted in disjunct refugia that has facilitated divergence among allopatric populations (Schneider et al., 1999; Hugal et al., 2002; Dolman, 2006; Mousalinnni et al., 2009). Population genetic structure reflecting secondary contact between isolated lineages has been identified, reflecting cycles of range contraction and subsequent expansion of forest habitat during Pliocene and Pleistocene glacial cycles (Hopkins et al., 1993; Schneider, 1999; Hugal et al., 2002; Hoskin, 2007; Mortiz et al., 2009). In comparison to studies of rainforest endemic fauna, responses of rainforest endemic plants to paleoclimatic shifts is not as well studied globally (Moritz et al., 2000; Morris and Shaw, 2018). Previously, limitations in molecular markers available for resolving relationship among closely related plant groups have hindered research (Beheregaray, 2008; Morris and Shaw, 2018). Traditionally, studies of intraspecific diversity within animals have utilised genetic markers from the mitochondrial genome. However, in plants, nucleotide substitutions within the mitochondrial and plastid genomes accumulate relatively slowly, providing little variation that can be utilised to resolve relationships among

closely related groups (Avise, 2009; Beheregaray, 2008; Schaal et al., 1998; Newton et al., 1999). Recent advances in high throughput sequencing methods provide new opportunities for generating genomic data to reconstruct evolutionary relationships among closely related plant taxa (McPherson et al., 2013; Van der Merwe et al., 2014; Fernández-Mazuecos et al., 2017; Jiang et al., 2019). In the Wet Tropics Bioregion, phylogeographic studies of plants have identified divergence across the BMC in several species (Rossetto et al., 2009; Burke et al., 2013; Simpson et al., 2018). However, previous studies were limited in their sampling within populations across the Wet Tropics and plant phylogeography within the Wet Tropics region remains poorly understood. New methods of genomic analysis provide an important opportunity to investigate phylogeographic patterns within Australia's tropical mountaintop endemic plants.

Bulbophyllum L. (Orchidaceae) presents a model group to investigate how past climatic shifts have influenced distributions and genetic diversity in Australia's tropical upland endemic taxa. The Wet Tropics Bioregion harbours 20 of 33 Australian *Bulbophyllum* species, and 50% of these species are endemic to the upland rainforests of the region (Jones, 2006). Being epiphytes, *Bulbophyllum* species may represent effective indicators of environmental change due to the unique environmental requirements of epiphytic species, particularly for moisture availability (Zotz and Bader, 2009). So far, no studies have been carried out reconstructing intraspecific diversity within Australasian *Bulbophyllum* species. This study aims to reconstruct the phylogeography of the Wet Tropics mountaintop endemic epiphyte *Bulbophyllum lageniforme* F.M. Bailey to understand responses to past climatic fluctuations and inform effective conservation management of the species. Phylogenetic analysis has shown *B. lageniforme* belongs to the argyropus clade within the Australasian *Bulbophyllum* lineage and divergence dating analyses inferred a Pliocene divergence from the closely related Wet Tropics mountaintop endemic species, *B. lilianae* (Chapter three, Fig. 3.1, Fig. 3.2). *Bulbophyllum lageniforme* occurs between 1000–1500m, in highly localised populations of the mountaintop rainforests of the central and northern Wet Tropics regions, to the north and south of the BMC. In the central Wet Tropics region, populations are known on mountain ranges of the Atherton Tablelands, Bellenden Ker, Mt Bartle Frere and the Lamb Range. In the northern Wet Tropics, the species occurs in populations on the southwestern ranges of the region, the Carbine and Windsor Tablelands.

The specific aims of this study were to use genomic methods to:

1. assess genetic diversity within *B. lageniforme*;
2. use geographic patterns of genetic diversity to evaluate evolutionary hypotheses explaining the current distribution of *B. lageniforme*; and
3. identify provenance sampling strategies to support ex situ conservation approaches that seek to preserve intraspecific genetic diversity.

4.2. Methods

4.2.1. Sampling

In total 65 samples of *B. lageniforme* were collected from seven populations across the known distribution of the species including Mt Bartle Frere, Mt Edith, Mt Haig, Lamb Range, Mt Lewis, Devils Thumb and Mt Windsor. From each population, 4–14 (mean=8) individuals were collected. Due to issues with extraction of high quality DNA from the sister species *B. lilianae*, sampling of this species has been limited to a single representative. Provenance and voucher information is provided in Table 1.

Table 4.1 Collection details for 65 individuals of B. lageniforme included in the study and two individuals of B. newportii and one individual of B. lilianae that comprised the outgroup.

Species	Voucher details	DNA number	Population
<i>B. lageniforme</i>	CNS: Simpson, L. 302A	CNS_G03716	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 301A	CNS_G03718	Devils Thumb
<i>B. lageniforme</i> F.M. Bailey	CNS: Simpson, L. 301C	CNS_G03719	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 296F	CNS_G03720	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 296A	CNS_G03721	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 287A	CNS_G03723	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 296G	CNS_G03724	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 283A	CNS_G03726	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 283A	CNS_G03727	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 283B	CNS_G05490	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 287B	CNS_G05491	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 294B	CNS_G05493	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 301F	CNS_G05494	Devils Thumb
<i>B. lageniforme</i>	CNS: Simpson, L. 180A	CNS_G03705	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 167G	CNS_G03706	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 167B	CNS_G03707	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163I	CNS_G03708	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163A	CNS_G03709	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 110	CNS_G03710	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 162C	CNS_G03712	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 162C	CNS_G03713	Lamb Range

Species	Voucher details	DNA number	Population
<i>B. lageniforme</i>	CNS: Simpson, L. 167C	CNS_G03714	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 167C	CNS_G03715	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163A	CNS_G04499	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 180A	CNS_G04502	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 180A	CNS_G04503	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163D	CNS_G05505	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 167E	CNS_G05507	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 180B	CNS_G05508	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163E	CNS_G05510	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163J	CNS_G05511	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 162B	CNS_G05512	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 163G	CNS_G05513	Lamb Range
<i>B. lageniforme</i>	CNS: Simpson, L. 432A	CNS_G03728	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432A	CNS_G03729	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432B	CNS_G03730	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 447	CNS_G03731	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432C	CNS_G03733	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 431B	CNS_G03734	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 430A	CNS_G03736	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 435	CNS_G03737	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 445	CNS_G03738	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 059	CNS_G03739	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432E	CNS_G04537	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432E	CNS_G04538	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 431D	CNS_G05498	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 431C	CNS_G05499	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 430B	CNS_G05500	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 432D	CNS_G05501	Mt Bartle Frere
<i>B. lageniforme</i>	CNS: Simpson, L. 133G	CNS_G03677	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 133A	CNS_G03678	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 133B	CNS_G03680	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 133B	CNS_G03681	Mt Edith

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Species	Voucher details	DNA number	Population
<i>B. lageniforme</i>	CNS: Simpson, L. 133E	CNS_G03682	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 137A	CNS_G03684	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 137B	CNS_G03686	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 133C	CNS_G05496	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 133B	CNS_G05497	Mt Edith
<i>B. lageniforme</i>	CNS: Simpson, L. 143	CNS_G03687	Mt Haig
<i>B. lageniforme</i>	CNS: Simpson, L. 149A	CNS_G03688	Mt Haig
<i>B. lageniforme</i>	CNS: Simpson, L. 149A	CNS_G03689	Mt Haig
<i>B. lageniforme</i>	CNS: Simpson, L. 149C	CNS_G03691	Mt Haig
<i>B. lageniforme</i>	CNS: Simpson, L. 149B	CNS_G03692	Mt Haig
<i>B. lageniforme</i>	CNS: Simpson, L. 220D	CNS_G03670	Mt Lewis
<i>B. lageniforme</i>	CNS: Simpson, L. 220D	CNS_G03671	Mt Lewis
<i>B. lageniforme</i>	CNS: Simpson, L. 220D	CNS_G03672	Mt Lewis
<i>B. lageniforme</i>	CNS: Simpson, L. 215	CNS_G03673	Mt Lewis
<i>B. lageniforme</i>	CNS: Simpson, L. 220C	CNS_G03674	Mt Lewis
<i>B. lageniforme</i>	Sanger, J. sn	CNS_G03850	Mt Lewis
<i>B. lageniforme</i>	Sanger, J. sn	CNS_G03852	Mt Lewis
<i>B. lageniforme</i>	CNS: Simpson, L. 181C	CNS_G03695	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 187	CNS_G03696	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 195D	CNS_G03697	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 192C	CNS_G03698	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 192A	CNS_G03699	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 195F	CNS_G03700	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 192B	CNS_G03701	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 195E	CNS_G03702	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 195A	CNS_G03703	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 195B	CNS_G03704	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 192A	CNS_G04498	Mt Windsor
<i>B. lageniforme</i>	CNS: Simpson, L. 196A	CNS_G04541	Mt Windsor
<i>B. lillianae</i> Rendle	CNS: Simpson, L. 465	CNS_G05659	
<i>B. newportii</i> (F.M. Bailey) Rolfe	CNS: Simpson, L. 442G	CNS_G03857	
<i>B. newportii</i>	CNS: Simpson, L. 313C	CNS_G05516	

4.2.2. DNA extraction and RAD library preparation

Total genomic DNA was extracted from ca. 40 mg of silica-dried leaf material. Extractions were carried out using the CTAB method (Doyle and Doyle, 1990), with modifications as described in Weising et al. (2005). Subsequent library preparation and sequencing were carried out at the Australian Genome Research Facility (Melbourne, Australia) based on the double digest restriction associated DNA (ddRAD) protocol described in Peterson et al. (2012). In the establishment phase the optimal restriction enzyme combination was selected from eight restriction enzyme combinations combining a six base cutter (*PstI* or *EcoRI*) and a four base cutter (*MspI*, *HpyCH4IV*, *MseI* or *NlaIII*). Restriction digests for each enzyme combination were carried out with a pooled DNA sample including one individual of *B. lageniforme* and two individuals of *B. newportii*, the second species selected for phylogeographic analysis (Chapter five). Barcode adaptors were ligated to the restriction fragments, which were purified and amplified with PCR to create libraries of restriction associated DNA (RAD) fragments. The libraries were assessed on a TapeStation instrument (Agilent technologies Inc, Santa Clara USA), and the *PstI*/*MspI* enzyme combination was identified as the optimal combination as it yielded the fewest repetitive sequences. To test the optimal size range of RAD fragments for batch processing, restriction digests using the *PstI*/*MspI* enzyme combination were carried out for three samples, barcode adaptors were ligated to the RAD fragments and samples were pooled and purified. Size selection of the RAD fragments was carried out with the BluePippin (SageScience, Beverly USA) testing both narrow and wide size selections. For each size range, the RAD libraries were amplified with PCR using indexed primers, and single end sequencing was carried out on a MiSeq sequencer (Illumina, San Diego USA) with 160 cycles.

For batch processing, restriction enzyme digests were carried out for 65 *B. lageniforme* individuals and 10% duplicate samples, with the *PstI*/*MseI* enzyme combination. Barcode adaptors were ligated to the RAD fragments and samples were pooled, purified and size selected for fragments in the narrow size range between 280–342bp. Amplification via PCR was carried out using indexed primers, and RAD fragments were sequenced with paired ends on a HiSeq sequencer (Illumina, San Diego USA) with 100 cycles at the Australian Genomic Research Facility, Melbourne.

4.2.3. Data filtering and management

Datasets of homologous RAD loci were generated with the pyRAD pipeline (Eaton, 2014). Sequence reads were demultiplexed based on unique barcodes assigned to each individual.

Adapter and primer sequences were removed, low quality base calls (phred Qscore <33) were recorded with the ambiguity code 'N' and reads with more than five ambiguous bases were excluded. Sequence reads were clustered *de novo* using vsearch (Rognes et al., 2016) and aligned with Muscle (Edgar, 2004a; 2004b) in ipyrad. Consensus allele sequences were estimated based on a binomial model and estimated sequence error rate and heterozygosity. Consensus sequences with more than ten ambiguous sites per locus were removed to avoid issues with clustering loci across individuals. Consensus sequences were clustered *denovo* across individuals using vsearch and aligned with Muscle in ipyrad. To filter out poor final alignments and potential paralogous, loci with more than 16 indels per locus, more than 50% shared polymorphic sites or greater than two unique alleles per individual were excluded. The final dataset was also filtered to loci which were present in a minimum number of samples (MS) (Eaton, 2014).

To assess the effect of filtering parameters implemented in the pyRAD pipeline, datasets were generated varying three key parameters for a subsample of individuals including 1–4 individuals from all populations and all replicate samples. The first parameter tested was the clustering threshold, indicating the proportion of identical base pairs required to generate a cluster in both the alignment of sequence reads into RAD loci within each individual, and in the alignment of RAD loci across individuals. Datasets were generated with a clustering threshold of 0.80, 0.85 and 0.90. For example, under a threshold of 0.80, reads that shared at least 80% identical base pairs would be aligned in a locus. The second parameter tested was the minimum depth of sequence reads at which statistical base calls could be made when generating consensus sequences for RAD loci within each individual. Datasets were generated with minimum read depth set to three, six and ten. The final parameter tested was the minimum number of samples (MS) present in a locus required for the inclusion in the final dataset. Datasets were generated including RAD loci present in at least 50% of individuals (MS50%) and at least 75% of individuals (MS75%). To identify the optimal parameter settings, ML phylogenetic trees were reconstructed from each dataset and the clustering of replicate samples, overall resolution and bootstrap support in the resulting trees was compared. Maximum likelihood (ML) phylogenetic trees were reconstructed from concatenated alignments of RAD loci generated from each dataset in the program RAxML v. 8.2.4 (Stamatakis, 2014), applying the rapid hill climbing algorithm and a GTR gamma nucleotide substitution model, with 100 bootstrap replicates.

For the final analyses including all individuals, six datasets were generated in ipyrad v. 0.7.23 (<https://github.com/dereneaton/ipyrad>). All datasets were generated with the optimal clustering threshold (0.9) and minimum read depth (six) determined from parameter tests based on the smaller subset of individuals. Final datasets were generated with three variations of the minimum samples per locus parameter: RAD loci present in at least one individual per population (MS1), RAD loci present in at least two individuals per population (MS2) and RAD loci present in at least eight individuals per population (MS8). For each variation of the minimum samples per locus parameter, datasets were produced both including and excluding the outgroup, which comprised one individual of *B. lilianae* and two individuals of *B. newportii*.

4.2.4. NeighborNet analyses

To detect potentially conflicting phylogenetic signal in the data sets NeighborNet (Bryant and Moulon, 2004) analyses of the SNP datasets were carried out in the software SplitsTree4, V.4.13.1 (Huson and Bryant, 2006), based on the proportion of nucleotide sites at which two individuals differ (uncorrected p distances, Nei and Kuma, 2000).

4.2.5. Maximum likelihood phylogenetic analysis

ML analyses were carried out with concatenated alignments of RAD loci in the program RAxML v. 8.2.4 (Stamatakis, 2014), with settings as described above (section 4.2.3). ML reconstructions were carried out both including and excluding an outgroup, comprising two individuals of *B. newportii* and one individual of *B. lilianae*.

4.2.6. TETRAD phylogenetic analyses

Within species, reticulation and recombination result in heterogeneity among the genealogy of loci. A concatenated ML approach does not allow for heterogeneity in the genealogy among loci and therefore resulting trees may not accurately reflect accurate relationships in the presence of incomplete lineage sorting. The reticulated nature of relationships within species can be reconstructed under the coalescent model which allows for heterogeneity in the genealogy among loci. To reconstruct intraspecific relationships among lineages under the coalescent model a single value decomposition (SVD) quartet tree was reconstructed in the program TETRAD provided in the ipyrad analysis toolkit (<https://ipyrad.readthedocs.io/analysis.html>). TETRAD applies the theory of phylogenetic invariants (Lake, 1987) to infer quartet trees which are then used to reconstruct a species tree using the software wQMC (Avni et al., 2015). This combined approach was first developed in the software SVD Quartets (Chifman and Kubatko, 2014). The approach is applied under a coalescent based model, allowing for heterogeneity in the genealogy of loci. Unlike summary

coalescent methods such as MP-est (Liu et al., 2010) and ASTRAL 2 (Mirarab and Warnow, 2015), it uses all the information provided in the dataset and provides an efficient method of analysis compared to Bayesian methods such as BEST (Edwards et al., 2007), *BEAST (Heled and Drummond, 2010) and BPP (Yang and Rannala, 2014), which are computationally expensive. Under low levels of incomplete lineage sorting, the SVD quartet method outperforms concatenated ML based approaches and is competitive with the best available summary coalescent methods (Chifman and Kabatko, 2014). The SVD quartet trees were reconstructed both including and excluding the outgroup based on unlinked SNPs (one SNP per locus), extracted from the MS2 datasets. The SVD algorithm was carried out in the program TETRAD with 100 bootstrap replicates.

4.2.7. STRUCTURE analyses

Bayesian cluster analyses as implemented in the software STRUCTURE V.2.3.2.1 to identify discrete intraspecific genetic groups and individuals with genetic admixture (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007; Hubisz et al., 2009). The STRUCTURE analysis was based on unlinked SNPs from dataset MS2, excluding outgroups. This dataset included RAD loci present in at least two individuals per population, sufficient sampling to accurately estimate *Fst* statistics based on large numbers of SNPs (>1500) (Nazareno et al., 2017). To estimate the highest number of distinct genetic groups (K) that best fit the data, initial runs of 40,000 generations were conducted, with the first 20,000 generations discarded as the burnin fraction. For *B. lageniforme*, initial runs were carried out with K values ranging from 2 to 6. An admixed ancestry and correlated allele frequency model were specified, as recommended for closely related groups (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007). Ten independent replicate runs were carried out for each K value with one SNP randomly sampled from each RAD locus in replicate runs. Results from replicate runs were summarised using the software clump, with the default settings (Jakobsson and Rosenberg, 2007). The best number of genetic groups (K) was determined using the ad hoc value ΔK , which is based on the second order rate of change of the likelihood function with respect to K (Evanno et al., 2005). The most appropriate number of genetic groups (K) was determined from the modal value of the resulting ΔK distribution, excluding runs that did not converge. Ten independent final runs were carried out with the same settings and 200,000 generations, and the first 100,000 generations discarded as the burn-in fraction, for K values up to the best K value determined from the initial runs plus one. The most appropriate K value was calculated again based on the final runs.

4.3. Results

4.3.1. Parameter testing/datasets/replicate clustering

4.3.1.1. *Parameter testing*

ML phylogenetic trees reconstructed from the dataset generated with a clustering threshold of 0.90 and a read depth of six showed the best clustering of replicate samples and overall bootstrap support. All trees reconstructed from datasets generated with a clustering threshold of 0.80 or 0.85 or with a minimum read depth of three failed to cluster replicate samples.

4.3.1.2. *Datasets*

For the final analyses including all samples, three datasets were generated (MS1, MS2, MS8). The number of loci, SNPs, and % missing data for each dataset is provided in Table 4.2. Variation between the number of loci and SNPs in datasets including and excluding outgroups is due to exclusion of loci in the final filtering step. For datasets excluding the outgroup, outgroup species were removed and the final steps of the ipyrad pipeline were repeated including realignment and filtering of loci that did not meet thresholds for minimum samples per locus, maximum indels per locus, and/or maximum shared heterozygous sites per locus. As a result, a decrease was observed in the number of loci present in datasets including the outgroup, which is largely due to the exclusion of loci that included a higher number of indels, exceeding the maximum indel threshold.

Table 4.2 Number of loci, SNPs, and % missing data in the six final datasets including RAD loci present in a minimum of: one sample per population (MS1), two samples per population (MS2), and eight samples per population (MS8), including and excluding the outgroup.

Dataset	Outgroup	No. of RAD loci	Concatenated alignment length (bp)	No. of SNPs	missing data (%)
MS1	included	17289	2623010	78122	45
	excluded	17379	2634565	51531	44
MS2	included	9998	1647962	55021	30
	excluded	10054	1655559	35150	29
MS8	included	4128	736917	28242	11
	excluded	4146	739416	17508	9

4.3.1.3. *Replicate clustering*

Analyses based on all datasets reconstructed 78% of the replicate samples in closely related groups. Two pairs of replicates, G03680/G03681 and G03714/G03715, did not cluster in a sister group relationship.

4.3.2. Phylogenetic networks

NeighbourNet diagrams based on all datasets reconstructed two main groups within *B. lageniforme*, Group A and Group B (Fig. 4.1, Appendices 4.1, 4.2). Group A included individuals from mountaintops in the northern Wet Tropics (Devils Thumb, Mt Lewis, Windsor Tableland). Group B was composed of individuals from mountaintops in the central Wet Tropics (Lamb Range, Mt Bartle Frere, Mt Edith, Mt Haig). Within group A, no subgroups were discernible; individuals from each mountaintop did not cluster together but were spread across Group A. Within group B, three sub-groups were found, B1, B2 and B3, and the composition of these subgroups was largely consistent for all three datasets. Subgroup B1 was composed of individuals from the Lamb Range (the network based on dataset MS1 also included one individual (G03677) from Mt Edith), subgroup B2 included individuals from Mt Bartle Frere and subgroup B3 consisted of individuals from mountaintops of the Atherton Tablelands (Mt Edith, Mt Haig). Within subgroup B3, individuals from each mountaintop did not cluster together. Small variations were observed between datasets for samples reconstructed in intermediate positions between the main clusters. In networks based on dataset MS1, one individual from the Lamb Range (represented by duplicate samples Ka_G03712 and Ka_G03713) was found in an intermediate position between subgroup B1 and B2 and one individual from Mt Edith (G03686) was found in an intermediate position between subgroup B2 and B3 (Appendix 4.1). In the network based on dataset MS8, four individuals from the Lamb Range (G05511, G03707, G03708, G03710) and one individual from Mt Haig (G03692) were shown in an intermediate position between Group A and Group B (Appendix 4.2).

Figure 4.1 NeighborNet diagram of 65 B. lageniforme individuals based on the uncorrected P distances (Nei and Kumar, 2000) derived from 35,150 SNPs contained in dataset MS2 excluding outgroups. Replicate samples are indicated with an asterisk. BF: Mt Bartle Frere, DT: Devils Thumb, Ed: Mt Edith, Ha: Mt Haig, Le: Mt Lewis, LR: Lamb Range, Wi: Windsor Tableland.

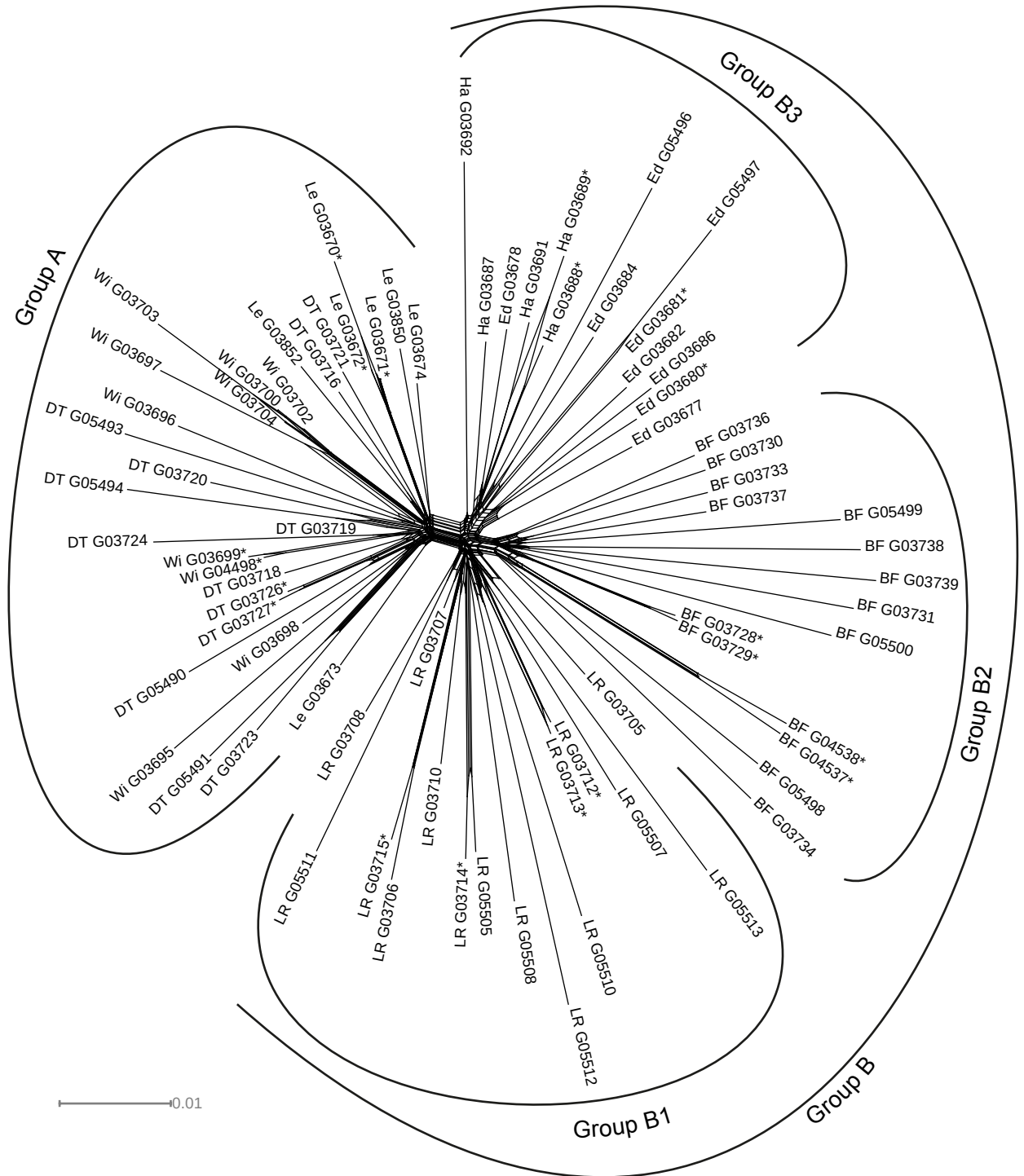


Fig 4.1

4.3.3. Phylogenetic reconstructions

Maximum likelihood phylogenetic reconstructions including the outgroup reconstructed all *B. lageniforme* individuals within a clade which received maximum support in all datasets (Fig. 4.2, Appendices 4.3, 4.4). *Bulbophyllum lageniforme* and *B. lilianae* were recovered in a highly supported sister group relationship (BS 100). Within the *B. lageniforme* clade, groups were resolved on short branches, and the backbone relationships remained unsupported. Individuals from the central Wet Tropics region were reconstructed in two clades corresponding to Mt Bartle Frere and the Atherton Tableland mountaintops (Mt Edith, Mt Haig) respectively, and a basal grade comprising individuals from the Lamb Range. This basal grade included one main clade of ten samples (unsupported) and four smaller clades of one or two samples each (the two clades of two samples each received maximum support). All individuals from the Atherton Tableland clustered together forming a weakly supported clade (BS 76) clade. Within this clade, individuals did not cluster according to the mountaintop from which they were sampled but were resolved in groups including a mixture of individuals from different mountaintops across the Atherton Tableland. All individuals from Mt Bartle Frere clustered together forming an unsupported clade. All individuals from the northern Wet Tropics region clustered together forming a highly supported clade (BS 96). Within this group individuals did not cluster according to the mountaintop from which they were sampled but were resolved in groups including a mixture of individuals from different mountaintops across the northern Wet Tropics region (Fig. 4.2).

Figure 4.2 Maximum likelihood reconstruction of 65 B. lageniforme individuals and two outgroup species based on 9,998 concatenated RAD loci from dataset MS2 including outgroups. Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset c. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Location of the Wet Tropics Bioregion. Inset c: Map of Wet Tropics Bioregion showing sampling locations.

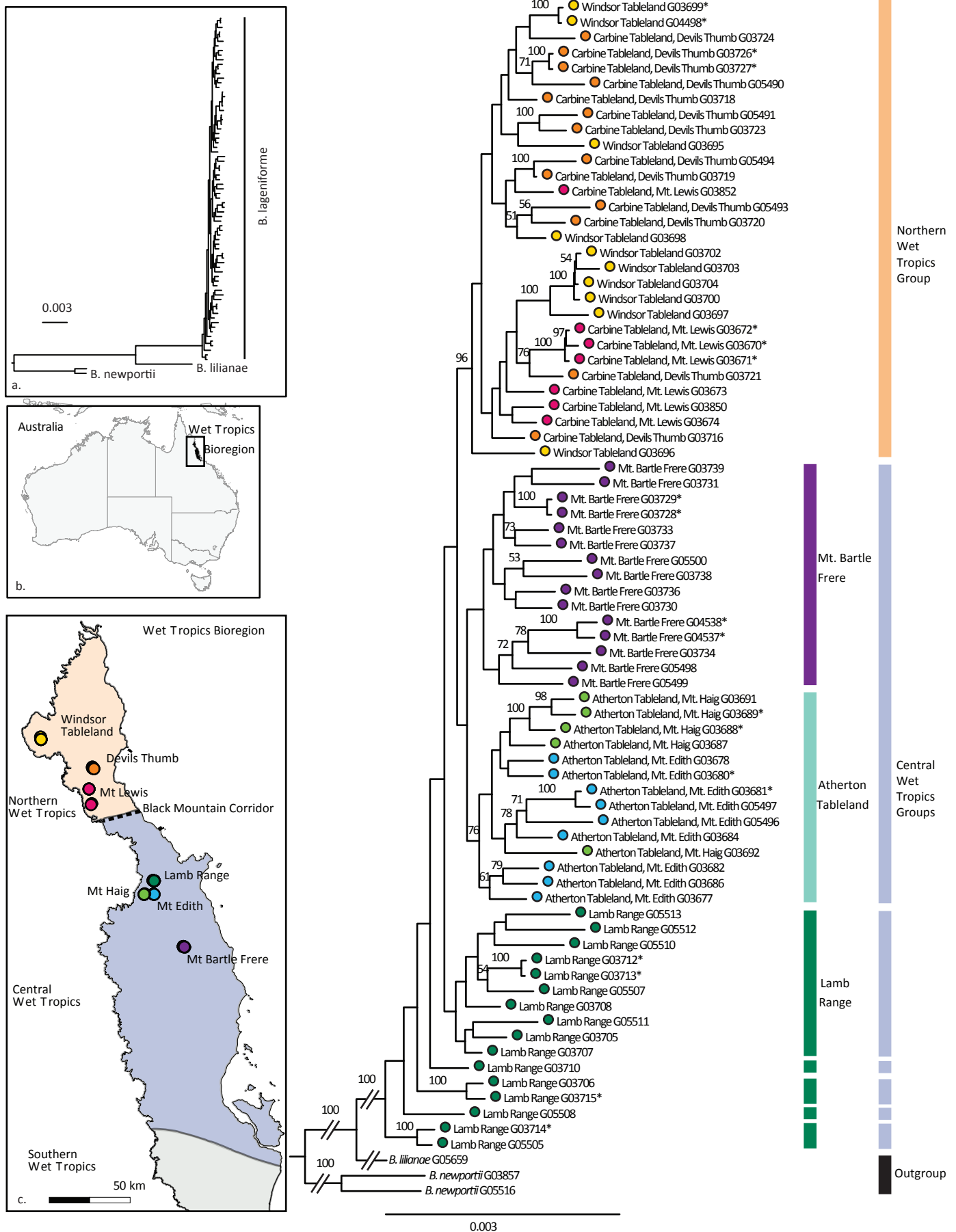


Fig 4.2

4.3.4. TETRAD

Phylogenetic reconstructions inferred under the coalescent model, including outgroups, reconstructed *B. lageniforme* individuals in a highly supported clade (BS 98) (Figure 4.3, Appendices 4.5, 5.6). *Bulbophyllum lageniforme* and *B. lilianae* were reconstructed in a highly supported sister group relationship (BS 100). Within the *B. lageniforme* clade, backbone relationships remained unsupported. Phylogenetic reconstructions resolved a broad topology within the *B. lageniforme* clade that was largely consistent in reconstructions based on all three datasets (Figure 4.3, Appendices 4.5, 4.6). Individuals from the central Wet Tropics region were resolved in a phylogenetic grade, with individuals from Mt Bartle Frere reconstructed in groups diverging at the base of the tree and individuals from the Lamb Range and the Atherton Tableland diverging later. However, backbone relationships remained unsupported. All individuals from the Atherton Tableland clustered together but without support. Within this clade, individuals from each mountaintop did not cluster together. All individuals from the northern Wet Tropics were resolved in a highly supported clade (BS 94) but relationships within this group were mostly unsupported and individuals from each mountaintop did not cluster together.

Figure 4.3 SVD quartet tree showing relationships among 65 individuals of B. lageniforme and two outgroup species, based on 9,998 SNPs derived from dataset MS2 including outgroups (one SNP per RAD locus). Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset b. Insert a: Location of the Wet Tropics Bioregion. Insert b: Map of Wet Tropics Bioregion showing sampling locations.

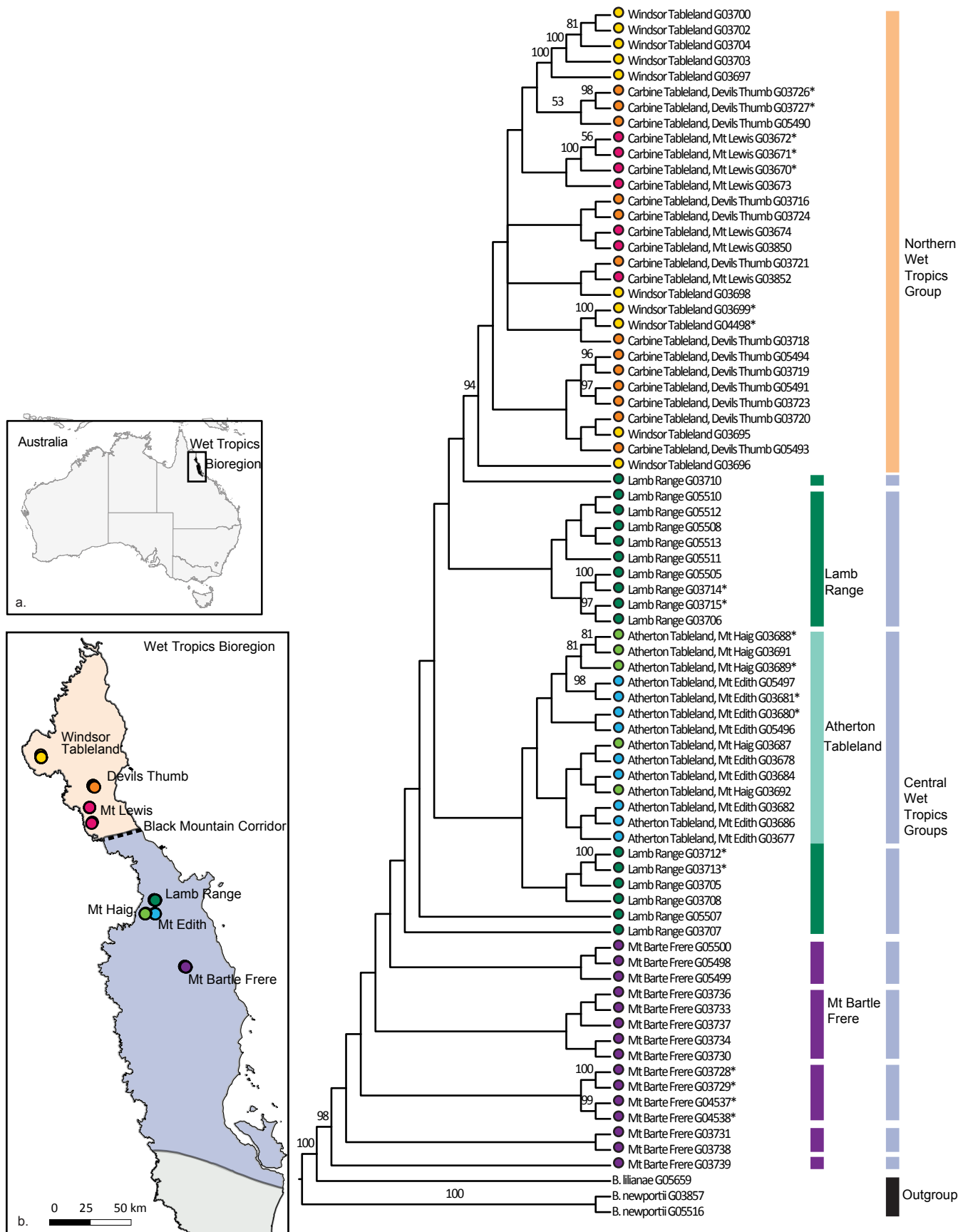


Fig 4.3

4.3.5. STRUCTURE

Bayesian clustering analysis in STRUCTURE did not recover population structure within *B. lageniforme* (Fig. 4.4). The modal value of delta K indicated a K value of four, assuming ancestry from four distinct genetic sources best fit the data (Appendix 4.7). However, all STRUCTURE plots, assuming ancestry from two to eight genetic groups, showed each *B. lageniforme* individual was predominantly characterised by the same genetic group with small proportions of admixture from other groups (Appendix 4.8). The membership coefficient plots for analyses assuming ancestry from four genetic sources showed the genetic profile of each individual was dominated by one genetic group and also included smaller proportions of the other three genetic groups (Fig. 4.4). A distinct difference in the proportion of genetic admixture within individuals was observed between the northern and central Wet Tropics populations. The genetic profiles of the individuals from the northern group are dominated by genetic group one with only a small fraction of admixture. The genetic profiles of individuals from the central Wet Tropics populations were also dominated by genetic group one but included higher proportions of genetic group two compared with the northern Wet Tropics individuals. The fraction of genetic groups three and four was very small in most *B. lageniforme* individuals. However, several individuals from Lamb Range were characterised by a more substantial fraction of genetic admixture from these groups.

Figure 4.4 Bar plots indicating genetic admixture of 65 individuals of B. lageniforme. These results are based on a Bayesian analysis of genetic structure as implemented in STRUCTURE based on 10,054 SNPs derived from dataset MS2 excluding outgroups (one SNP per RAD locus) and assuming K=4. Replicate samples are indicated with an asterisk. Bar plot is displayed adjacent to the maximum likelihood phylogeny from Fig. 4.2.

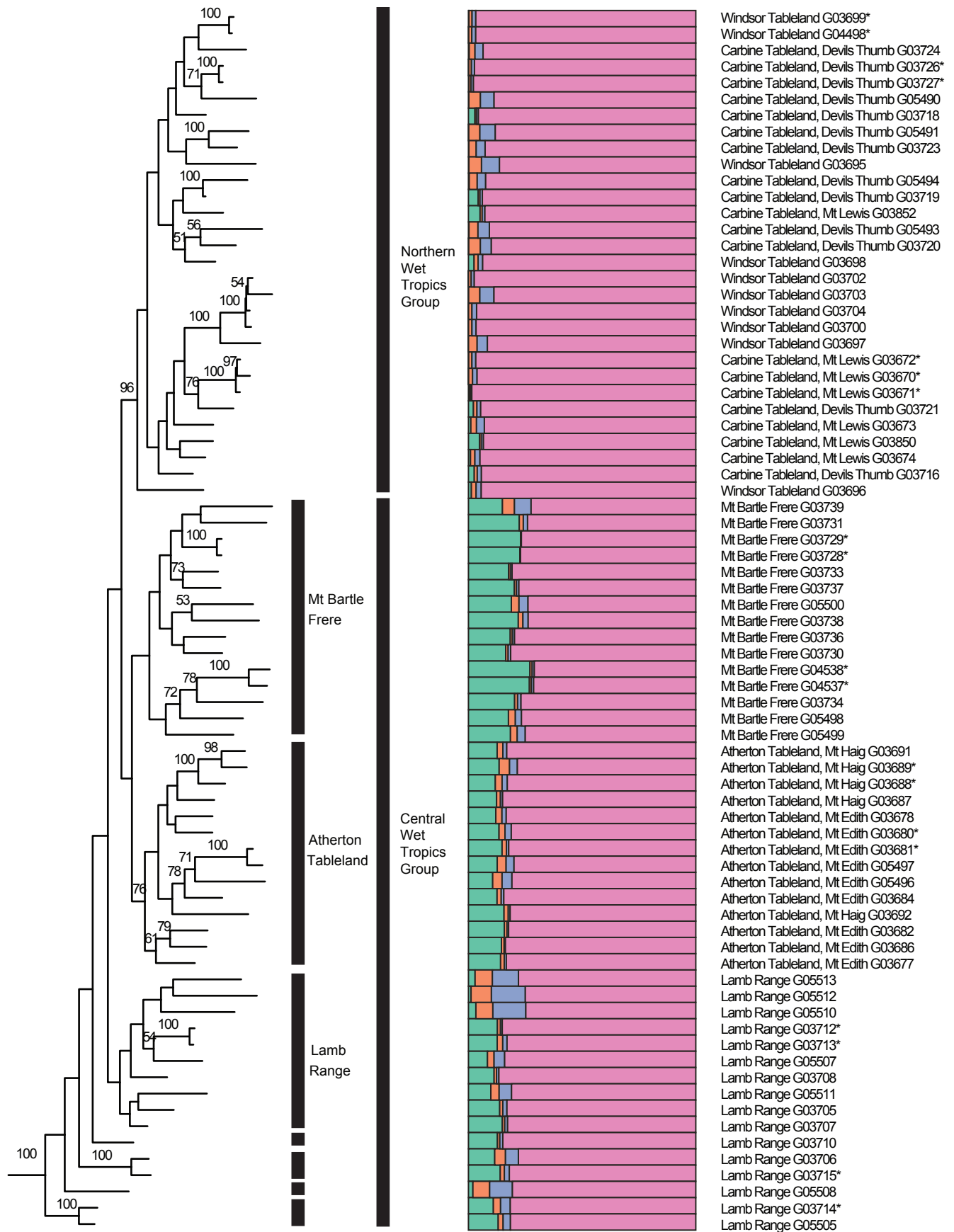


Fig. 4.4

4.4. Discussion

4.4.1. Interspecific relationships

The phylogenetic analyses showed *B. lageniforme* individuals formed a divergent and highly supported group resolved in a sister group relationship to *B. lilianae* in both ML and coalescent-based analyses based on all datasets (Figs 4.2, 4.3, Appendices 4.3-4.6). Together *B. lageniforme* and *B. lilianae* formed a highly supported sister group relationship to a clade including *B. newportii*. The relationships reconstructed from RAD loci are consistent with the interspecific relationships reconstructed from ML and Bayesian phylogenetic analyses based on two highly informative plastid coding regions and three nuclear ribosomal regions (Chapter three). However, given sampling of *B. lilianae* in the population level analysis was limited to a single individual and Bayesian clustering analyses included only *B. lageniforme* individuals, it remains beyond the scope of this study to assess the level of gene flow between *B. lageniforme* and *B. lilianae*.

4.4.2. Phylogeographic structure within *B. lageniforme*

Phylogenetic reconstructions and NeighborNet diagrams each revealed phylogeographic structure within *B. lageniforme* (Figs 4.1-4.3). NeighborNet diagrams differentiated two main groups comprising individuals from: 1) the northern Wet Tropics region and 2) the central Wet Tropics region (Fig. 4.1, Appendices 4.1, 4.2). The central Wet tropics group was further delineated into three subgroups including individuals from: 1) Lamb Range, 2) Atherton Tableland and 3) Mt Bartle Frere. ML and coalescent based phylogenetic reconstructions based on all datasets resolved individuals from the northern Wet Tropics region within a moderate to high supported clade providing evidence for divergence between the northern Wet Tropics and the central Wet tropics populations (Fig. 4.2, 4.3, Appendices 4.3-4.6). The northern and central Wet Tropics regions are separated by a recognised biogeographic barrier for rainforest specialists, the BMC.

The BMC is characterised by savannah communities extending from the coast westward, dissecting the northern and central Wet Tropics rainforest blocks (Moritz et al. 2009). Under current climatic conditions, the corridor of savannah habitat is relatively narrow, separating the northern and central rainforest blocks by 50–60 km. However, modelling of rainforest and savannah communities under paleoclimates shows these habitats expanded and contracted dynamically in response to climatic fluctuations, that were particularly pronounced during the

glacial cycles of the Quaternary (Graham et al. 2006, Hilbert et al. 2007, Vanderwal et al. 2009). With the expansion of savannah habitat during drier climates such as the last glacial maximum, the BMC is likely to have presented a stronger biogeographic barrier. Divergence across the BMC has been previously identified in phylogeographic studies of rainforest endemic reptiles and invertebrates (Hugall et al., 2002; Dolman and Moritz 2006; Moussalli et al., 2009; Edwards and Melville 2010; Bell et al., 2010; Hoskin et al., 2011; Bell et al., 2012). While the influence of the BMC on plants is not as well studied, evidence suggests it may be more permeable for plant species than for animal species. Evidence for divergence across the BMC has been identified within a widespread epiphytic and lithophytic orchid adapted to open and closed forest types (*Dendrobium speciosum* Sm.), however, evidence of gene flow indicated the barrier was semi-permeable (Burke et al., 2013; Simpson et al., 2018). Comparative phylogeographic studies of rainforest trees (*Elaeocarpus* L., Elaeocarpaceae) showed the influence of the barrier varied between species due to subtle trait and habitat related differences that promoted independent patterns of geneflow and migration across the Wet Tropics (Rossetto et al., 2009). Among the trees studied, the strongest genetic structure across the BMC was identified in species restricted to the upland rainforests.

The results from phylogenetic reconstructions and NeighbourNet diagrams of *B. lageniforme* provided evidence of divergence across the BMC within an endemic rainforest epiphyte species. However, the short stem branch of the northern Wet Tropics clade indicated divergence from the central Wet Tropics populations is relatively shallow and the Bayesian clustering analysis of *B. lageniforme* indicated only shallow population genetic structure within the Wet Tropics, including across the BMC. The Bayesian clustering analyses may be unable to reconstruct population substructure due to low levels of population differentiation among the geographic groups delineated in the phylogenetic analyses and networks (Latch et al., 2006). Whilst the phylogeographic analyses provided evidence for weak phylogeographic structure, overall the results indicate low levels of intraspecific genetic diversity within *B. lageniforme*.

4.4.3. Does the current distribution of *B. lageniforme* represent refugial populations or recent colonisations?

Divergence dating analyses estimated a Pliocene divergence between *B. lageniforme* and its sister species *B. lilianae*, both of which are endemic to high altitude rainforests of the Wet Tropics (Chapter three). However, the shallow phylogeographic structure and lack of population structure identified within *B. lageniforme* does not reflect the genetic diversity

expected among refugial populations that have persisted through repeated cycles of rainforest contraction and fragmentation in the Wet Tropics Bioregion during the Pliocene and Pleistocene. The low diversity identified within *B. lageniforme* is in contrast to phylogeographic studies that have found evidence for highly structured diversity within Wet Tropics rainforest endemic fauna species, which is thought to be the result of long term persistence in rainforest refugia that have become isolated during restrictive climates, facilitating allopatric divergence (Hugall et al., 2002; Dolman and Moritz, 2006; Moussalli et al., 2009; Edwards and Melville, 2010; Bell et al., 2010; Hoskin et al., 2011; Bell et al., 2012).

Two hypotheses are considered to explain the low genetic diversity identified within *B. lageniforme*: 1) high rates of gene flow between disjunct populations or 2) recent colonisation from a refugial population. In plants gene flow is mediated by a myriad of variables, including pollen and ovule export. *Bulbophyllum* orchids are pollinated by several families of fruit flies, however their pollination biology remains poorly understood. Orchidaceae is characterised by their minute seeds lacking an endosperm. Evidence for long distance dispersal (LDD) has been frequently inferred in higher level evolutionary studies of orchids (Givnish et al., 2016; Perez-Escobar and Chomicki et al., 2017). However, the rate of LDD events inferred was relatively low, therefore, over shorter evolutionary timescales dispersal over long distances is likely to be relatively rare (Givnish et al., 2016). Empirical studies of orchid seed dispersal have shown dispersal distance decreases with increasing distance from the parent plant, with only a small proportion of seeds dispersing long distances (Chung, et al., 2004; Jersakova and Mallinova, 2007; Brozosko, 2017). Effective LDD is further limited by the complex requirements for establishment of orchid seeds, for example their dependence on fungal mycorrhiza (Arditti and Ghani, 2000; Jersáková and Malinová, 2007; McCormick et al., 2012). The rates of seed dispersal and migration of insect pollinators of *B. lageniforme* remains unclear.

Alternatively, the low diversity found within *B. lageniforme* can be explained by a process of recent colonisations from a single refugial population, which have not had sufficient time to diverge from the source population. Low diversity has been previously identified in phylogeographic studies of rainforest specialists, suggesting the genetic structure of these species has been shaped by processes of localised extinction and recolonisation in response to environmental changes associated with climatic fluctuations (Williams et al., 1997; Schneider et al., 1998; Bowyer et al., 2000; Schneider and Williams, 2005; Oza et al., 2012). The narrow

environmental range and epiphytic niche occupied by *B. lageniforme* indicates this species is also likely to have been highly vulnerable to localised extinction during restrictive climates. The low diversity identified within this species may reflect a relatively recent range expansion from a single refugial population. Given the restraints on seed dispersal, establishment and migration of small insect pollinators in Orchidaceae, it seems unlikely that gene flow among disjunct populations across the Wet Tropics would be sufficient to maintain low genetic diversity. Therefore, based on current evidence, a hypothesis of recent colonisation from a refugial population is preferred.

Within *B. lageniforme*, higher genetic diversity was identified within the central relative to the northern Wet Tropics region, suggesting the species may have maintained a refugial distribution in the central Wet Tropics region with a relatively recent expansion into the northern Wet Tropics region. NeighborNet analyses reconstructed three geographic groups within the central Wet Tropics region composed of individuals from: 1) the Atherton Tablelands, 2) Lamb Range and 3) Mt Bartle Frere. In contrast, individuals from the northern Wet Tropics region did not cluster together based on the mountaintop from which they were sampled, and this group was characterised by lower genetic diversity. The lower genetic diversity observed for the northern Wet Tropics, relative to the central Wet Tropics populations could result from higher rates of gene flow between mountaintops in the northern Wet Tropics region, or more recent colonisation events. However, populations within either the northern or central Wet Tropics are similar in their geographic proximity, separated by lowland barriers that appear similar in their capacity to limit dispersal. Therefore, higher rates of gene flow among the northern Wet Tropics populations seems unlikely. Alternatively, the weak phylogeographic structure reconstructed among populations in the central Wet Tropics region can be explained under a scenario where *B. lageniforme* has maintained a refugial distribution in the central Wet Tropics region, while the northern Wet Tropics region has been colonised relatively recently with insufficient time having elapsed for disjunct populations to diverge.

Modelling studies have shown the mountaintops of central Wet Tropics region such as Mt Bartle Frere and Mt Bellenden Ker have provided the most stable upland rainforest refugia in the Wet Tropics region throughout the climatic cycles of the Quaternary (Vanderwal et al. 2009, Graham et al. 2010). The high species endemism and high number of unique lineages found in these regions indicates the long-term stability of these refugia (Mortiz et al., 2009; Williams et al., 2010). Modelling of rainforest habitat under paleoclimatic conditions indicated

rainforest habitat was unlikely to have persisted on Mt Windsor during restrictive climates such as the last glacial maximum and therefore the Mt Windsor populations are most likely to have been derived from recent colonisations from other northern Wet Tropics areas that maintained habitat stability throughout the Quaternary such as the Carbine Tableland (Graham et al. 2010).

Today, *B. lageniforme* occupies a restricted distribution, found only on mountaintops of the central and northern Wet Tropics regions. However, it remains unclear if the species previously occupied a wider distribution across the Wet Tropics (such as the southern or the extreme northern Wet Tropics) or persisted in multiple refugial populations within its current range. Considering the estimated Pliocene divergence of the species and the dispersal potential of minute wind-dispersed seeds, it is possible this species has previously established populations across the Wet Tropics, which have subsequently succumbed to localised extinction. Rainforest refugia of the northern and southern Wet Tropics regions are reconstructed as smaller patches and were found to be more unstable over the Quaternary, relative to the major refugia of the central Wet Tropics regions (Vanderwal et al., 2009; Graham et al., 2010). The narrow environmental range and epiphytic niche that the species currently occupies indicates *B. lageniforme* would have been highly vulnerable to localised extinction with the severe contraction of the high-altitude rainforest habitats during cooler, drier climatic periods such as the last glacial maximum. Highly stable refugia provided suitable habitat during these restrictive climates and likely played a critical role in preserving upland rainforest specialist taxa.

4.4.4. Conservation

The narrow environmental range of *B. lageniforme* in high altitude rainforest and its low genetic diversity indicates this species is likely to be at elevated risk of extinction due to climatic shifts predicted for the Wet Tropics region, including a reduction in moisture availability to upland rainforest associated with an increase in average global temperatures (Still et al., 1999; Foster, 2001; Williams et al., 2012). Environmental niche modelling of tree species endemic to mountaintops of the Wet Tropics under future climatic conditions predicted that habitat suitability will be drastically reduced by 2080 (Costion et al., 2015; Gagul et al., 2018). An assessment of the conservation status of *B. lageniforme* is urgently required and should consider the highly localised range of the species and impacts of predicted climatic shifts. However, insufficient data on generation time, population sizes and range under

contemporary and future climatic conditions is available to assess *B. lageniforme* against the IUCN red list criteria (IUCN 2012, 2017). So far, climate change has not been sufficiently considered in red listing of Australian orchids (Wraith and Pickering, 2019) and yet recent studies have shown many orchids in Australia are threatened by climate change (Reiter et al., 2016, Simmons et al., 2018, Simpson et al., 2018). Environmental niche modelling is required to predict the impact of climatic shifts and complete an IUCN red list assessment for *B. lageniforme*.

For *ex situ* conservation of *B. lageniforme* it is recommended that sampling is informed by the results from the phylogeographic analysis of the species. These results of this study indicate that to conserve the genetic diversity present within the species, sampling should include representatives from both the northern and southern Wet Tropics groups and where resources are available sampling within the central Wet Tropics should include representatives of the Lamb Range, Atherton Tablelands and Mt Bartle Frere groups.

4.5. Chapter summary

- A phylogeographic study was carried out to assess intraspecific genetic diversity with *Bulbophyllum lageniforme*, a narrow range epiphytic species endemic to rainforests of Australia's tropical mountaintops
- 65 individuals were sampled from seven mountaintops in the Wet Tropics Bioregion spanning the distribution of the species with 1–14 (mean=8) individuals sampled per population
- Genomic data was generated using double-digest restriction site-associated DNA (ddRAD) sequencing and three genomic datasets were generated with varying levels of filtering stringency allowing for different proportions of loci with missing data, resulting in datasets ranging from 17379 to 4146 RAD loci.
- Maximum likelihood, coalescent and distance-based phylogenetic reconstructions showed evidence of weak phylogeographic structure across a recognised biogeographic barrier for rainforest endemic species, the Black Mountain Corridor.
- Bayesian clustering analyses revealed low genetic diversity and no evidence of population structure across the entire dataset.
- Overall the results from the phylogenetic and Bayesian clustering analyses were consistent with the current distribution having resulted from relatively recent expansion from a single refugial population.
- To conserve the genetic diversity present within *B. lageniforme* *ex situ* conservation efforts should include representatives from both the northern and southern Wet Tropics groups at a minimum. Where resources allow, sampling within the central Wet Tropics should include representatives of the Lamb Range, Atherton Tablelands and Mt Bartle Frere groups.

Chapter five: Comparative phylogeography of two closely related mountaintop endemic epiphytes from tropical Australia with varied ecological niches (*Bulbophyllum newportii* (F.M.Bailey) Rolfe and *Bulbophyllum lageniforme* F.M. Bailey, Orchidaceae)

5.1. Introduction

Australia's tropical mountaintops harbour a unique composition of endemic diversity including relic populations of Gondwanan derived rainforest lineages mixed with more recent arrivals of Asian origins (Sniderman and Jordan, 2011, Crayn et al., 2015). Studies have indicated this unique endemic diversity may be at threat with significant climatic changes predicted for Australia's tropical mountaintops (Hilbert et al., 2001; Hilbert and Williams, 2003; Williams, 2003; Shoo et al., 2005a; 2005b; Williams and Hilbert, 2006; Costion et al., 2015). *Ex situ* conservation action has been recommended and initiated for mountaintop endemic plants to mitigate against extinction (Costion et al., 2015; Mountner, 2019). To increase long term viability, sampling for *ex situ* conservation should aim to preserve intraspecific genetic diversity and effective sampling strategies should be informed by an understanding of the spatial distribution of genetic diversity within these communities. However, limited studies are available that document phylogeographic patterns within Australia's tropical montane communities that can inform conservation actions (Moritz et al., 2001). Comparative phylogeographic studies have shown variation in the spatial distribution of genetic diversity within species, even among closely related taxa, indicating varied responses to past environmental changes (Schneider et al., 1998; Rossetto et al., 2009; Bell et al., 2012; Burke et al., 2013; Simpson et al., 2018). Phylogeographic studies have largely focused on fauna (Williams et al., 1997; Schneider et al., 1998; Bowyer et al., 2000; Hugall et al., 2002; Schneider and Williams, 2005; Dolman and Moritz 2006; Moussalli et al., 2009; Bell et al., 2010; Edwards and Melville 2010; Hoskin et al., 2011; Bell et al., 2012; Oza et al., 2012) and further studies are required to assess the genetic diversity present in Australia's tropical upland endemic flora.

This study aims to reconstruct the phylogeography of an epi-lithophytic species endemic to Australia's tropical upland mesic forests, *Bulbophyllum newportii*, to infer its response to past climatic changes and inform effective sampling strategies for *ex situ* conservation action.

Bulbophyllum newportii occupies a widespread distribution across the tropical mountaintops of Australia's eastern coastal ranges. *Bulbophyllum newportii* (F.M. Bailey) Rolfe is closely related to the rainforest endemic species *B. lageniforme* F.M. Bailey, providing a useful study system to compare responses to past climatic shifts among closely related, co-occurring species. Both species belong to the Australasian *Bulbophyllum* lineage (Chapter three, Fig. 3.1, 3.2). Molecular dating analysis estimated *B. newportii* and *B. lageniforme* each diverged from their sister species during the

Pliocene (Chapter three). Both species are restricted to tropical upland mesic habitats but occupy different environmental niches. *Bulbophyllum lageniforme* is a narrow range rainforest endemic species found between 1000–1500m. *Bulbophyllum newportii* occupies a broader environmental niche, occurring at mid to high altitudes (600m–1600m), in open or closed mesic habitats including rainforest, wet sclerophyll forest, and exposed rocky outcrops (Jones, 2006). The species is predominantly found in the montane forests of the Wet Tropics Bioregion, a zone of mesic forest communities distributed for 450km along a series of coastal ranges reaching 1600m elevation. The northern distributional limit of the species is marked by disjunct populations in the Iron and McIlwraith Ranges in Australia's Cape York Peninsula Bioregion, which harbour small areas of upland mesic forest communities along ranges reaching 800m elevation. The southern distributional limit of the species is marked by a disjunct population on the Clarke Range in the Central Mackay Coast Bioregion. The Clarke Range harbours a small patch of upland cloud forest habitat at an elevation of about 800m.

The distribution of upland mesic forest that *B. newportii* occupies is highly fragmented. The Iron and McIlwraith Ranges to the north and the Clarke Range to the south are each separated from the montane forests in the Wet Tropics Bioregion by at least 800 km of lowland dry forest communities. Within the Wet Tropics Bioregion, the upland forests form an archipelago, fragmented by lowland forest communities. Several major disjunctions are known which separate the northern, central and southern upland forest habitats of the region. The BMC is a recognised dry biogeographic barrier that separates the rainforests of the northern and central Wet Tropics regions, while the Herbert River valley is a lowland corridor that separates the upland forests of the central and southern Wet Tropics regions (Crisp et al., 2017). Palynological, paleoclimatic modelling and phylogeographical studies showed that Australia's upland mesic forests have undergone a series of expansions and contractions in response to climatic fluctuations during the glacial cycles of the Pliocene and Pleistocene. Through these fluctuations, large areas of rainforest refugia persisted in the central Wet Tropics regions and smaller fragments were maintained in the northern and southern Wet tropics regions (Hilbert et al., 2007; VanDerWal et al., 2009).

The specific aims of this study are to use genomic methods to:

1. assess genetic diversity within *B. newportii*;
2. use geographic patterns of genetic diversity to evaluate evolutionary hypotheses explaining the current distribution of *B. newportii*;

3. compare phylogeographic structure between *B. newportii* and the closely related rainforest endemic species *B. lageniforme*; and
4. identify provenance sampling strategies to support ex situ conservation approaches that seek to preserve intraspecific genetic diversity.

5.2. Methods

5.2.1. Sampling and DNA extraction

In total 91 *B. newportii* samples from 13 populations across the known distribution of the species were analysed. Plants were collected under the Australian Tropical Herbarium Queensland parks and wildlife collecting permit. As per conditions of the collecting permit, for the Thornton Peak and Mt Finnigan areas a research proposal was presented to Jabalbina Yalanji Aboriginal Corporation as the prescribed body corporate, and further permission was sought from the Traditional Owners for these regions. Based on the wishes and consultation with Traditional Owners from the Thornton Peak region, samples from this area have not been included in this study. Sampling localities included nine mountaintops in the Wet Tropics (Mt Bartle Frere, Mt Edith, Mt Haig, Carrington Falls, Lamb Range, Black Mountain, Devils Thumb, Mt Windsor and Mt Finnigan), Clarke Range in Central Mackay Coast Bioregion and Mt Carter in the Cape York Peninsula Bioregion. From each population one to 13 (M=7) individuals were collected. Due to limited resources available for field work on Australia's southern east coast, sampling of the sister species *B. exiguum* was limited to a single representative. Provenance and voucher information are provided in Table 5.1.

Table 5.1 Collection details for 91 individuals of B. newportii included in the study and two individuals of B. lageniforme and one individual of B. exiguum that comprised the outgroup.

Species	Voucher details	DNA number	Population
<i>B. exiguum</i> F. Muell	CANB: Clements, M.A. 12008	CNS_G05503	
<i>B. lageniforme</i> F.M. Bailey	CNS: Simpson, L. 296F	CNS_G03720	
<i>B. lageniforme</i>	CNS: Simpson, L. 432E	CNS_G04537	
<i>B. newportii</i>	CNS: Simpson, L. 121F	CNS_G03826	Carrington Falls
<i>B. newportii</i>	CNS: Simpson, L. 121F	CNS_G03827	Carrington Falls
<i>B. newportii</i>	CNS: Simpson, L. 121E	CNS_G03834	Carrington Falls
<i>B. newportii</i>	CNS: Simpson, L. 121B	CNS_G03835	Carrington Falls
<i>B. newportii</i>	CNS: Simpson, L. 121H	CNS_G03836	Carrington Falls
<i>B. newportii</i>	CNS: Simpson, L. 395A	CNS_G03740	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 395A	CNS_G03741	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 377	CNS_G03743	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 377	CNS_G03744	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 383	CNS_G03747	Clarke Range

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Species	Voucher details	DNA number	Population
<i>B. newportii</i>	CNS: Simpson, L. 396C	CNS_G03749	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 382C	CNS_G03751	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 516	CNS_G03837	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 396E	CNS_G04521	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 395H	CNS_G05525	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 395F	CNS_G05527	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 382B	CNS_G05528	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 395I	CNS_G05529	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 396D	CNS_G05530	Clarke Range
<i>B. newportii</i>	CNS: Simpson, L. 270	CNS_G04489	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 270	CNS_G04490	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 281A	CNS_G04491	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 277	CNS_G04492	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 305A	CNS_G04495	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 313A	CNS_G04497	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 284D	CNS_G04509	Devils thumb
<i>B. newportii</i>	CNS: Simpson, L. 284D	CNS_G04511	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 315	CNS_G05514	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 313C	CNS_G05516	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 311	CNS_G05517	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 305B	CNS_G05518	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 308B	CNS_G05519	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 272	CNS_G05521	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 289	CNS_G05522	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 284B	CNS_G05523	Devils Thumb
<i>B. newportii</i>	CNS: Simpson, L. 157E	CNS_G03753	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 155E	CNS_G03754	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 155C	CNS_G03755	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 171	CNS_G03757	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 157C	CNS_G03759	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 157D	CNS_G03761	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 157A	CNS_G05475	Lamb Range

Species	Voucher details	DNA number	Population
<i>B. newportii</i>	CNS: Simpson, L. 157B	CNS_G05476	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 155B	CNS_G05477	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 102	CNS_G05534	Lamb Range
<i>B. newportii</i>	CNS: Simpson, L. 517	CNS_G03843	Longlands Gap, Herberton
<i>B. newportii</i>	CNS: Simpson, L. 077	CNS_G03854	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 442D	CNS_G03855	Mt Bartle Frere
<i>B. newportii</i> (F.M. Bailey) Rolfe	CNS: Simpson, L. 442F	CNS_G03856	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 442G	CNS_G03857	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 442C	CNS_G03858	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 442A	CNS_G03859	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 442H	CNS_G04478	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 080	CNS_G05531	Mt Bartle Frere
<i>B. newportii</i>	CNS: Simpson, L. 515	CNS_G03844	Mt Black Mountain
<i>B. newportii</i>	CNS: Simpson, L. 518	CNS_G03841	Mt Carter
<i>B. newportii</i>	CNS: Simpson, L. 140	CNS_G03828	Mt Edith
<i>B. newportii</i>	CNS: Simpson, L. 140	CNS_G03829	Mt Edith
<i>B. newportii</i>	CNS: Simpson, L. 132A	CNS_G03833	Mt Edith
<i>B. newportii</i>	CNS: Simpson, L. 327A	CNS_G03812	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 327A	CNS_G03813	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 341A	CNS_G03814	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 343A	CNS_G03816	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 334A	CNS_G03817	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 334A	CNS_G03818	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 326A	CNS_G03821	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 334B	CNS_G05479	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 343F	CNS_G05480	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 323A	CNS_G05481	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 322B	CNS_G05482	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 332C	CNS_G05483	Mt Finnigan

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Species	Voucher details	DNA number	Population
<i>B. newportii</i>	CNS: Simpson, L. 326B	CNS_G05486	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 325B	CNS_G05488	Mt Finnigan
<i>B. newportii</i>	CNS: Simpson, L. 153A	CNS_G03824	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 153A	CNS_G03825	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 147B	CNS_G03830	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 146	CNS_G03831	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 153B	CNS_G03832	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 147A	CNS_G05478	Mt Haig
<i>B. newportii</i>	CNS: Simpson, L. 520	CNS_G03839	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 519	CNS_G03840	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 211B	CNS_G03845	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 185F	CNS_G03846	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 185E	CNS_G03847	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 185C	CNS_G03848	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 185A	CNS_G03849	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 185D	CNS_G05532	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 184	CNS_G05533	Mt Windsor
<i>B. newportii</i>	CNS: Simpson, L. 417A	CNS_G04481	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 399A	CNS_G04484	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 399A	CNS_G04485	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 411A	CNS_G04486	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 407A	CNS_G04487	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 404A	CNS_G04488	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 404B	CNS_G04550	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 404B	CNS_G04553	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 404C	CNS_G05467	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 417B	CNS_G05468	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 418B	CNS_G05469	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 407B	CNS_G05470	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 399B	CNS_G05471	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 414B	CNS_G05473	Paluma Range
<i>B. newportii</i>	CNS: Simpson, L. 411B	CNS_G05474	Paluma Range

5.2.2. DNA extraction and RAD library preparation

DNA extraction and RAD library preparation were carried out as described previously (Chapter four, section 4.2.2).

5.2.3. Data filtering and management

Datasets of homologous RAD loci were generated with the pyRAD pipeline (Eaton, 2014) and preliminary analysis was carried out to identify optimal parameters as described previously (Chapter four, section 4.2.3). For the final analyses including all individuals of *B. newportii*, six datasets were generated in ipyrad v. 0.7.23. All datasets were generated with the optimal clustering threshold (0.8) and minimum read depth (six) determined from parameter tests based on the smaller subset of individuals. Final datasets were generated with three variations of the minimum samples per locus parameter: RAD loci present in at least one individual per population (MS1), RAD loci present in at least two individuals per population (MS2) and RAD loci present in at least eight individuals per population (MS8). For each variation of the minimum samples per locus parameter, datasets were produced both including and excluding the outgroup, comprising one individual of *B. exiguum* and two individuals of *B. lageniforme*.

5.2.4. Phylogenetic analyses

To detect potentially conflicting phylogenetic signal in the data sets, NeighborNet analyses (Bryant and Moulton, 2004) were carried out on the SNP datasets, excluding the outgroup in SplitsTree4, V.4.13.1 (Huson and Bryant, 2006), based on the proportion of nucleotide sites at which two individuals differ (uncorrected p distances: Nei and Kumar, 2000).

To reconstruct relationships among individuals ML analyses were carried out with concatenated alignments of RAD loci in the program RAxML v. 8.2.4 (Stamatakis, 2014), with settings as described previously (Chapter four, section 4.2.3). ML reconstructions were carried out both including and excluding the outgroup, which comprised two individuals of *B. exiguum* and one individual of *B. lageniforme*.

To reconstruct relationships among individuals under the coalescent model, SVD quartet trees were reconstructed in the program TETRAD, implemented within ipyrad v 0.7.23. Analyses were run both including and excluding the outgroup, based on unlinked SNPs (one SNP per locus), extracted from the MS2 dataset, with settings as described previously (Chapter four, section 4.2.6).

5.2.5. Population analyses

To identify discrete intraspecific genetic groups and individuals with genetic admixture, Bayesian cluster analyses were run for each species excluding the outgroup using STRUCTURE V.2.3.2.1 (Falush et al., 2003, Falush et al., 2007, Hubisz et al., 2009, Pritchard et al., 2000). Initial and final runs were carried out as described previously (Chapter four, section 4.2.7). For *B. newportii*, initial runs to identify the highest number of distinct genetic groups (K) best fitting the data were carried out with K values ranging from two to ten.

5.3. Results

5.3.1. Parameter testing/datasets/replicate clustering

5.3.1.1. Parameter testing

ML phylogenetic trees reconstructed from the dataset generated with a clustering threshold of 0.8 and a read depth of six showed the best clustering of replicate samples and highest overall bootstrap support. All trees reconstructed from datasets generated with a clustering threshold of 0.9 or 0.85 or with a minimum read depth of three or ten failed to cluster replicate samples.

5.3.1.2. Datasets

For the final analyses including all samples, three datasets were generated, MS1, MS2 and MS8. The number of loci, SNPs, and % missing data for each dataset is provided in Table 5.2. Variation between the number of loci and SNPs in datasets including and excluding outgroups is due to exclusion of loci in the final filtering step (Chapter four, section 4.3.1).

Table 5.2 Number of loci, SNPs, and % missing data in the six final datasets including RAD loci present in a minimum of: one sample per population (MS1), two samples per population (MS2), and eight samples per population (MS8), including and excluding the outgroup.

Dataset	outgroup	No. of RAD loci	Concatenated alignment length (bp)	No. of SNPs	missing data (%)
MS1	included	7010	1024261	49944	41
	excluded	7398	1085315	42897	39
MS2	included	4349	705546	36990	29
	excluded	4633	752089	31540	28
MS8	included	1476	264990	15490	10
	excluded	1582	284066	13077	9

5.3.1.3. Replicate clustering

Analyses based on all datasets clustered 88% of the replicate samples in closely related groups. One replicate pair (G03812/G03813) did not cluster together in all analyses.

5.3.2. Phylogenetic networks

NeighbourNet diagrams based on all datasets reconstructed two main groups within *B. newportii*, Group A and Group B (Fig. 5.1 Appendices 5.1, 5.2). Group A included the individual from Mt Carter,

Cape York Peninsula and all individuals from mountaintops in the northern Wet Tropics, namely Mt Finnigan, Devils Thumb and the Windsor Tableland. Group B was composed of individuals from Clarke Range in Central Mackay Coast Bioregion, Paluma range in the southern Wet tropics and mountaintops from the central Wet Tropics including the Lamb Range, Mt Bartle Frere, Mt Edith, Mt Haig, Longlands Gap, Carrington Falls and also Black Mountain located between the northern and central regions of the Wet Tropics. Thus, Group A and B comprise individuals from north and south of the BMC, respectively. Within Group A, individuals from Mt Windsor and Devils Thumb did not form sub-groups. Individuals from Mt Finnigan clustered together but this group was poorly differentiated from the other taxa within Group A. The single individual from Mt Carter in the Cape York Peninsula Bioregion included in the study was reconstructed on a long branch and clustered with the northern Wet Tropics individuals. Within Group B, four subgroups were differentiated. Subgroups B1 and B2 clustered together, including individuals from the Paluma Range in the southern Wet Tropics region, and from the Clarke Range in Mackay Coast Bioregion respectively. Subgroup B2 was reconstructed on a long branch. Subgroups B3 and B4 were reconstructed in an intermediate position between Group A and subgroups B1 and B2. In all datasets, subgroups B3 and B4 included a mixture of individuals from mountaintops of the central Wet Tropics region. However, the composition of individuals in the two groups varied in reconstructions based on different datasets. In reconstructions based on dataset MS2 (Fig. 5.1), subgroup B3 included all individuals from Mt Bartle Frere, Longlands Gap and Black Mountain as well as some individuals from Carrington Falls, Mt Edith and Lamb Range. Subgroup B4 included all individuals from Mt Haig and the remaining individuals from Carrington Falls, Mt Edith and Lamb Range.

Figure 5.1 NeighborNet phylogenetic network of 91 B. newportii individuals. These results are based on uncorrected P distances derived from 31,540 SNPs contained in dataset MS2 excluding outgroups. Replicate samples are indicated with an asterisk. BF: Mt Bartle Frere, BM: Black Mountain, Ca: Carrington Falls, CR: Clarke Range, DT: Devils Thumb, Ed: Mt Edith, Fi: Mt Finnigan, Ha: Mt Haig, LR: Lamb Range, MC: Mt Carter, LG: Longlands Gap, Pa: Paluma Range, Wi: Windsor Tableland.

Fig. 5.1

5.3.3. Phylogenetic reconstructions

In maximum likelihood phylogenetic reconstructions including the outgroup, all *B. newportii* individuals fell within a clade which received maximum support in all datasets (Fig. 5.2, Appendices 5.3, 5.4). *Bulbophyllum newportii* and *B. exiguum* were reconstructed in a highly supported sister group relationship (BS 100). Within the *B. newportii* clade a phylogenetic grade was reconstructed with the northern populations diverging initially, while the most derived groups were composed of the southern-most populations. Within the *B. newportii* clade, the individual from Mt Carter (Cape York Peninsula) is reconstructed as sister to the remaining *B. newportii* individuals which form a highly supported clade (datasets MS1 & MS2: BS 100, MS8: BS 95). Within the main *B. newportii* group backbone relationships were largely unsupported in analyses based on all datasets. Individuals from the northern Wet Tropics region were resolved in several unsupported groups that were reconstructed in a phylogenetic grade. Individuals did not cluster together based upon the mountaintops from which they were sampled, and groups usually included a mixture of individuals from different mountaintops across the northern Wet Tropics region. At the tip of the phylogenetic grade of northern Wet Tropics groups, a highly to weakly supported group (datasets MS1: BS 90, MS2: BS 63, MS8: <50) was reconstructed comprising all individuals in the southern range of the species, from: 1) Black Mountain, located in the BMC that separates the northern and southern Wet Tropics regions; 2) the central Wet Tropics region; 3) Paluma Range in the southern Wet Tropics, and 4) the Clarke Range in the Central Mackay Coast Bioregion.

Figure 5.2 Maximum likelihood phylogenetic reconstruction of 91 B. newportii individuals based on 4,349 concatenated RAD loci from dataset MS2, including outgroups. Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Map of Australia showing location of the Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset c: Map showing Mt Carter in the Cape York Peninsula bioregion. Inset d: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset e: Map showing Clarke Range in the Central Mackay Coast Bioregion.

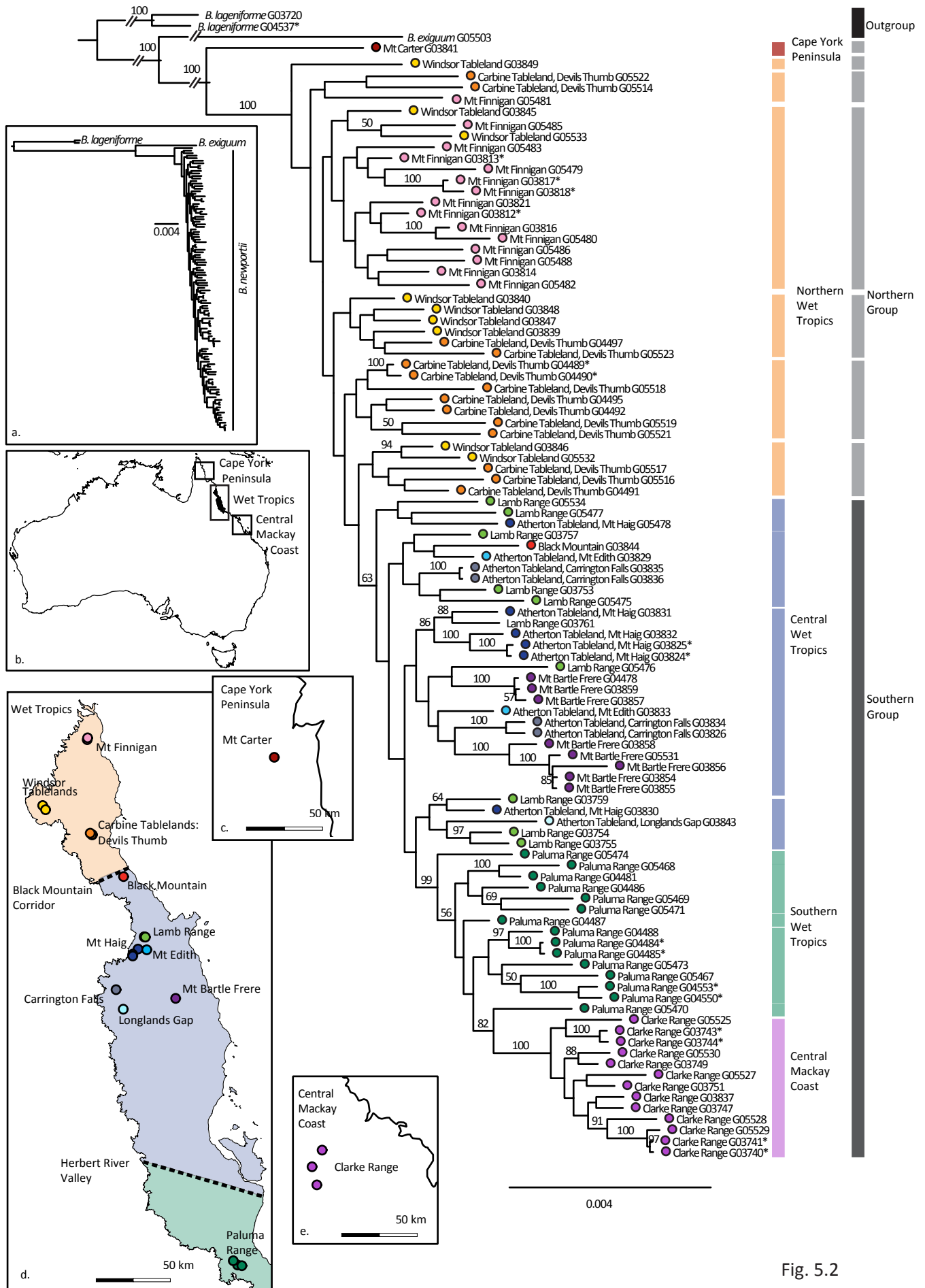


Fig. 5.2

Within the southern group, individuals from Black Mountain and the central Wet Tropics region were resolved in several unsupported groups that were reconstructed in a phylogenetic grade. Individuals did not cluster together based upon the mountaintops from which they were sampled, and groups usually included a mixture of individuals from different mountaintops across the central Wet Tropics region. At the tip of the phylogenetic grade of central Wet Tropics groups, a highly supported group (datasets MS1: BS 100, MS2: BS 99, MS8: BS 55) was reconstructed comprising all individuals from the southern Wet Tropics and the Clarke Range. Individuals from the Paluma Range in the southern Wet Tropics region were resolved in several unsupported groups that were reconstructed in a phylogenetic grade, with the most derived group composed of all individuals from the Clarke Range receiving maximum support in reconstructions based on all datasets.

5.3.4. Tetrad

Phylogenetic reconstructions inferred under the coalescent model reflected similar relationships to those depicted in the ML reconstructions. In reconstructions inferred under the coalescent model, including outgroups, all *B. newportii* individuals were reconstructed in a clade which received moderate support in analyses based on datasets MS1 (BS 86) (Appendix 5.5) and MS2 (BS 80) (Fig. 5.3) and low support in the reconstruction based on dataset MS8 (BS 70) (Appendix 5.6).

Bulbophyllum newportii and *B. exiguum* were reconstructed in a highly supported sister group relationship in reconstructions based on all datasets (dataset MS1: BS 98, MS2: BS 100, dataset MS8: 96). Within the *B. newportii* clade, the individual from Mt Carter (Cape York Peninsula) was reconstructed in a sister position to all other *B. newportii* individuals which formed a clade that received moderate support in reconstructions based on dataset MS1 (BS 81) and weaker support in analyses based on datasets MS2 (BS 73) and MS8 (BS <50). Within the main *B. newportii* clade backbone relationships remained largely unsupported in reconstructions based on all datasets.

Figure 5.3 SVD quartet tree of 91 B. newportii individuals based on 4,349 SNPs derived from dataset MS2 (one SNP per RAD locus). Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Map of Australia showing location of the Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset b: Map showing Mt Carter in the Cape York Peninsula bioregion. Inset c: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset d: Map showing Clarke Range in the Central Mackay Coast Bioregion.

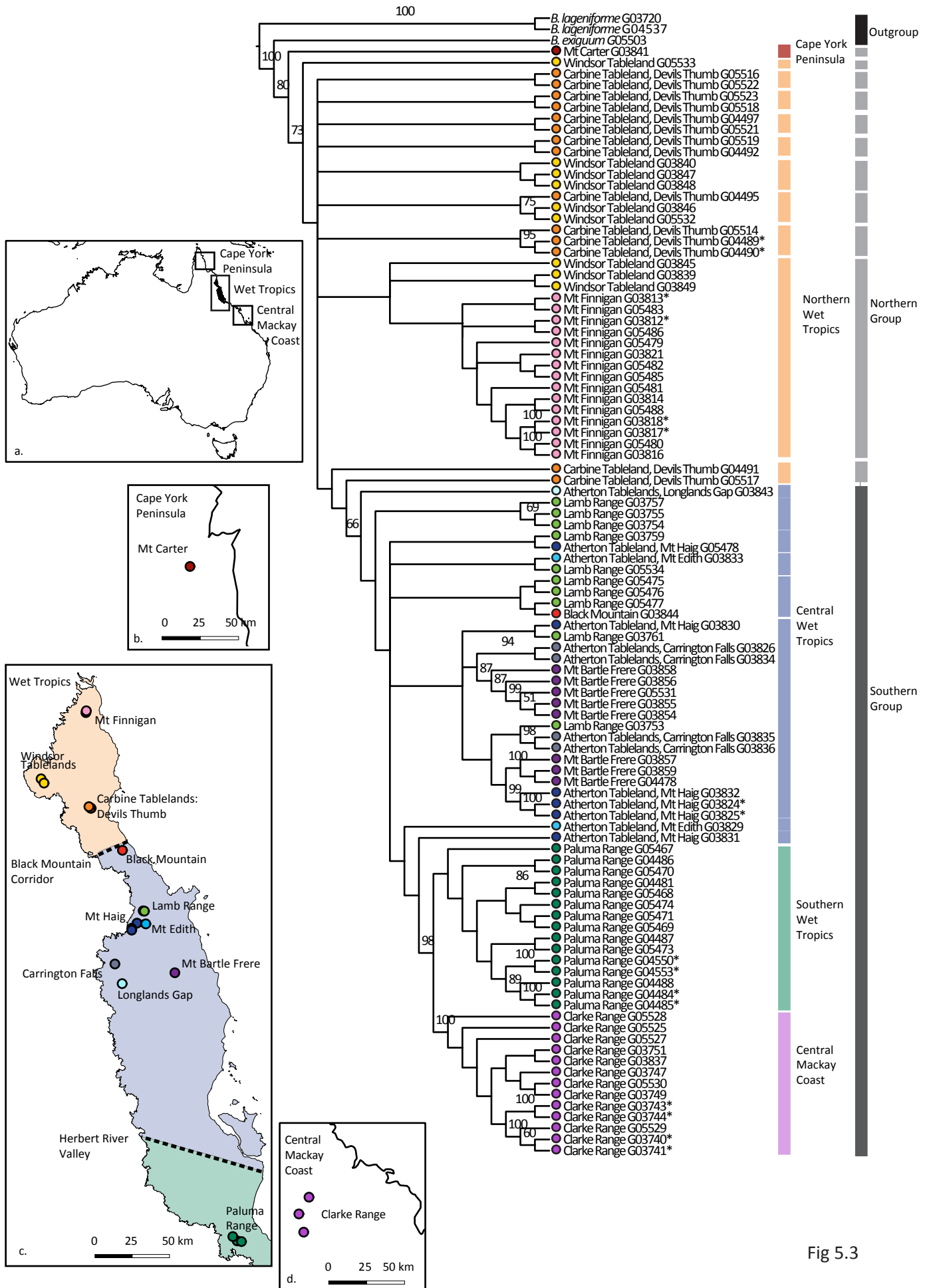


Fig 5.3

Individuals from the northern Wet Tropics region were resolved in several unsupported groups that were reconstructed in a phylogenetic grade. Individuals did not cluster together based upon the mountaintops from which they were sampled, and groups usually included a mixture of individuals from different mountaintops across the northern Wet Tropics region. At the tip of the phylogenetic grade of northern Wet Tropics groups, a highly to weakly supported group (datasets MS1: BS 90, MS2: BS 66, MS8: <50) was reconstructed comprising all individuals in the southern range of the species, from Black Mountain, mountaintops in the central Wet Tropics, Paluma Range in the southern Wet Tropics, and the Clarke Range in the Central Mackay Coast Bioregion. Within the southern group, individuals from Black Mountain and the central Wet Tropics region were resolved in several unsupported groups that were reconstructed in a phylogenetic grade. Individuals did not cluster together based upon the mountaintops from which they were sampled, and groups usually included a mixture of individuals from different mountaintops across the central Wet Tropics region. At the tip of the phylogenetic grade of central Wet Tropics groups, a highly to weakly supported group was reconstructed (datasets MS1: BS 98, MS2: BS 98, MS8: BS 74), which split into two lineages including individuals from: 1) the Paluma Range, unsupported in reconstructions based on all datasets and 2) individuals from the Clarke Range which received maximum support in reconstructions based on all datasets.

5.3.5. STRUCTURE

Bayesian clustering analysis using STRUCTURE revealed three main genetic groups within *B. newportii* (Fig. 5.4, Appendices 5.7, 5.8). The modal value of delta K indicated a K value of four best fitted the data. The membership coefficient plots for the analysis assuming K = 4 show three main genetic groups within *B. newportii* and a fourth group composed of a single individual from Mt Bartle Frere (G03856) (Fig. 5). The first group included the Northern Group reconstructed in the phylogenetic analyses, including individuals from the northern Wet Tropics and the individual from Mt Carter on Cape York Peninsula. The second group included individuals from the central and southern Wet Tropics regions. The third group was composed of individuals from Clarke Range in the Central Mackay Coast Bioregion.

Figure 5.4 Bar plots indicating genetic admixture of 91 individuals of B. newportii. These results were reconstructed using Bayesian clustering analyses implemented in the software STRUCTURE based on 4,633 SNPs derived from dataset MS2 excluding outgroups (one SNP per RAD locus) and assuming K=4. Replicate samples are indicated with an asterisk. Plots are displayed next to the maximum likelihood phylogenetic reconstruction from Fig. 5.2.

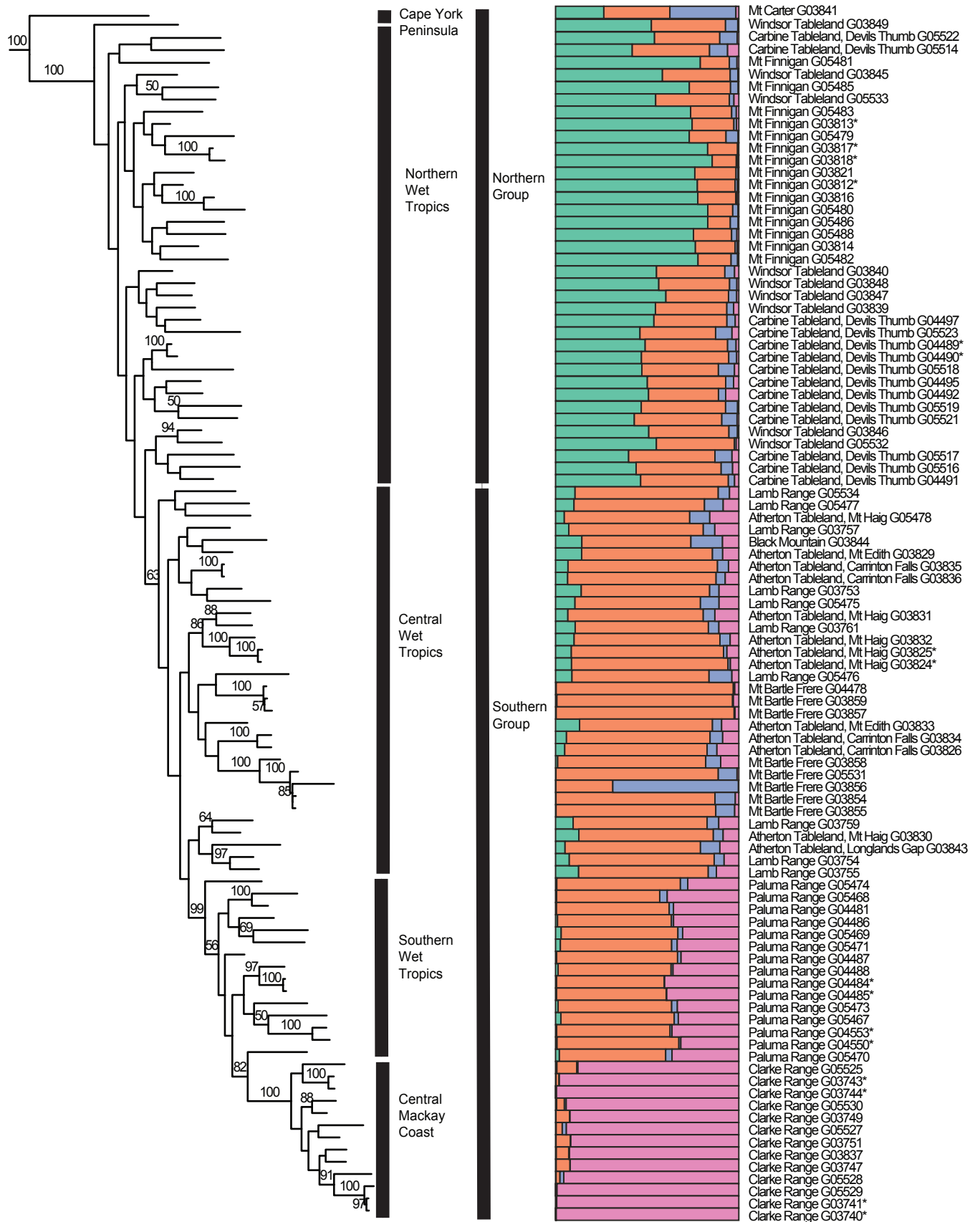


Fig 5.4

Within each of these three main groups many individuals also exhibited some genetic admixture. Individuals from the Northern Group were characterised by genetic admixture from the central/southern Wet Tropics group and the Clarke Range group. The southwestern populations of the northern Wet Tropics region, the Devils Thumb and Windsor Tableland were characterised by higher proportions of genetic admixture from the central/southern Wet tropics group relative to the north-eastern populations of the northern Wet Tropics such as Mt Finnigan. Within the central and southern Wet Tropics group, individuals were characterised by genetic admixture from the northern group and the Clarke Range group. Within the central Wet Tropics region populations from Lamb Range and Atherton Tablelands, genetic admixture from the northern group, and to a lesser degree from the Clarke Range group, was evident. However, the clustering analysis indicated the Mt Bartle Frere population was relatively pure and found no evidence of genetic admixture with the northern group and only small proportions of admixture with the Clarke Range group. Individuals from the southern Wet Tropics region contained no or low levels of genetic admixture from the northern group and high proportions of genetic admixture from the Clarke Range group. The Clarke Range population was shown to be a relatively genetically pure population with only small proportions of genetic admixture found in some individuals.

5.4. Discussion

5.4.1. Interspecific relationships

All *B. newportii* individuals were reconstructed in a highly supported clade, in a sister group relationship to *B. exiguum* in both ML and coalescent-based analyses based on all datasets (BS 100, PP 70–1) (Fig. 5.2, Fig. 5.3, Appendices 4.3-4.6). Together *B. newportii* and *B. exiguum* formed a sister group relationship to *B. lageniforme*. The relationships reconstructed from RAD loci support the interspecific relationships reconstructed from ML and Bayesian phylogenetic analyses based on two highly informative plastid protein-coding regions and three nuclear ribosomal regions (Chapter three). However, given sampling of *B. exiguum* in the population level analysis was limited to a single individual and Bayesian clustering analyses included only *B. newportii* individuals, it remains beyond the scope of this study to assess the level of gene flow between *B. newportii* and *B. exiguum*.

5.4.2. Phylogeographic structure within *B. newportii*

Within *B. newportii*, phylogeographic structure was identified in phylogenetic, NeighbourNet and Bayesian clustering analyses (Figs 5.1-5.4). NeighbourNet diagrams differentiated two main groups: 1) a northern group, comprising populations from the northern Wet Tropics region and the individual from Mt Carter in the Cape York Peninsula Bioregion; and 2) a southern group, including populations from the central and southern Wet Tropics regions and the Clarke Range in the Central Mackay Coast Bioregion. ML and coalescent based phylogenetic reconstructions resolved all individuals from the southern group in weakly to highly supported lineage providing evidence for divergence between the southern and northern groups (ML analyses, datasets: MS1: BS90, MS2:BS63, MS8: unsupported; coalescent based analyses, datasets: MS1: BS90, MS2:BS66, MS8: <50). The northern and southern group were separated by a major biogeographic disjunction for rainforest species in the Wet Tropics, the BMC. The results support previous phylogeographic studies that have identified divergence across the BMC in reptiles and invertebrates (Schneider et al., 1998; Hugall et al., 2002; Dolman and Moritz, 2006; Moussalli et al., 2009; Edwards and Melville, 2010; Bell et al., 2010; 2012; Hoskin et al., 2011; Bell et al., 2012), within widespread epiphytic orchid species (Burke et al., 2013; Simpson et al., 2018), and high-altitude rainforests endemic tree species (Rossetto et al., 2009; Mellick et al., 2014) and epiphyte orchid *B. lageniforme* (Chapter four). The divergence found between the northern and southern groups within *B. newportii* adds to previous evidence showing this major biogeographic disjunction restricts gene flow within epiphytic orchid species.

Within the Southern group, further phylogeographic structure was reconstructed. The Paluma Range population from the southern Wet Tropics together with the Clarke Range populations from the Central Mackay Coast were reconstructed in a highly supported lineage in ML and coalescent based phylogenetic reconstructions (ML analyses, datasets: MS1: BS100, MS2: BS99, MS8: BS55; coalescent based analyses, datasets: MS1:BS98, MS2: BS98, MS8: BS74). Within this lineage, the Central Mackay Coast population formed a group receiving maximum support in all analyses (Figs 3, 4). The divergence of these groups was also evident in the NeighbourNet analyses which reconstructed the southern Wet Tropics group separated from the central Wet Tropics taxa on a short branch and the Central Mackay Coast population separated from the southern Wet Tropics population on a long branch (Fig. 2). Populations of *B. newportii* in the southern Wet Tropics and Central Mackay Coast regions are each isolated by lowland barriers intersecting the high-altitude forest communities occurring along the Great Dividing Range that run perpendicular to Australia's east coast. The Central Mackay Coast Bioregion is separated from the Wet Tropics Bioregion by the Burdekin Basin, a lowland barrier of dry habitats spanning approximately 350km. Within the Wet Tropics Bioregion, the southern and central Wet Tropics regions are separated by the Herbert River Valley (Crisp et al., 2017). The results provide evidence of divergence of the southern Wet Tropics and Clarke Range lineage from the central Wet Tropics, and to a greater degree divergence of the Central Mackay Coast lineage from the southern Wet Tropics populations (Figs 5.2, 5.3), indicating limited gene flow across these lowland barriers has facilitated allopatric divergence. Evidence for divergence within mesic adapted species has previously been identified across the Burdekin Gap in reptiles and mammals (Moussalli et al., 2005; Dolman and Moritz, 2006; Edwards and Melville, 2010; Bryant and Fuller, 2014).

5.4.3. Spatio-temporal evolution

Divergence dating analyses estimated *B. newportii* diverged from closely related species *B. exiguum* during the Pliocene (Chapter three), following an extensive period of aridification that took place on the Australian continent during the Miocene (Hill et al., 2017). Pollen records indicate that by the Pliocene the mesic forests that previously had spread across the Australian continent had contracted to a fragmented distribution along the east coast and underwent dynamic cycles of expansion and contraction in response to intensified glacial cycles that took place during the Pliocene and Pliocene (Graham et al., 2006; Hilbert et al., 2007; Vanderwal et al., 2009). *Bulbophyllum newportii* is currently restricted to upland mesic forests and its range is likely to have shifted dynamically with these repeated cycles of contraction and expansion. Two hypotheses of range evolution are considered to explain the phylogeographic structure present within *B. newportii*: 1) vicariance

resulting from the contraction and fragmentation of mesic refugia during restrictive climates and 2) range expansion across a fragmented distribution of mesic forests via long distance dispersal.

The phylogeographic structure identified within *B. newportii* may be the result of vicariance in a widespread population along Australia's northern east coast, caused by reduced gene flow across expanded biogeographic barriers during restrictive climates, facilitating allopatric divergence. Under this scenario, the genetic admixture reconstructed within individuals in the Bayesian clustering analysis may reflect geneflow between two or more divergent ancestral populations, which may have occurred during range expansions under favourable climates facilitating secondary contact among divergent populations (Simpson et al., 2018). Three main genetic groups were differentiated within *B. newportii* (Fig. 5.4). The first group corresponded to the northern lineage, the second group included individuals from the central and southern Wet Tropics and the third group was composed of the central Queensland lineage. Assuming these populations each represent ancestral lineages, the genetic admixture between these three groups, found in nearly all individuals, suggests gene flow between these populations. For example, genetic admixture between the northern Wet Tropics groups and the central/ southern Wet Tropics group is consistent with gene flow between the two main northern and southern lineages of *B. newportii* across the BMC. Within the northern Wet Tropics populations, admixture from the central/southern group was found in all individuals, with the highest proportions found in the Carbine and Windsor Tablelands populations relative to the proportion of admixture found in the Mt Finnigan populations. This pattern suggests higher rates of gene flow between the central Wet Tropics populations and the closest populations in the northern Wet Tropics (i.e. the Carbine and Windsor Tablelands), than to the more isolated northern populations such as Mt Finnigan. Among populations in the central Wet Tropics region, the Mt Bartle Frere population was found to exhibit very low proportions of genetic admixture, while the Lamb Range and Atherton Tableland populations contained higher proportions of genetic admixture from both the northern Wet Tropics group and the Clarke Range group. The pattern indicates that the Mt Bartle Frere population is genetically isolated from other populations, but that the Atherton Tablelands and the Lamb Range may represent a zone of secondary contact between populations in the northern, central and southern Wet Tropics regions. The Lamb Range has been previously identified as a suture zone between northern and central Wet Tropics lineages (Moritz et al., 2009; Baba, 2014).

Alternatively, the phylogeographic structure reconstructed within *B. newportii* may be the result of cumulative founder events as the species range expanded via dispersal across a fragmented

landscape of mesic forest habitat. The phylogenetic analyses resolved a phylogenetic grade from north to south, with individuals from the Mt Carter (Cape York Peninsula) and the northern Wet Tropics diverging from the most basal nodes and the most derived groups composed of individuals from southern populations in the southern Wet Tropics and Central Mackay Coast regions. The results are consistent with a scenario of range expansion by a series of colonisations from north to south, such that the central Wet Tropics populations were derived from the northern Wet tropics, the southern Wet tropics were derived from the central Wet Tropics and the Central Mackay Coast population were derived from the southern Wet Tropics populations. Population genetic theory hypothesises that in expanding populations, populations at the expansion front may be subject to founder effects due to the reduction in the effective population size, strengthening processes of genetic drift and accelerating divergence in isolated populations (Waters et al., 2013; Peter and Slatkin, 2015). The high support reconstructed for 1) the southern Wet Tropics + Central Mackay Coast lineage (ML MS1: BS 100, MS2: BS 99; TETRAD MS1 and MS2: BS 98) and 2) the Central Mackay Coast lineage (BS 100 in ML and TETRAD reconstructions based on MS1 and MS2) may reflect cumulative founder effects that have strengthened the effects of genetic drift in these isolated populations.

Under this scenario, the genetic admixture reconstructed within individuals in the Bayesian clustering analyses may reflect ancestral genetics of the source population that is retained in the colonising population, rather than gene flow between the two populations subsequent to their divergence (Lawson et al., 2018). For example, in the Bayesian clustering analysis, individuals from the southern Wet Tropics region were characterised by genetic admixture from the central Wet Tropics genetic group and from the Central Mackay Coast genetic group. Under a scenario where the southern Wet Tropics has been colonised by individuals from the central Wet Tropics, the genetic admixture from the central Wet Tropics genetic group may reflect ancestral genetics inherited from the source population in the central Wet Tropics. Similarly, genetic admixture from the Central Mackay Coast genetic group may reflect ancestral genetics that has been inherited in the Central Mackay Coast population, rather than gene flow from the Central Mackay Coast population subsequent to its divergence. The differentiation of the Central Mackay Coast lineage as a distinct and relatively pure population could be explained by strong genetic drift within this isolated population.

When developing hypotheses of vicariance and dispersal, it is important to consider broader evidence such as the age and extent of biogeographic barriers and the long-term stability of refugia.

Paleoenvironmental studies indicate that an extensive period of aridification took place on the Australian continent during the Miocene and mesic forests across the continent contracted significantly (Hill et al., 2017). Evidence of fragmentation of mesic forests along Australia's north east coast has been identified from genetic divergence across the Burdekin Gap between mesic adapted species or congeners dated to the Pliocene (Mousaalli et al., 2005; Dolman and Moritz, 2006; Edwards and Melville, 2010). Given divergence dating analyses placed the origin of *B. newportii* during the Pliocene both vicariance and dispersal scenarios are plausible hypotheses for the early evolution of the species.

Within the Wet Tropics region, paleodistribution models of upland rainforest reconstructed the major refugia in the central Wet Tropics region as occurring along and adjacent to the central Wet Tropics escarpment and in the northern Wet Tropics region, along the Carbine Tableland (Hilbert et al., 2007; Vanderwal et al., 2009; Graham et al., 2010). The BMC, which separates refugia in the northern and central Wet Tropics has been identified as a major biogeographic barrier for mesic adapted species with evidence of divergence across the barrier dating to as early as the early Pliocene (Dolman and Moritz, 2006). However, reconstructions show that the distribution of Wet Tropics rainforests has expanded and contracted dynamically in response to climatic fluctuations that took place during the Quaternary (Hilbert et al., 2007; Vanderwal et al., 2009). Phytogeography studies showed the BMC does not mark major species disjunctions, while phylogeography studies showed evidence of gene flow across the barrier and secondary contact among divergent lineages (Moritz et al., 2009; Crisp et al., 2017). These studies indicate the BMC is a semi-permeable barrier that has shifted dynamically as rainforests have expanded and contracted. During optimum climatic periods expansion of mesic habitat likely reduced the extent of the BMC and therefore its strength as a biogeographic barrier to the movement of minute wind dispersed orchid propagules.

Mountain ranges in the Cape York Peninsula and Central Mackay Coast Bioregions and the southern part of the Wet Tropics Bioregion are of lower altitude (ca. 800m), limiting interception of orographic clouds that provide critical moisture to upland mesic communities and the long-term stability of upland rainforest refugia in these regions remains unclear. Locally endemic species and lineages have been identified within the southern Wet Tropics region, indicating the long-term persistence of rainforest refugia (Hoskin et al., 2003; Dolman and Hugall, 2008; Moussalli et al., 2009; Bell et al., 2010). However, the lower endemic diversity of rainforest taxa in the southern Wet Tropics, Cape York Peninsula and Central Mackay Coast suggests that these areas do not contain rainforest refugia that have been stable over the long term. ML and coalescent based phylogenetic

analyses of *B. newportii* reconstructed (with moderate support) the Cape York Peninsula individual as diverging from a basal node which suggests this population may represent a refugial lineage. However, this result should be interpreted with caution due to limited sampling from this group, which included only one representative, and the high level of divergence identified in all analyses, which may be indicative of a sequencing artefact. Increased sampling is required to clarify the relationship of the Cape York Peninsula populations to the Wet Tropics lineages.

The phylogenetic position of the Central Mackay Coast + southern Wet Tropics lineages nested within the central Wet Tropics clade indicates that these lineages were founded from the central Wet Tropics region. The pattern is consistent with phylogeographic structure found within upland mesic adapted species that indicate the southern Wet Tropics region has been colonised from refugial populations in the central Wet Tropics region (Joseph et al., 1995; Schneider et al., 1998; Schneider et al., 1999; Hoskin et al., 2011).

The phylogeographic pattern identified within *B. newportii* taken together with evidence from a broad range of studies, suggests that the most likely evolutionary hypotheses for the species includes a combination of both vicariance and dispersal processes. It is likely that *B. newportii* has persisted in multiple refugia in the northern and central Wet Tropics and occupied a widespread distribution across this region during optimal climatic periods, with expansion of the BMC during restrictive climates resulting in vicariance between populations in the northern and central Wet Tropics regions. Whilst the evolutionary origins of the Cape York Peninsula population remain unclear, the derived phylogenetic position of the southern Wet Tropics and Central Mackay Coast lineages and limited evidence for long term stable mesic refugia in these regions, indicate that these populations have been established by dispersal from the central Wet Tropics region.

Given the estimated Pliocene divergence of *B. newportii* and the dispersal potential of its minute wind dispersed seeds, it is possible that during periods of rainforest expansion *B. newportii* established populations across North Queensland that succumbed to localised extinction during restrictive climates and were recolonised subsequently. The legacy of lineages that have succumbed to localised extinction are not recorded in the genetic signature of extant lineages and therefore hypotheses of prior occupation remain speculative. However, comparative phylogeographic studies of rainforest endemic species have identified a common pattern of localised extinction and recolonization (Schneider et al., 1998; Moritz et al., 2000; 2001). To test alternative evolutionary hypotheses, future work should utilise coalescent based modelling and environmental niche modelling.

5.4.4. Comparative phylogeography

The phylogeographic structure reconstructed within *B. newportii* indicates this species has survived in multiple refugial populations. The phylogeographic study of the closely related species, *B. lageniforme*, did not find evidence for long term persistence of multiple populations in rainforest refugia across the Wet Tropics but instead recovered low genetic diversity and lack of population structure, indicating disjunct populations in the northern and central Wet Tropics were colonised relatively recently (Chapter four). Both species are restricted to Australia's upland tropical mesic forests but occupy different ecological niches. *Bulbophyllum lageniforme* is a narrow range species endemic to high altitude rainforests between 1000–1500m in the northern and central regions of the Wet Tropics. *Bulbophyllum newportii* occupies a broader environmental niche occurring at mid to high altitudes (600m–1600m), in a range of open and closed mesic habitats from rainforest, wet sclerophyll forests to exposed rocky outcrops.

Some comparative phylogeographic studies have observed higher structure among upland specialists, compared to more broadly distributed species (Rossetto et al., 2009; Bell et al., 2010). However, comparisons between *B. newportii* and *B. lageniforme* demonstrates the more broadly distributed species, *B. newportii*, harbours structured genetic diversity, while the narrow range upland rainforest endemic was characterised by low genetic diversity and population structure. The contrast in the phylogeographic pattern between *B. lageniforme* and *B. newportii* is consistent with the intermediate climatic niche hypothesis, which predicts higher genetic diversity and population structure in species occupying an intermediate environmental niche, relative to highly specialised taxa (Moussalli et al., 2009). Under restrictive climates species adapted to a broader environmental niche are expected to persist despite environmental changes, however their distributions become fragmented due to some degree of niche conservatism, facilitating divergence in allopatry. *Bulbophyllum newportii*, which occupies a broader range, is characterised by higher genetic diversity and population structure relative to the narrow range endemic, *B. lageniforme*. The broader environmental niche has enabled *B. newportii* to persist in multiple refugial populations suggesting this species may also have a greater resilience to predicted future climatic changes. A comparison of phylogeography within these two epiphyte species endemic to Australia's tropical upland mesic forests, supports previous studies which identified subtle differences in niche breadth and habitat preference as important factors in predicting responses to environmental change, even among very closely related species (Moritz et al., 2009; Rossetto et al., 2009).

5.4.5. Conservation

Bulbophyllum newportii is restricted to mesic forests between 600m–1600m and is likely to be at risk from climatic changes predicted for Australia’s tropical upland forests, including a reduction in moisture availability associated with an increase in average global temperatures (Still et al., 1999, Foster, 2001, Williams et al., 2012). An assessment of the conservation status of *B. newportii* is urgently required, however, insufficient data are available to assess the species against the IUCN red list criteria (IUCN 2012, 2017). Environmental niche modelling is required to predict the impact of climatic shifts and complete an IUCN red list assessment. If *ex situ* conservation action were undertaken for *B. newportii*, sampling should be informed by the phylogeographic analysis of the species. The results indicate that to conserve the diversity present within the species, sampling should include representatives from the northern group and the southern group including representatives from the Paluma Range and the Clarke Range. The highly divergent population from Mt Carter, Cape York Peninsula should also be sampled. The Cape York Peninsula populations are already exposed to a warmer climate and may harbour genetic diversity enabling greater tolerance for warmer climatic conditions.

5.5. Chapter summary

- A phylogeographic study was carried out to assess intraspecific genetic diversity with *Bulbophyllum newportii*, a species endemic to a broad range of mesic forest habitats of Australia's tropical mountaintops.
- 93 individuals were collected from 13 mountaintops in the Wet Tropics, Cape York Peninsula and Central Mackay Coast Bioregions, spanning the distribution of the species with one to 13 (M=7) individuals sampled per population.
- Genomic data was generated based on double-digest restriction site-associated DNA (ddRAD) sequencing and three genomic datasets were generated with varying levels of filtering stringency allowing for different proportions of loci with missing data, resulting in datasets ranging from 7398 to 1582 RAD loci.
- Maximum likelihood, coalescent and distance-based phylogenetic reconstructions and Bayesian clustering analyses revealed structured genetic diversity within *B. newportii* that is consistent with multiple refugial populations and divergence across biogeographic disjunctions such as the Black Mountain Corridor, Herbert River Valley, Normanby Basin, and Burdekin Basin.
- Two hypotheses of range evolution are considered to explain the phylogeographic structure present within *B. newportii*: 1) vicariance resulting from the contraction and fragmentation of mesic refugia during restrictive climates and 2) range expansion across a fragmented distribution of mesic forests via dispersal.
- Comparisons between *B. newportii* and *B. lageniforme* demonstrates the more broadly distributed of the two species, *B. newportii*, harbours structured genetic diversity, while the narrow range upland rainforest endemic, *B. lageniforme*, was characterised by low genetic diversity and population structure. The results support previous studies which identified subtle differences in niche breadth and habitat preference as important factors in predicting responses to environmental change, even among very closely related species.
- To preserve the genetic diversity present within *B. newportii* *ex situ* conservation efforts should sample representatives from the northern, central and southern Wet Tropics, Cape York Peninsula and Central Mackay Coast Bioregions.

Chapter six: General conclusions

The broad aim of this thesis was to improve understanding of evolutionary relationships within *Bulbophyllum*, both among and within species, with a focus on the diverse and poorly understood Asian and Australasian taxa, and to use the results to infer historical biogeography, identify phylogenetically informative morphological characters and inform taxonomy and conservation efforts. During this research I have greatly improved sampling of the Asian and Australian taxa. With the application of advanced sequencing methods, I have reconstructed phylogenetic relationships and the spatio-temporal evolution within *Bulbophyllum* at three hierarchical phylogenetic scales: among sections, among species and among populations.

In the first study (Chapter two), I reconstructed broad infrageneric relationships among 148 *Bulbophyllum* species. I have included 124 samples from the Asian and Australasian region representing 63% (42) of the Asian and Australasian sections, a substantial increase from previous studies of *Bulbophyllum* that included between 4.5–31% of Asian and Australasian sections. I have also substantially increased sampling of the genome compared to previous studies, which were based on seven or fewer loci (Fischer et al., 2007; Smidt et al., 2011; Gamisch et al. 2015; Hu, 2018). Sanger sequencing methods were utilised to generate sequence data from the nuclear ribosomal regions (ITS1, 5.8s, ITS2) for 66 *Bulbophyllum* species, including 87% of Australasian species. Genome skimming with subsequent high throughput sequencing was carried out for 53 *Bulbophyllum* taxa including representatives for 55% of Asian and Australasian sections, generating sequences for 70 plastid coding regions and three nuclear ribosomal regions (ITS1, 5.8s, ITS2).

In the second study (Chapter three), I reconstructed broad infrageneric relationships and intraspecific relationships within the Australasian lineage based on 34 *Bulbophyllum* species including representatives of major infrageneric lineages. All described species of sect. *Adelopetalum* and six species from sect. *Minutissima*, including all described Australasian species, were sampled with one to three representatives per species. Sanger sequencing of two plastid and three nuclear ribosomal regions was carried out for 24 samples and the data combined with sequences from 70 plastid coding regions and five nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) for 25 *Bulbophyllum* species representing major lineages. The sampling represents a considerable increase compared to previous molecular phylogenetic studies of *Bulbophyllum*, which did not sample Australasian taxa (Fischer et al., 2007;) or included limited sampling with at most eight Australasian species (Gravendeel et al., 2004; Smidt et al., 2011; Gamisch et al., 2015; 2019; Hu, 2018). In total, my studies have generated 4401 new sequences that will be made publicly available through the GenBank genetic database for future research.

6.1. Infrageneric relationships within *Bulbophyllum*

Phylogenetic reconstructions based on sequences of 70 plastid coding regions reconstructed three highly supported biogeographical clades: 1) an Australasian clade (BS 95, PP 1) including *Bulbophyllum* sect. *Adelopetalum* and sect. *Minutissima*, 2) a large Asian clade (BS 100, PP 1), and 3) an Afrotropical/Neotropical clade (BS 100, PP 1) (Chapter two, Chapter three). Analyses based on three nuclear ribosomal regions (ITS1, 5.8s, ITS2) reconstructed an Afrotropical/Neotropical clade with moderate to high support (BS 85, PP 0.99) but poor support for the Asian (BS <50, PP 0.77) and Australasian (BS <50, PP < 0.5) clades (Chapter two). However, analyses based on five nuclear ribosomal regions (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS) reconstructed high support for the Australasian (BS 94, PP1), and Asian (BS 92, PP 1) clades, and moderate support for the Afrotropical/Neotropical clade (BS 85, PP 0.98) (Chapter three).

Relationships among the three major lineages remains unclear due to topological incongruence between the plastid and nuclear phylogenies. Reconstructions based on 70 plastid coding regions recover the Australasian clade in a sister relationship to a highly supported clade (BS 83, PP1) including the Afrotropical, Neotropical and Asian lineages. Reconstructions based on seven nuclear ribosomal regions instead recovered the Afrotropical/Neotropical lineage in a sister relationship to a low to moderately supported clade (BS 80, PP 0.92) composed of the Australasian and Asian lineages (Chapter three). Nevertheless, the results advance understanding of the major lineages that comprise *Bulbophyllum*. Among the major Afrotropical, Neotropical and Asian biogeographic lineages identified in previous molecular phylogenetic studies, an early diverging Australasian clade was identified and highly supported in both plastid and nuclear phylogenies. The large, predominantly Asian clade, that was unsupported or poorly supported in previous molecular phylogenetic studies (Gravendeel et al., 2004; Fischer et al., 2007; Smidt et al., 2011; Gamisch et al. 2015), is reconstructed with high support in both plastid and nuclear phylogenies, providing evidence for the monophyly of this group. The high support for the Afrotropical/Neotropical in both plastid and nuclear phylogenies supports the findings of previous studies (Fischer et al., 2007; Smidt et al., 2011; Gamisch et al. 2015). Phylogenetic reconstructions have identified sections nine sections as polyphyletic (sections *Cirrhopetalum*, *Desmosanthes*, *Rhytionanthos*, *Hoplandra*, *Leopardinae*, *Macrocaulia*, *Minutissima*, *Polymeres* and *Sestochilus*) and three sections as paraphyletic (*Lemniscata*, *Beccariana*, *Stenochilus*), highlighting the need for further sectional revision within *Bulbophyllum*.

The divergence dating and ancestral range estimation have provided new insights into the evolution of *Bulbophyllum* and provided the first reconstruction of the spatio-temporal evolution of the genus within the Asian and Australasian region. Divergence dating analyses estimated the genus diverged from its sister genus, *Dendrobium*, in the early Oligocene, ca. 33 Ma. and the Australasian, Asian and Afrotropical/Neotropical lineages diverged in the early evolution of the genus, from the late Oligocene to early Miocene (ca. 23 Ma). These estimates were reconstructed from a 70 plastid cds dataset and are slightly older than those estimated by previous divergence dating analyses based on the nuclear ribosomal ITS region (Gamisch et al., 2015, Gamisch and Comes, 2019). The estimated age of the genus may provide some valuable insights regarding the broader treatment of *Bulbophyllum* at the generic rank or higher. The ages of a broad range of plant and animal lineages recognised at the generic rank has been shown to be remarkably consistent (Hedges *et al.*, 2015). The mode age of 1,308 angiosperm genera was 14.1 Ma (12.3–16.2 Ma). By comparison *Bulbophyllum* is a relatively old genus. A treatment that recognises several younger genera within the sub-tribe Bulbophyllinae, as was traditionally described in various forms (Lindley, 1840; Reichenbach, 1861; Benthams and Hooker, 1883; Pfitzer, 1889; Schlechter, 1913 Holttum, 1953; Seidenfaden, 1973; 1979; Vermeulen 1987, Dressler 1993, Garay et al., 1994; Szlachetko and Margonska, 2001; Clements and Jones, 2002) may be more consistent with classifications of other angiosperm genera and more appropriate for the complexity of this diverse group.

The divergence dates estimated for *Bulbophyllum* and the major biogeographic lineages indicate the genus evolved and spread between Asia, Australasia and the Afrotropics subsequent to the dispersal of continental landmasses following the break-up of Gondwana (McLoughlin, 2001). Therefore, a vicariance explanation for divergence among the major lineages, associated with the breakup of Gondwana in the Mesozoic, is rejected. Alternatively, LDD is postulated between Australasia, Asia and the Afrotropics, giving rise to the major biogeographic lineages. The specific geographic origins of the genus remain unclear and further studies are required to resolve the direction of dispersal. The results provided new insights on the origins of the Australian *Bulbophyllum* taxa. The divergence dating and ancestral range analyses based on the 70 plastid cds dataset estimate that *Bulbophyllum* has been present in Australasia since the late Oligocene. Ten independent origins for the evolution of *Bulbophyllum* taxa in Australia were inferred from the phylogenetic analyses, with subsequent diversification taking place in Australia in taxa from sections *Adelopetalum*, *Minutissima*, *Polyblepharon*, *Polymeres* and *Oxysepala*.

Within the Asian clade, two major lineages were resolved, one predominantly composed of taxa from temperate and tropical Asia and the other predominantly made up of taxa from tropical Asia,

Papuasia, Australasian and the Pacific. These groups received high support in Bayesian analyses based on the 70 plastid cds dataset. The results provide evidence for the monophyly of ‘predominantly eastern and western Malesian lineages’ that were resolved without support in previous studies (Pridgeon et al., 2014). Multiple range expansions were inferred within the Asian clade from tropical Asia to temperate Asia, Papuasia, Australasia and the South Pacific regions, implicating LDD in range expansions to Papuasia, Australasia and the South Pacific regions. The ancestral range analysis reconstructed ancestral ranges in Papuasia from the early Miocene which coincides with emergence of New Guinea. The diversification among lineages within the predominantly Papuasian Clade B6 coincided with the uplift of the central New Guinea mountain ranges.

The results advance understanding of the spatio-temporal evolution of *Bulbophyllum* and contribute to a growing body of knowledge elucidating the complex biogeographic history of the Asian and Australian region. Early Miocene divergence between the Asian and Australasian *Bulbophyllum* clades coincides with the collision of the Asian and Australasian plates and dispersal inferred between Asia and Australasia at this time, and between tropical Asia and Papuasia from the late Miocene, are among the earliest dispersal events postulated based on genetic disjunctions across Wallacea (Crayn et al., 2015). These results highlight the exceptional dispersal potential of the minute Orchidaceae seeds and support previous work that recognised the important role that this dispersal potential has played in the diversification of the family (Givnish et al., 2016). The diversification among lineages within the predominantly Papuasian Clade B6 that coincides with the uplift of the central New Guinea mountain ranges supports the hypotheses that mountain ranges are a significant driver of diversification within Orchidaceae, facilitating allopatric speciation between heterogeneous habitats at different elevations or in lineages occurring on isolated ranges (Givnish et al., 2015).

6.2. Intraspecific relationships within the Australasian *Bulbophyllum* clade

Reconstructions based on both the plastid and nuclear datasets found section *Adelopetalum* and Australasian species of section *Minutissima* form the highly supported Australasian clade. Section *Minutissima* was shown to be highly polyphyletic with the Australasian species, including the type for the section reconstructed within the Australasian clade, while Asian *Minutissima* species were nested within the Asian clade, supporting results of previous morphological analyses (Jones and Clements, 2001) and indicating sectional revision is required. Two species, *B. pygmaeum* (sect. *Minutissima*) and *B. weinthalii* (sect. *Adelopetalum*) were identified to be of putative hybrid origin

based on incongruence between plastid and nuclear phylogenies. The phylogenetic reconstructions considerably advance understanding of interspecific relationships from cladistic studies of *Bulbophyllum* sect. *Adelopetalum* that reconstructed relationships from morphological traits, where several species were not included because either material was not available (*B. boonjee*, *B. lingulatum*), taxa were regarded as a synonym (*B. corythium* and *B. tuberculatum*), or the species was not classified in the section due to highly divergent morphology (*B. weinthalii*) (Vermeulen, 1993). The phylogenetic and ancestral character reconstructions found strong support for the grouping of *B. lageniforme*, *B. lilianae*, *B. lingulatum*, and *B. newportii* in Vermeulen's (1993) cladistic analysis, which corresponds to the *newportii* clade in the phylogenetic reconstructions. However, the sister group relationships between *B. bracteatum* and *B. argyropus* was not supported by molecular evidence and ancestral character reconstructions identified that this relationship is based on homoplasious morphological characters.

The divergence dating and ancestral range reconstruction show that the Australasian lineage emerged on the Australian continent in the late Oligocene, ca. 23.7 Ma, and diversified mainly on the Australian continent during the Miocene. The reconstruction of spatio-temporal evolution within the Australasian clade provides evidence for Miocene diversification within Australia's mesic forests, supporting the hypotheses that diversification and fragmentation of forest habitats in Australia has been an important driver of lineage divergence in Australia's mesic biome (Byrne et al., 2011). At least four independent dispersal events were inferred from the Australian mainland eastwards to Lord Howe and Norfolk islands, New Caledonia and New Zealand, giving rise to new species. The pattern of eastward dispersal is consistent with dispersal patterns observed in other angiosperms from diverse families including *Dendrobium*, *Abrotanella*, *Dracophyllum*, *Hebe*, *Korthalsella*, *Leucopogon*, *Northofagus*, *Oreobolus*, and *Rytidosperma* (Linder, 1999; Molvray et al., 1999; Lavarack et al., 2000; Swenson et al., 2001; Wagstaff et al., 2010, 2006, 2002; Chacón et al., 2006; Puente-Lelièvre et al., 2013). The results further support observations of an eastward bias in dispersal from Australia, which is likely to be facilitated by the westerly winds that dominate wind currents in the southern hemisphere (Sanmartin et al., 2007).

6.3. Relationships within species endemic to Australia's tropical upland forests

In the third and fourth studies (Chapters four and five), I have reconstructed intraspecific diversity within two *Bulbophyllum* species endemic to Australia's tropical upland forests to infer responses to past climatic changes and to inform sampling strategies for ex situ conservation. To reconstruct phylogeographic structure within each species, 65 samples of *B. lageniforme* and 91 samples of

B. newportii, were collected from 13 mountaintops, across the known distributions of the two species in Australia's Wet Tropics, Central Mackay Coast and Cape York Peninsula Bioregions. RAD sequencing methods were utilised to generate highly informative genomic datasets with up to 17379 RAD loci for *B. lageniforme* and 7398 RAD loci for *B. newportii*. Within *B. lageniforme*, phylogeographic reconstructions revealed weak genetic structure across a major biogeographic barrier in the Wet Tropics Bioregion, the BMC. However, Bayesian clustering analyses were unable to differentiate discrete genetic groups within this species. Therefore, the results did not find evidence for long-term persistence of multiple refugial populations across the Wet Tropics and it is postulated the low genetic diversity is due to relatively recent radiation from a single refugial population. Within *B. newportii*, phylogenetic and population genetic reconstructions identified evidence for divergence across the BMC, the Herbert River Valley, Burdekin Basin and Normanby Basin. The results indicate *B. newportii* has survived in multiple refugial populations in both the northern and central regions of the Wet Tropics and populations in the southern Wet Tropics and Central Mackay Coast Bioregions have been established from refugial populations in the central Wet Tropics. The evolutionary origin of the Cape York Peninsula population remains unclear.

A comparison of phylogeographic structure within *B. lageniforme* and *B. newportii* reveals contrasting evolutionary histories. Differences in phylogeographic structure is hypothesised to be the result of different impacts of past environmental changes and variation in the niche breadth occupied by the two species is identified as an important indicator of their relative resilience to environmental change. *Bulbophyllum lageniforme* is a narrow range high altitude rainforest endemic, while *B. newportii* occupies a broader environmental niche. It is hypothesised that the narrow environmental tolerances of *B. lageniforme* have made this species vulnerable to localised extinctions during restrictive climates during the Pliocene and Pleistocene, while the broad niche occupied by *B. newportii* has allowed this species to persist in multiple refugia.

These studies are among the first detailed plant phylogeographic studies utilising RAD sequencing methods to be completed and the first to be carried out in the Wet Tropics Bioregion. They provide a valuable contribution to the field of phylogeography where studies of plants have been limited by a lack of suitable genetic markers for resolving relationships among closely related taxa. The phylogeographic structure found within *B. newportii* and lack of genetic diversity found in *B. lageniforme* is not consistent with comparative phylogeographic studies that have observed deeper structure among upland specialists, compared to more broadly distributed species (Rossetto 2009; Bell et al., 2010). The results are consistent with the intermediate climatic niche hypothesis,

which predicts higher genetic diversity and population structure in species occupying an intermediate environmental niche, relative to highly specialised taxa (Moussalli et al., 2009). The results support previous studies showing Australia's tropical montane taxa have persisted in refugia and that narrow range rainforest endemics are highly vulnerable to localised extinctions with changing environmental conditions. A comparison of phylogeographic structure between *B. lageniforme* and *B. newportii* supports previous studies which identified subtle differences in niche breadth and habitat preference as important factors in predicting responses to environmental change, even among closely related species (Moritz et al., 2009; Rossetto et al., 2009).

6.4. Future directions

6.4.1. Resolving evolutionary relationships within *Bulbophyllum* to underpin a robust taxonomy

Well supported evidence of evolutionary relationships is required to underpin a robust taxonomy for *Bulbophyllum*. Evidence of paraphyletic and polyphyletic sections identified in this thesis and previous studies highlights the need for further infrageneric revision among the Asian and Australian taxa. Recent studies have made progress for the *Cirrhopetalum* alliance (Hu, 2018). However, much work remains to be done. Further phylogenetic studies are required, including representative sampling within all described sections and former genera within the subtribe Bulbophyllinae. The plastid phylogeny presented here provides a well resolved and supported phylogenetic framework, a critical resource for developing sampling priorities for future studies, either to fill sampling gaps to complete a broad framework or to identify evolutionary groups on which to focus more fine scale phylogenetic analyses.

My phylogenetic analyses reconstructed from the nuclear ribosomal ITS region have provided valuable insights, reconstructing several highly supported clades. However, the nuclear phylogeny remained overall poorly supported with relationships frequently unsupported or poorly to moderately supported and discordance was observed between the plastid and nuclear phylogenies. For example, moderately supported relationships resolved among the Asian, Australian and Afrotropical/Neotropical lineages based on nuclear ribosomal regions were discordant with highly supported relationships resolved among these lineages in reconstructions based on the 70 gene plastid phylogeny. Within the Australasian clade relationships among the major lineages remain unclear in reconstructions based on both plastid and nuclear datasets. Increased sampling from the nuclear genome is required to further clarify relationships among major lineages within *Bulbophyllum* and within the Australasian clade. Target enrichment high throughput sequencing

methods provide an exciting new opportunity to increase sampling of low copy nuclear genes in future studies of *Bulbophyllum* (Meyer and Kircher, 2010; Cronn et al., 2012). Increasing sampling from the nuclear genome may be especially critical for resolving relationships among species due to processes that result in heterogeneity between gene trees among closely related taxa, such as incomplete lineage sorting and hybridisation. During my research on *Bulbophyllum*, a target capture project has been initiated and marker development specific to *Bulbophyllum* is currently underway to provide a customised bait set to facilitate the application of target enrichment methods in future studies of *Bulbophyllum* (Nargar et al., 2016). The suitability of target enrichment methods for small amounts of degraded DNA provides exciting new opportunities to incorporate a vast resource of *Bulbophyllum* collections from herbaria around the world in future molecular phylogenetic studies (Bieker and Martin, 2018).

6.4.2. Conservation of Australia's tropical montane endemic taxa

Australia's tropical montane habitats are considered highly vulnerable to predicted climatic changes (Still et al., 1999, Foster, 2001, Williams et al., 2012; Costion et al., 2015) and *ex situ* conservation programs are underway to mitigate against the threat of localised or complete extinction in endemic taxa (Mountner, 2019). Environmental niche modelling of distributions under future climates is urgently required to assess the conservation status of Australia's tropical montane taxa. During sampling expeditions carried out for this research more than 115 occurrence records were collected for *B. lageniforme* and 153 occurrence records for *B. newportii*, providing the high-resolution data required for environmental niche modelling. A collaboration has been established to undertake such analyses and thus include *B. lageniforme* and *B. newportii* in an expanding suite of species (Costion et al., 2015; Rooble, 2018) being compiled to better understand ecosystem level climate impacts. The distribution of Australia's tropical montane endemic species should be monitored, and occurrence data collated in this thesis provides an important baseline dataset from which to monitor change in *B. lageniforme* and *B. newportii*. Additionally, studies carried out under controlled environmental conditions are also required to assess species resilience to warming climatic conditions. These studies will require considerably more investment in time and resources and phylogeographic studies provide a critical resource for understanding variation in responses that may be associated with genetic variation. To best preserve the intraspecific genetic diversity present within these species in *ex situ* conservation efforts, the following sampling strategies are recommended based on the phylogeographic structure identified within *B. lageniforme* and *B. newportii*. For *B. lageniforme*, sampling should include representatives from both the northern and southern Wet Tropics groups at a minimum and where resources are available sampling within

the central Wet Tropics group should include representatives of the Lamb Range, Atherton Tablelands and Mt Bartle Frere groups. For *B. newportii*, sampling should include representatives from the northern Wet Tropics ranges, the central Wet Tropics ranges, Paluma Range (southern Wet Tropics Bioregion), Clarke Range (Central Mackay Coast Bioregion) and Mt Carter (Cape York Peninsula Bioregion).

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Appendices

Appendix 2.1

MrModelTest output for a preliminary alignment of the nuclear ribosomal ITS1, 5.8s, ITS2 regions (62 taxa, 760bp). -lnL: negative log likelihood, K: number of estimated (free) parameters, AIC: Akaike Information Criterion, delta: Akaike difference, weight: Akaike weight, cumWeight: cumulative Akaike weight.

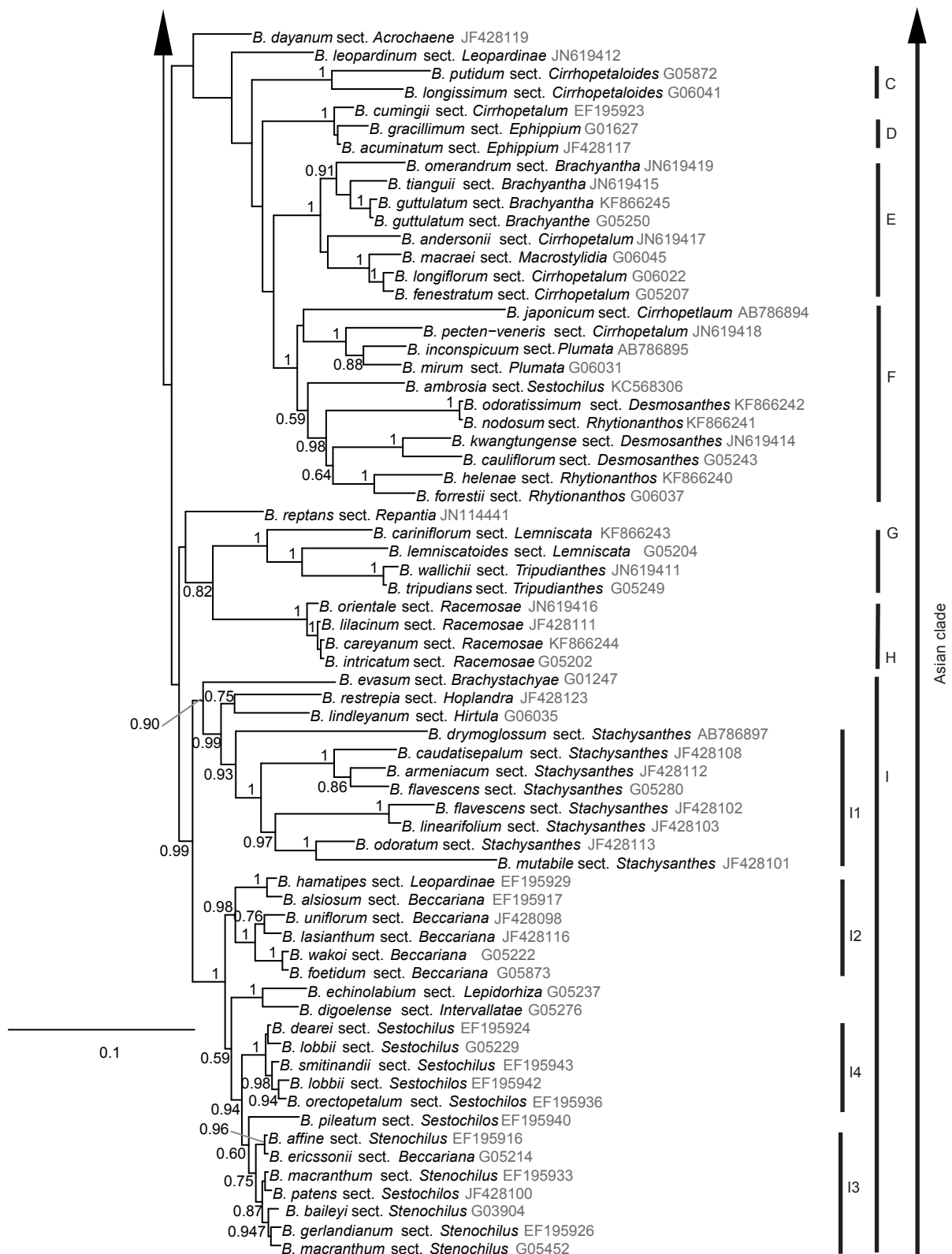
Model	-lnL	K	AIC	delta	Weight	CumWeight
GTR+I+G	8276.9746	10	16573.9492	0.0000	0.9999	0.9999
GTR+G	8286.9590	9	16591.9180	17.9688	0.0001	1.0000
HKY+I+G	8293.6719	6	16599.3438	25.3945	3.06e-06	1.0000
HKY+G	8301.8740	5	16613.7480	39.7988	2.28e-09	1.0000
SYM+I+G	8306.3564	7	16626.7129	52.7637	3.49e-12	1.0000
K80+I+G	8312.9980	3	16631.9961	58.0469	2.48e-13	1.0000
SYM+G	8313.0459	6	16638.0918	64.1426	1.18e-14	1.0000
K80+G	8319.8057	2	16643.6113	69.6621	7.46e-16	1.0000
GTR+I	8404.0547	9	16826.1094	252.1602	0.00e+00	1.0000
HKY+I	8437.5898	5	16885.1797	311.2305	0.00e+00	1.0000
SYM+I	8466.8867	6	16945.7734	371.8242	0.00e+00	1.0000
K80+I	8480.1777	2	16964.3555	390.4062	0.00e+00	1.0000
F81+I+G	8677.6709	5	17365.3418	791.3926	0.00e+00	1.0000
F81+G	8686.0801	4	17380.1602	806.2109	0.00e+00	1.0000
JC+I+G	8692.5537	2	17389.1074	815.1582	0.00e+00	1.0000
JC+G	8700.1484	1	17402.2969	828.3477	0.00e+00	1.0000
GTR	8742.0635	8	17500.1270	926.1777	0.00e+00	1.0000
HKY	8793.7080	4	17595.4160	1021.4668	0.00e+00	1.0000
F81+I	8815.2764	4	17638.5527	1064.6035	0.00e+00	1.0000
SYM	8816.5400	5	17643.0801	1069.1309	0.00e+00	1.0000
JC+I	8840.3594	1	17682.7188	1108.7695	0.00e+00	1.0000
K80	8843.5645	1	17689.1289	1115.1797	0.00e+00	1.0000
F81	9164.5049	3	18335.0098	1761.0605	0.00e+00	1.0000
JC	9191.4229	0	18382.8457	1808.8965	0.00e+00	1.0000

MrModelTest output for a preliminary plastid supermatrix alignment including 70 genes (61707bp). -
 lnL: negative log likelihood, K: number of estimated (free) parameters, AIC: Akaike Information
 Criterion, delta: Akaike difference, weight: Akaike weight, cumWeight: cumulative Akaike weight.

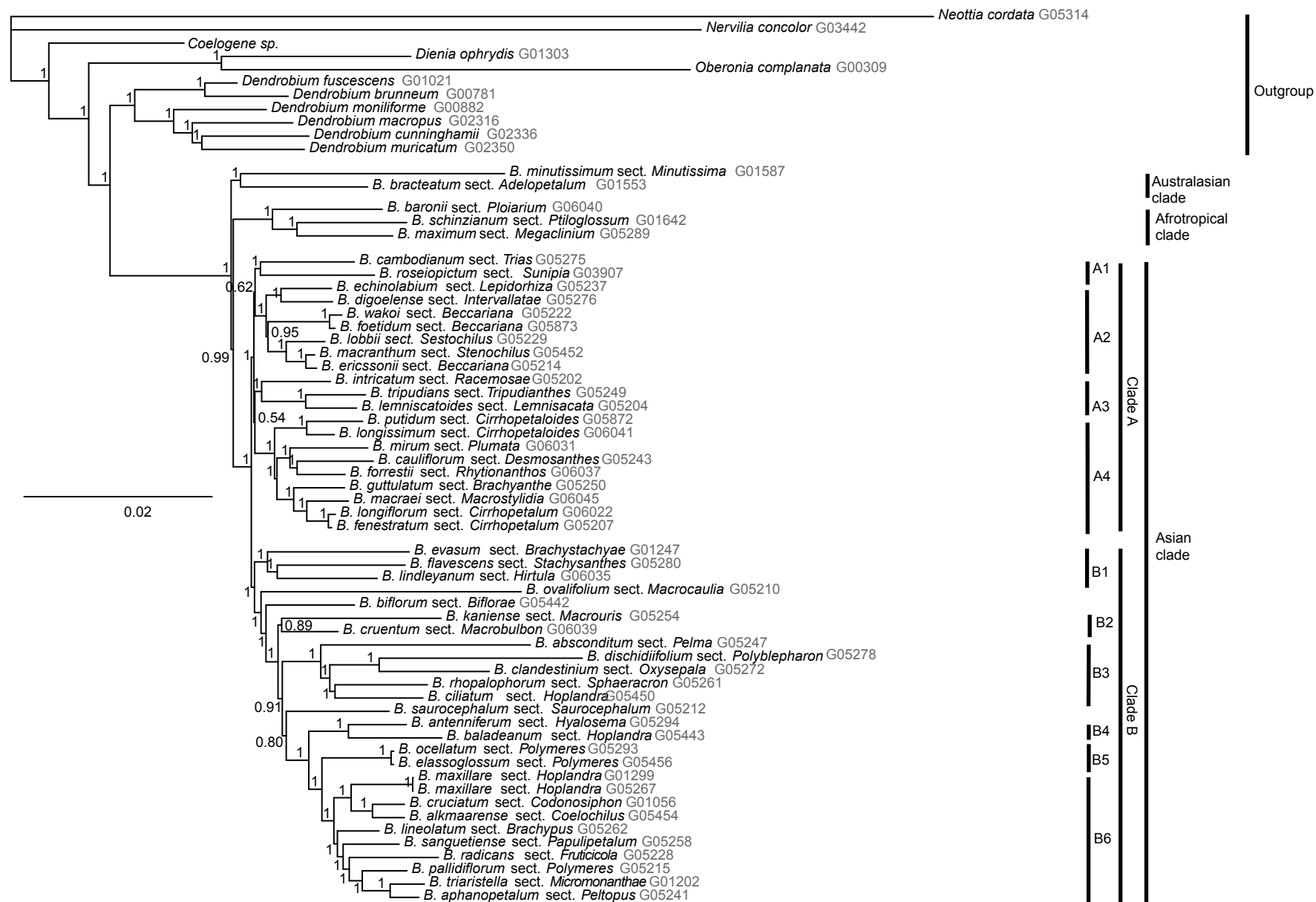
Model	-lnL	K	AIC	delta	Weight	CumWeight
GTR+I+G	184449.7344	10	368919.4688	0.0000	1.0000	1.0000
GTR+G	184947.3281	9	369912.6562	993.1875	0.00e+00	1.0000
GTR+I	185102.9531	9	370223.9062	1304.4375	0.00e+00	1.0000
HKY+I+G	185561.3594	6	371134.7188	2215.2500	0.00e+00	1.0000
HKY+G	186090.0625	5	372190.1250	3270.6562	0.00e+00	1.0000
HKY+I	186204.8438	5	372419.6875	3500.2188	0.00e+00	1.0000
SYM+I+G	186815.0938	7	373644.1875	4724.7188	0.00e+00	1.0000
SYM+G	187437.4219	6	374886.8438	5967.3750	0.00e+00	1.0000
SYM+I	187491.9219	6	374995.8438	6076.3750	0.00e+00	1.0000
F81+I+G	187534.5938	5	375079.1875	6159.7188	0.00e+00	1.0000
K80+I+G	187867.8438	3	375741.6875	6822.2188	0.00e+00	1.0000
F81+G	188079.0938	4	376166.1875	7246.7188	0.00e+00	1.0000
F81+I	188169.5781	4	376347.1562	7427.6875	0.00e+00	1.0000
K80+G	188474.1562	2	376952.3125	8032.8438	0.00e+00	1.0000
K80+I	188534.7031	2	377073.4062	8153.9375	0.00e+00	1.0000
JC+I+G	189775.6562	2	379555.3125	10635.8438	0.00e+00	1.0000
GTR	189838.8281	8	379693.6562	10774.1875	0.00e+00	1.0000
JC+G	190367.9062	1	380737.8125	11818.3438	0.00e+00	1.0000
JC+I	190429.3750	1	380860.7500	11941.2812	0.00e+00	1.0000
HKY	191148.2656	4	382304.5312	13385.0625	0.00e+00	1.0000
SYM	192889.2656	5	385788.5312	16869.0625	0.00e+00	1.0000
F81	193247.5781	3	386501.1562	17581.6875	0.00e+00	1.0000
K80	193887.0312	1	387776.0625	18856.5938	0.00e+00	1.0000
JC	195751.7969	0	391503.5938	22584.1250	0.00e+00	1.0000



Appendix 2.2 Bayesian phylogeny of 145 *Bulbophyllum* taxa reconstructed from the nuclear ITS region. Posterior probabilities >50% are displayed near nodes.



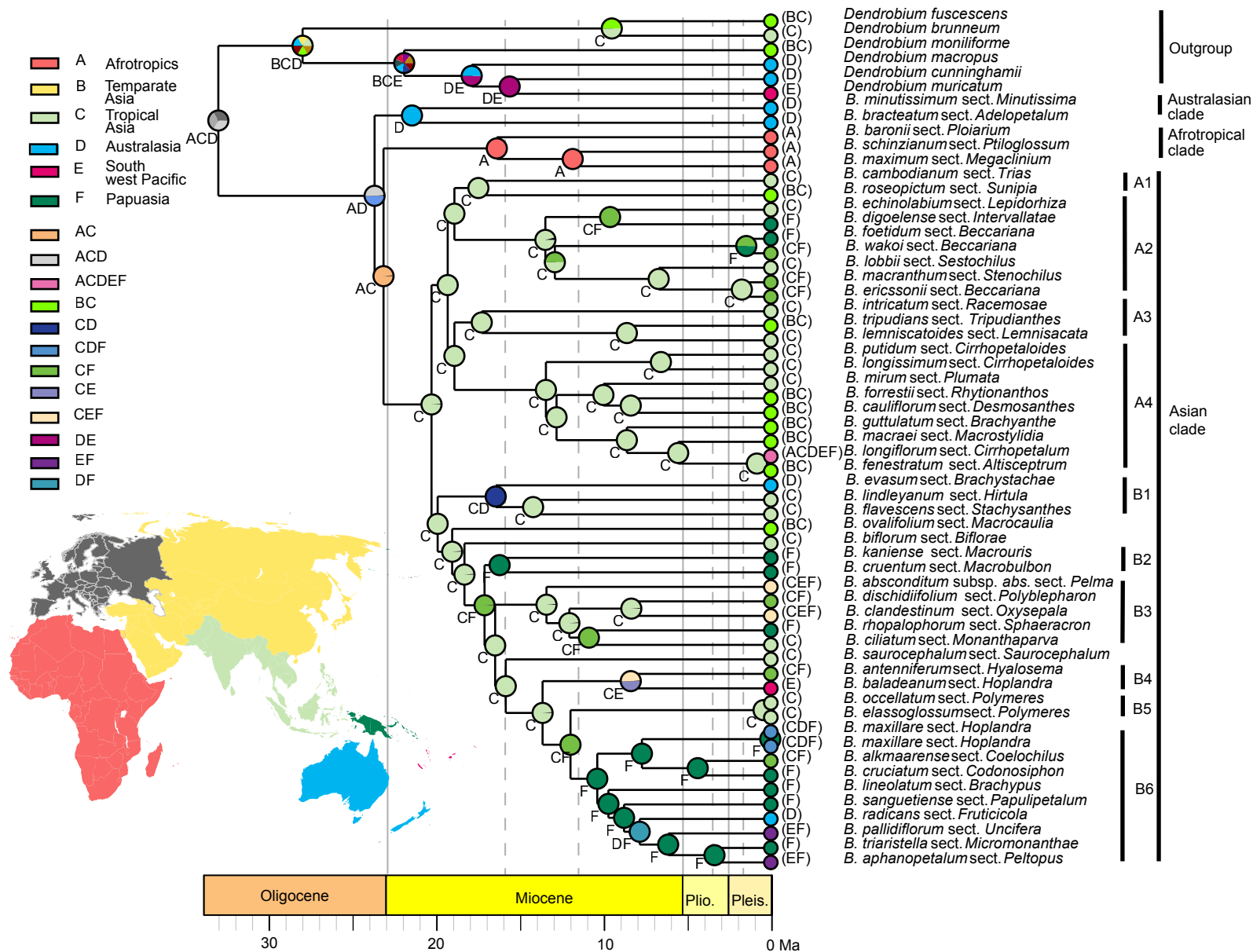
Appendix 2.2 Bayesian phylogeny of 145 *Bulbophyllum* taxa reconstructed from the nuclear ITS region. Posterior probabilities >50% are displayed near nodes.



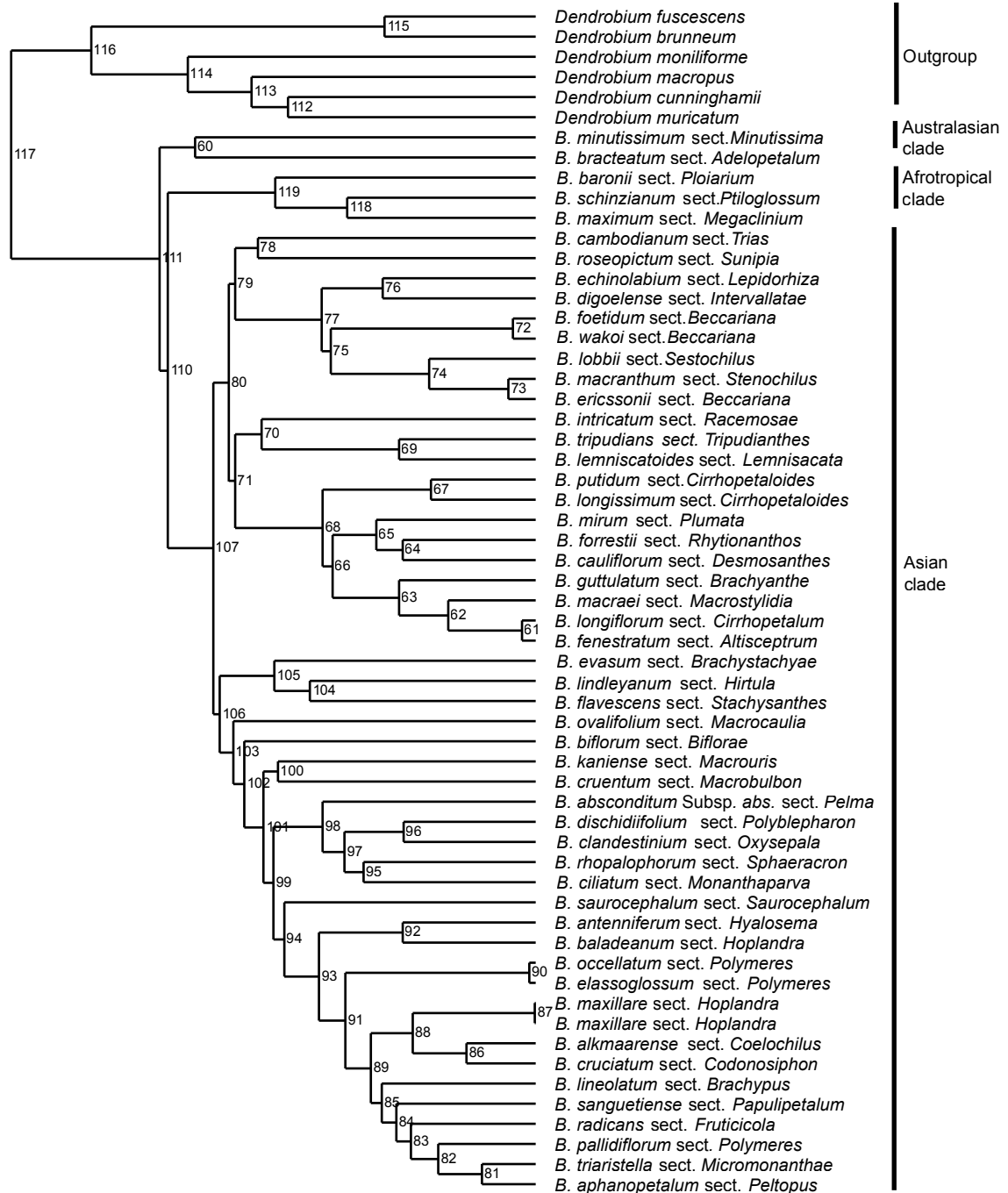
Appendix 2.3 Bayesian phylogeny of 53 *Bulbophyllum* taxa reconstructed from 70 plastid coding regions. Posterior probabilities >50% are displayed near nodes.

Appendix 2.4. Model test comparing DIVALIKE and DEC models of range evolution

	LnL	numparams	d	e	AICc	AICc_wt
DEC	-178	2	0.009	0.0027	360.2	2.20E-09
DIVALIKE	-186.2	2	0.01	2.00E-09	376.7	5.80E-13



Appendix 2.5 Historical biogeography of 53 *Bulbophyllum* species inferred from the DIVALIKE model of range evolution.



Appendix 2.6 Cladogram indicating node ID for the ancestral range analyses.

Appendix 2.7. Range probabilities (expressed as percentages) at each node from the DEC analysis

Node	Range probabilities
node 60	D 100.00
node 61	ACDEF 72.23 ACDF 5.74 ACEF 5.74 ACDE 5.74 CDEF 5.73 C 1.32 BC 0.41 CEF 0.35 CDF 0.35 ACF 0.35 ACD 0.35 ACE 0.35 CDE 0.35 CD 0.25 CE 0.25 AC 0.25 CF 0.25
node 62	C 80.82 BC 7.96 AC 1.54 CE 1.54 CD 1.54 CF 1.54 ACDEF 1.40 ACEF 0.48 ACDF 0.48 ACDE 0.48 CDEF 0.48 ACF 0.26 ACE 0.26 ACD 0.26 CDF 0.26 CDE 0.26 CEF 0.26 ABCEF 0.05 ABCDF 0.05 ABCDE 0.05 BCDEF 0.05
node 63	C 92.31 BC 7.58 ACDEF 0.11
node 64	C 91.66 BC 8.34
node 65	C 100.00
node 66	C 100.00
node 67	C 100.00
node 68	C 100.00
node 69	C 90.85 BC 9.15
node 70	C 100.00
node 71	C 100.00
node 72	CF 100.00
node 73	CF 68.35 C 31.65
node 74	C 88.58 CF 11.42
node 75	C 99.88 CF 0.12
node 76	CF 82.62 C 17.38
node 77	C 81.00 CF 19.00
node 78	C 100.00
node 79	C 100.00
node 80	C 100.00
node 81	F 87.58 EF 12.42
node 82	F 96.43 EF 3.58
node 83	DF 59.90 F 40.10
node 84	F 94.72 DF 5.28
node 85	F 100.00
node 86	F 69.16 CF 30.84

node 87	CDF 100.00
node 88	CF 58.55 F 32.80 CDF 8.65
node 89	CF 64.08 F 35.92
node 90	C 100.00
node 91	CF 52.11 C 47.15 F 0.74
node 92	CE 50.16 CEF 33.42 EF 8.40 C 7.95 F 0.07
node 93	C 67.64 CF 30.22 F 1.62 CE 0.45 CEF 0.07
node 94	C 82.77 CF 17.23
node 95	CF 68.18 C 23.17 F 8.65
node 96	C 48.15 F 28.79 CF 22.47 CEF 0.55 CE 0.05
node 97	C 42.36 CF 39.53 F 18.11
node 98	CF 43.37 C 42.99 F 13.58 CEF 0.06
node 99	CF 58.25 C 41.75
node 100	F 100.00
node 101	CF 98.19 C 1.63 F 0.19
node 102	C 83.92 CF 16.08
node 103	C 96.31 CF 3.69
node 104	C 100.00
node 105	CD 56.97 C 43.03
node 106	C 76.21 CD 23.79
node 107	C 83.87 CD 13.69 CF 2.44
node 108	A 100.00
node 109	A 100.00
node 110	AC 84.30 ACD 12.65 ACF 2.94 C 0.12
node 111	ACD 96.66 ACDF 2.80 CD 0.54
node 112	DE 66.96 D 32.96 CDE 0.08
node 113	D 62.60 DE 35.99 CD 1.33 CDE 0.08
node 114	CD 48.20 BCD 15.21 CDE 11.82 BD 11.32 C 7.17 CE 4.38 BDE 1.11 D 0.41 BCE 0.26 B 0.14
node 115	C 68.03 BC 31.97
node 116	C 66.93 CD 20.82 B 6.41 BCD 4.03 BC 0.63 CDE 0.45 BD 0.43 CE 0.19 BCDE 0.09

node 117	ACD 43.86 CD 21.69 AC 11.07 ABCD 10.17 C 5.06 BCD 5.00 ACDF 2.05 CDE 0.32 ACDE 0.30 ABC 0.18 D 0.07 AD 0.07 CDF 0.06 ABD 0.04 BC 0.03
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Appendix 2.8. Range probabilities (expressed as percentages) at each node from the DIVALIKE analysis

Node	Range probabilities
node 60	D 100
node 61	C 100
node 62	C 100
node 63	C 100
node 64	C 100
node 65	C 100
node 66	C 100
node 67	C 100
node 68	C 100
node 69	C 100
node 70	C 100
node 71	C 100
node 72	F 50, CF 50
node 73	C 100
node 74	C 100
node 75	C 50, CF 50
node 76	CF 100
node 77	C 96, CF 4
node 78	C 100
node 79	C 100
node 80	C 100
node 81	F 100
node 82	F 100
node 83	DF 100
node 84	F 100
node 85	F 100

node 86	F 100
node 87	F 100
node 88	F 100
node 89	F 100
node 90	C 100
node 91	CF 100
node 92	CE 49.5, CEF 49.5, EF 1
node 93	C 98.5, F 1.5
node 94	C 100
node 95	CF 100
node 96	C 98.5, F 1.5
node 97	C 98.5, F 1.5
node 98	C 98.5, F 1.5
node 99	C 100
node 100	F 100
node 101	CF 98.5, F 1.5
node 102	C 98.5, CF 1.5
node 103	C 98.67, BF 0.67, B CF 0.67
node 104	C 100
node 105	CD 100
node 106	C 100
node 107	C 99, BCF 1
node 108	A 100
node 109	A100
node 110	AC 98.67, ABF 0.67, ABCF 0.67
node 111	AD 49.5, ACD 49.5, ABDF 0.5, ABCDF 0.5
node 112	DE 100
node 113	DE 50, D 50
node 114	BCE 14.29, CD 14.29, BCDE 14.29, BDE 14.29, BD 14.29, BE 14.29, BCD 14.29
node 115	C 50, BC 50
node 116	BCD 16.67, BC 16.67, BCE 16.67, BCDE 16.67, B 16.67, C 16.67
node 117	ACD 33.17, ABCD 33.17, ABCDE 33.17, ABCDF 0.5

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Appendix 3.1 Molecular regions for each sample included in supermatrices are marked ‘x’ and will be replaced with GenBank accession numbers with the revised manuscript. DNA numbers from which new sequences have been generated for this study are indicated in bold. Accessions included in divergence age estimations are indicated with an asterix.

Species	DNA / GenBank No.	Sequencing method		GenBank accession numbers							
		Illumina	Sanger	<i>accD</i>	<i>atpA</i>	<i>atpB</i>	<i>atpE</i>	<i>atpF</i>	<i>atpH</i>	<i>atpI</i>	<i>ccsA</i>
<i>B. argyropus</i> (Endl.) Rchb.f.	CNS_G00333	—	x	—	—	—	—	—	—	—	—
<i>B. argyropus</i> (Endl.) Rchb.f.	CNS_G03566*	x	—	x	x	x	x	x	x	x	x
<i>B. baronii</i> Ridl.	CNS_G06040*	x	—	x	x	x	x	x	x	x	x
<i>B. boonjee</i> B.Gray & D.L.Jones	CNS_G03563	—	x	—	—	—	—	—	—	—	—
<i>B. boonjee</i> B.Gray & D.L.Jones	CNS_G07175*	x	—	x	x	x	x	x	x	x	x
<i>B. bracteatum</i> (Fitzg.) F.M.Bailey	CNS_G00383	—	x	—	—	—	—	—	—	—	—
<i>B. bracteatum</i> (Fitzg.) F.M.Bailey	CNS_G01553*	x	—	x	x	x	x	x	x	x	x
<i>B. ciluliae</i> Bianch. & J.A.N.Bat.	ITS: GQ339698*	—	x	—	—	—	—	—	—	—	—
<i>B. corythium</i> N.Hallé	CNS_G00370*	—	x	—	—	—	—	—	—	—	—
<i>B. cylindrobulbum</i> Schltr.	CNS_G05215*	x	—	x	x	x	x	x	x	x	x
<i>B. elisae</i> (F.Muell.) Benth.	CNS_G01548	—	x	—	—	—	—	—	—	—	—
<i>B. elisae</i> (F.Muell.) Benth.	CNS_G01549	—	x	—	—	—	—	—	—	—	—
<i>B. elisae</i> (F.Muell.) Benth.	CNS_G01550*	—	x	—	—	—	—	—	—	—	—
<i>B. evasum</i> T.E.Hunt & Rupp	CNS_G01247*	x	—	x	x	x	x	x	x	x	x
<i>B. exiguum</i> F.Muell.	CNS_G00234	—	x	—	—	—	—	—	—	—	—
<i>B. exiguum</i> F.Muell.	CNS_G00322	—	x	—	—	—	—	—	—	—	—
<i>B. exiguum</i> F.Muell.	CNS_G01558*	—	x	—	—	—	—	—	—	—	—
<i>B. globuliforme</i> Nicholls	CNS_G00392	—	x	—	—	—	—	—	—	—	—
<i>B. globuliforme</i> Nicholls	CNS_G01584*	—	x	—	—	—	—	—	—	—	—
<i>B. hoehnei</i> E.C.Smidt & Borba	ITS: GQ339700*	—	x	—	—	—	—	—	—	—	—
<i>B. humblotii</i> Rolfe	ITS: EF195986* <i>ycf1</i> : KJ558543*	—	x	—	—	—	—	—	—	—	—

Species	DNA / GenBank No.	Illumina	Sanger	<i>accD</i>	<i>atpA</i>	<i>atpB</i>	<i>atpE</i>	<i>atpF</i>	<i>atpH</i>	<i>atpI</i>	<i>ccsA</i>
<i>B. intricatum</i> Seidenf.	CNS_G05202*	x	—	x	x	x	x	x	x	x	x
<i>B. keekee</i> N.Hallé	CNS_G07329*	x	—	x	x	x	x	x	x	x	x
<i>B. lageniforme</i> F.M.Bailey	CNS_G04503	—	x	—	—	—	—	—	—	—	—
<i>B. lageniforme</i> F.M.Bailey	CNS_G03707*	x	—	x	x	x	x	x	x	x	x
<i>B. lageniforme</i> F.M.Bailey	CNS_G01630	—	x	—	—	—	—	—	—	—	—
<i>B. lilianae</i> Rendle	CNS_G01632	—	x	—	—	—	—	—	—	—	—
<i>B. lilianae</i> Rendle	CNS_G01561	—	x	—	—	—	—	—	—	—	—
<i>B. lilianae</i> Rendle	CNS_G07754*	x	—	x	x	x	x	x	x	x	x
<i>B. lingulatum</i> Rendle	CNS_G06046*	x	—	x	x	x	x	x	x	x	x
<i>B. lobbii</i> Lindl.	CNS_G05229*	x	—	x	x	x	x	x	x	x	x
<i>B. longissimum</i> (Ridl.) J.J.Sm.	CNS_G06041*	x	—	x	x	x	x	x	x	x	x
<i>B. maxillare</i> (Lindl.) Rchb.f.	CNS_G05267*	x	—	x	x	x	x	x	x	x	x
<i>B. maximum</i> (Lindl.) Rchb.f.	CNS_G05289*	x	—	x	x	x	x	x	x	x	x
<i>B. minutissimum</i> (F.Muell.) F.Muell.	CNS_G01585	—	x	—	—	—	—	—	—	—	—
<i>B. minutissimum</i> (F.Muell.) F.Muell.	CNS_G01587*	x	—	x	x	x	x	x	x	x	x
<i>B. moniliforme</i> E.C.Parish & Rchb.f.	CNS_G03763*	x	—	x	x	x	x	x	x	x	x
<i>B. mucronatum</i> (Blume) Lindl.	CNS_G05440*	x	—	x	x	x	x	x	x	x	x
<i>B. newportii</i> (F.M.Bailey) Rolfe	CNS_G01562	—	x	—	—	—	—	—	—	—	—
<i>B. newportii</i> (F.M.Bailey) Rolfe	CNS_G01565	—	x	—	—	—	—	—	—	—	—
<i>B. newportii</i> (F.M.Bailey) Rolfe	CNS_G03754*	x	—	x	x	x	x	x	x	x	x
<i>B. nummularia</i> (H.Wendl. & Kraenzl.) Rolfe	CNS_G05275*	x	—	x	x	x	x	x	x	x	x
<i>B. ocellatum</i> Cootes & M.A.Clem.	CNS_G05293*	x	—	x	x	x	x	x	x	x	x
<i>B. pervillei</i> Rolfe ex Scott-Elliot	ITS: EF196049* ycf1: KJ558551*	—	x	—	—	—	—	—	—	—	—
<i>B. pygmaeum</i> (Sm.) Lindl.	CNS_G04599*	x	—	x	x	x	x	x	x	x	x

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Species	DNA / GenBank No.	Illumina	Sanger	<i>accD</i>	<i>atpA</i>	<i>atpB</i>	<i>atpE</i>	<i>atpF</i>	<i>atpH</i>	<i>atpI</i>	<i>ccsA</i>
<i>B. pygmaeum</i> (Sm.) Lindl.	CNS_G03988	—	x	—	—	—	—	—	—	—	—
<i>B. rhopalophorum</i> Schltr.	CNS_G05261*	x	—	x	x	x	x	x	x	x	x
<i>B. schinzianum</i> Kraenzl.	CNS_G01642*	x	—	x	x	x	x	x	x	x	x
<i>B. tuberculatum</i> Colenso	CNS_G03993	—	x	—	—	—	—	—	—	—	—
<i>B. tuberculatum</i> Colenso	CHR572204*	—	x	—	—	—	—	—	—	—	—
<i>B. weinthalii</i> R.S. Rogers	CNS_G01624	—	x	—	—	—	—	—	—	—	—
<i>B. weinthalii</i> R.S. Rogers	CNS_G03564	—	x	—	—	—	—	—	—	—	—
<i>B. weinthalii</i> R.S. Rogers	CNS_G06024*	x	—	x	x	x	x	x	x	x	x
<i>Coelogyne flaccida</i> Lindl.	n/a	—	x	—	x	x	x	x	x	x	x
<i>Dendrobium brunneum</i> Schuit. & Peter B.Adams	CNS_G00781*	x	—	x	x	x	x	x	x	x	x
<i>Dendrobium cunninghamii</i> Lindl.	CNS_G02336*	x	—	x	x	x	x	x	x	x	x
<i>Dendrobium fuscescens</i> (Griff.) Summerh.	CNS_G01021*	x	—	x	x	x	x	x	x	x	x
<i>Dendrobium macropus</i> (Endl.) Rchb.f.	CNS_G02316*	x	—	x	x	x	x	x	x	x	x
<i>Dendrobium moniliforme</i> (L.) Sw.	CNS_G00882*	x	—	x	x	x	x	x	x	x	x
<i>Dendrobium muricatum</i> Finet	CNS_G02350*	x	—	x	x	x	x	x	x	x	x
<i>Dienia ophrydis</i> (J.Koenig) Seidenf.	CNS_G01303*	x	—	x	x	x	x	x	x	x	x
<i>Neottia cordata</i> (L.) Rich.	CNS_G05314*	x	—	x	x	x	x	x	x	x	x
<i>Nervilia concolor</i> (Blume) Schltr.	CNS_G03442*	x	—	x	x	x	x	x	x	x	x
<i>Oberonia complanata</i> (A.Cunn.) M.A.Clem. & D.L.Jones	CNS_G00309*	x	—	x	x	x	x	x	x	x	x

[illegible]

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[illegible]

223 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]

[illegible]

225 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]

[illegible]

227 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]

[illegible]

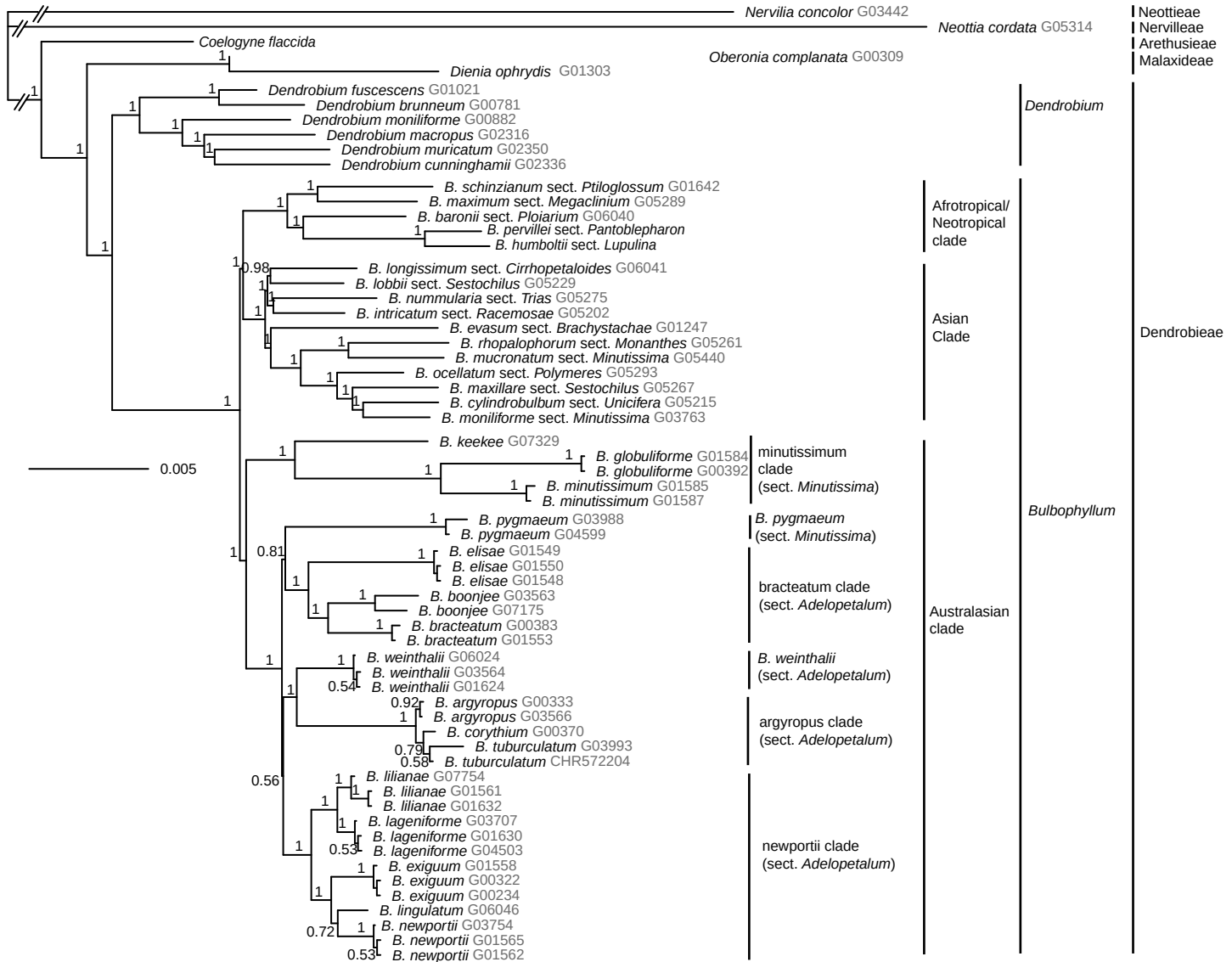
229 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]

DNA / GenBank No.	<i>rps11</i>	<i>rps12</i>	<i>rps14</i>	<i>rps15</i>	<i>rps16</i>	<i>rps18</i>	<i>rps19</i>	<i>ycf1</i>	<i>ycf2</i>	<i>ycf3</i>	<i>ycf4</i>	<i>ycf68</i>
CNS_G00333	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G03566*	X	X	X	X	X	X	X	X	X	X	X	X
CNS_G06040*	X	X	X	X	X	X	X	X	X	X	X	X
CNS_G03563	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G07175*	X	X	X	X	X	X	X	X	X	X	X	X
CNS_G00383	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01553*	X	X	X	X	X	X	X	X	X	X	X	X
ITS: GQ339698*	—	—	—	—	—	—	—	—	—	—	—	—
CNS_G00370*	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G05215*	X	X	X	X	X	X	X	X	X	X	X	X
CNS_G01548	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01549	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01550*	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01247*	X	X	X	X	X	X	X	X	X	X	X	X
CNS_G00234	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G00322	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01558*	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G00392	—	—	—	—	—	—	—	X	—	—	—	—
CNS_G01584*	—	—	—	—	—	—	—	X	—	—	—	—
ITS: GQ339700*	—	—	—	—	—	—	—	—	—	—	—	—
ITS: EF195986* <i>ycf1</i> : KJ558543*	—	—	—	—	—	—	—	KJ558543	—	—	—	—

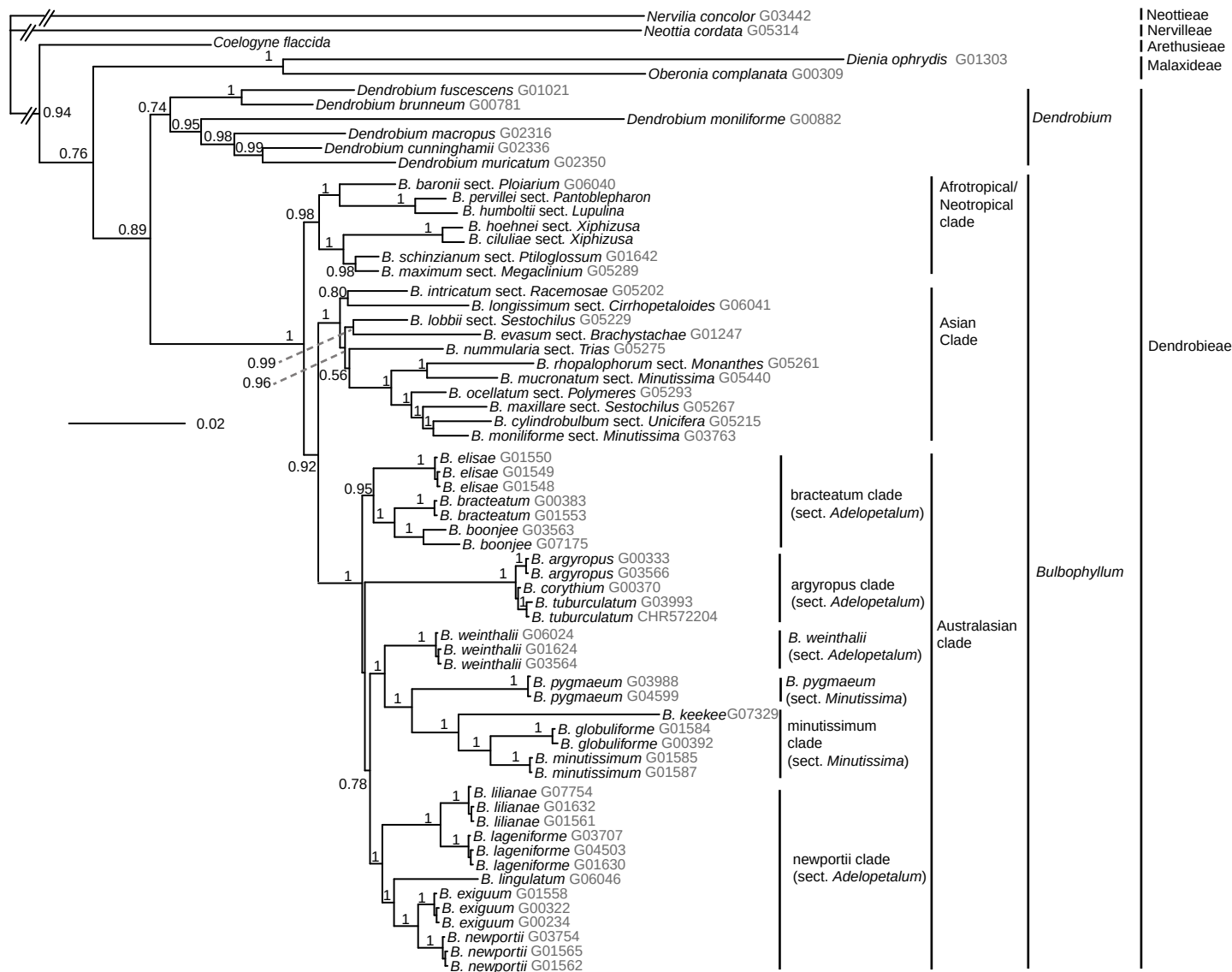
231 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]

[illegible]

233 SPATIO-TEMPORAL EVOLUTION OF AUSTRALASIAN *BULBOPHYLLUM*[illegible]



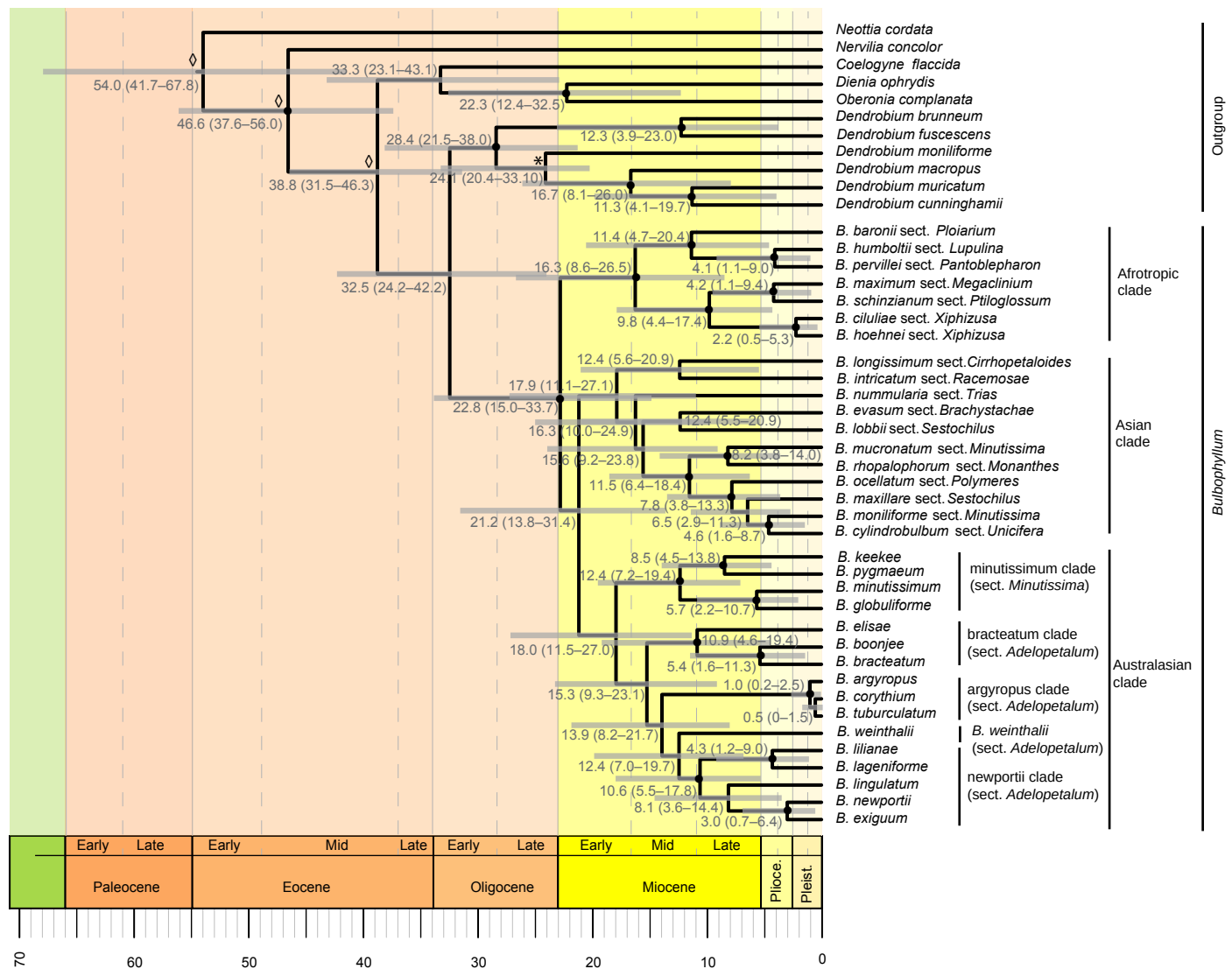
Appendix 3.2 Bayesian reconstruction of 51 *Bulbophyllum* based on the supermatrix of 70 plastid coding regions. Posterior probabilities are displayed adjacent to nodes.



Appendix 3.3 Bayesian reconstruction of 53 *Bulbophyllum* based on seven nuclear ribosomal markers (5'ETS, 18s, ITS1, 5.8s, ITS2, 28s, 3'ETS). Posterior probabilities are displayed adjacent to nodes.

Appendix 3.4. Model comparison estimating the AICM. SE estimated with 100 bootstrap replicates. Lower AICM values indicate better model fit. Differences between AICM are reported. Positive values indicate better relative model fit of the rows model compared to the columns model.

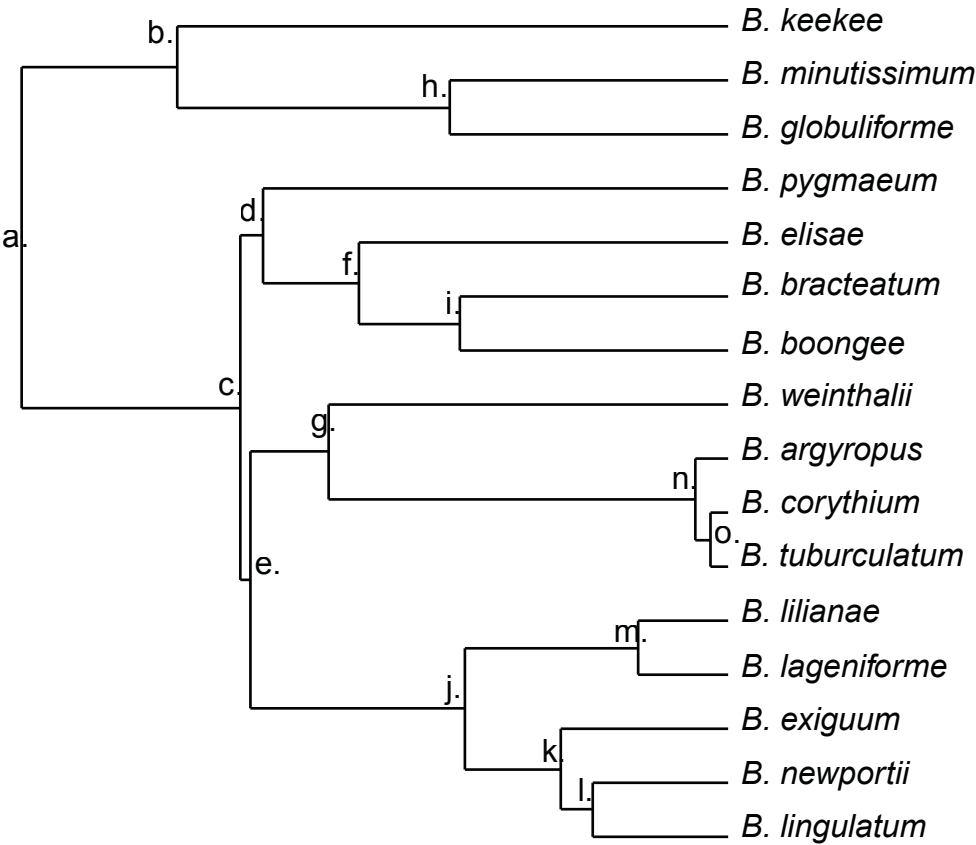
Trace	AICM	S.E.	Plastid, relaxed lognormal clock, birth death	Plastid, strict clock, birth death	Plastid, relaxed lognormal clock, Yule
Plastid, relaxed lognormal clock, birth death	362329.312	+/- 0.115	-	23231.403	2.154
Plastid, strict clock, birth death	385560.715	+/- 0.099	-23231.403	-	-23229.249
Plastid, relaxed lognormal clock, Yule	362331.466	+/- 0.184	-2.154	23229.249	-



Appendix 3.5 Maximum clade credibility chronogram for *Bulbophyllum* based on three nuclear ribosomal regions (ITS1, 5.8s, ITS2). Black circles indicate highly supported nodes (PP > 0.95). Divergence dates and 95% highest posterior density values are indicated adjacent to nodes. Grey bars indicate 95% highest posterior density. The asterisk denotes the node constrained with a fossil calibration point; the diamond denotes nodes which were constrained by secondary calibration points.

Appendix 3.6. Ancestral range reconstruction model test. LnL: log of the likelihood; AICc: corrected Akaike Information Criterion; d: dispersal rate; e: extinction rate; AICc_wt: corrected Akaike Information Criterion weight

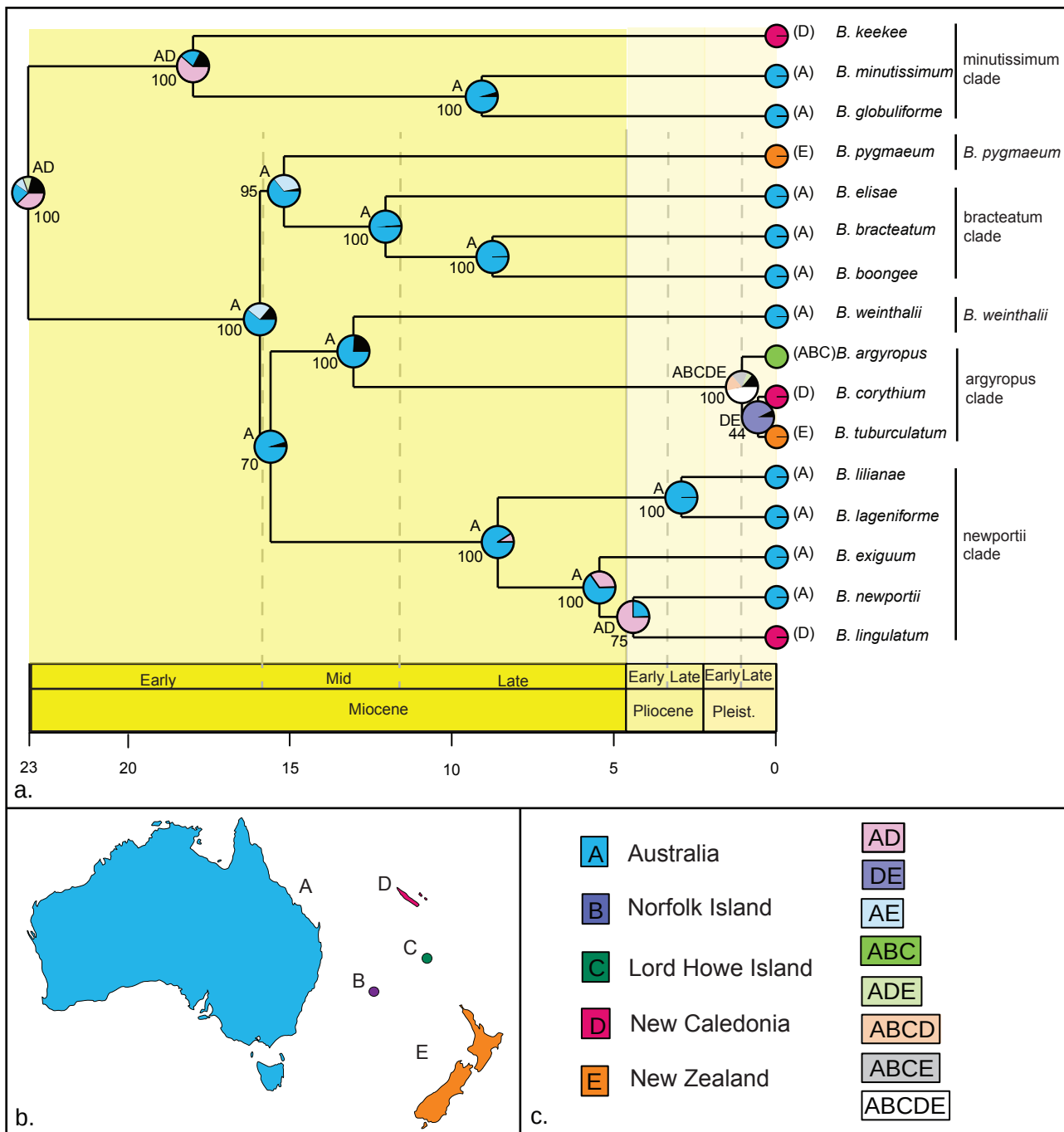
	LnL	Number of parameters	d	e	AICc	AICc_wt
DEC	-34.28	2	0.0092	0.0094	73.48	0.0006
DIVALIKE	-29.94	2	0.01	0.0056	64.8	0.045



Appendix 3.7 Cladogram indicating Node ID for the ancestral range analyses

Appendix 3.8. Range probabilities (expressed as percentages) at each node determined by the dispersal-vicariance model

Node ID	Probability of alternative ranges
node a.	A 61.71 AD 24.26 ADE 5.00 ACD 2.09 ABD 2.09 AE 0.86 ABDE 0.62 ACDE 0.62 AC 0.50 AB 0.50 DE 0.36 ABCD 0.31 ABCDE 0.19 ABE 0.16 ACE 0.16 D 0.12 ABC 0.10 BD 0.06 CD 0.06 CDE 0.06 BDE 0.06 ABCE 0.05 BCDE 0.02 BCD 0.02 E 0.01 CE 0.01 BE 0.01
node b.	AD 70.28 A 18.65 D 3.38 ABD 1.16 ACD 1.16 ADE 1.08 CD 0.63 BD 0.63 AC 0.61 AB 0.61 DE 0.61 AE 0.59 ABCD 0.07 ACDE 0.06 ABDE 0.06 BCD 0.05 C 0.05 B 0.05 E 0.05 BDE 0.05 CDE 0.05 ABC 0.03 ACE 0.02 ABE 0.02 BC 0.02 BE 0.02 CE 0.02 ABCDE 0.01
node c.	A 93.05 AE 5.09 ABE 0.33 ACE 0.33 ABCE 0.24 ABCDE 0.16 ADE 0.14 ABDE 0.11 ACDE 0.11 AC 0.11 AB 0.11 ABC 0.06 AD 0.05 ABCD 0.03 ACD 0.02 ABD 0.02 E 0.01
node d.	A 59.79 AE 38.81 E 0.49 AC 0.20 AB 0.20 AD 0.19 ACE 0.07 ABE 0.07 CE 0.06 BE 0.06 ADE 0.03 DE 0.02
node e.	A 99.85 AC 0.03 AB 0.03 ABC 0.02 AD 0.02 AE 0.01 C 0.01 B 0.01 ABD 0.01 ACD 0.01
node f.	A 99.88 AE 0.07 AC 0.02 AB 0.02 AD 0.01
node g.	A 91.68 AE 1.54 AD 1.36 AB 1.27 AC 1.27 ABC 0.38 ACD 0.32 ABD 0.32 ADE 0.30 ABE 0.30 ACE 0.30 ABCD 0.22 ABCE 0.15 ACDE 0.11 ABDE 0.11 ABCDE 0.10 E 0.07 C 0.04 B 0.04 D 0.02 BCD 0.02 BC 0.01 BCE 0.01 BCDE 0.01 BD 0.01 CD 0.01 CE 0.01 BE 0.01
node h.	A 99.45 AD 0.25 AB 0.09 AC 0.09 AE 0.08 D 0.01
node i.	A 99.97 AE 0.01 AB 0.01 AC 0.01 AD 0.01
node j.	A 99.90 AD 0.08
node k.	A 97.59 AD 2.36 ACD 0.01 ADE 0.01 ABD 0.01
node l.	AD 83.38 A 16.13 D 0.23 AB 0.05 AE 0.05 AC 0.05 ACD 0.03 ADE 0.03 ABD 0.03
node m.	A 99.99
node n.	ABCDE 71.95 ABDE 9.72 ACDE 9.72 ADE 1.99 ABCD 1.98 ABCE 1.22 BCDE 1.02 ABD 0.54 ACD 0.54 ABE 0.31 ACE 0.31 AD 0.20 BDE 0.14 CDE 0.14 AE 0.13 BCD 0.04 BCE 0.02 DE 0.02 BD 0.01 CD 0.01
node o.	DE 99.91 E 0.03 D 0.03 ADE 0.01 BDE 0.01 CDE 0.01



Appendix 3.9 Historical biogeography of the Australasian clade.

- a) ancestral area reconstruction based on a dispersal, extinction, cladogenesis model;
 b) geographic regions delineated in the biogeographic analysis;
 c) legend of colour-coded geographic regions and shared ancestral areas.
 Values adjacent to nodes indicate bootstrap support.

Appendix 3.10. Range probabilities (expressed as percentages) at each node determined by the dispersal, extinction, cladogenesis model.

Node ID	Probability of alternative ranges
node a.	AD 37.74 A 22.11 AE 9.65 ADE 9.31 ABD 3.68 ACD 3.68 AB 1.84 AC 1.84 ABDE 1.67 ACDE 1.67 ACE 1.08 ABE 1.08 ABCDE 1.05 ABCD 0.98 DE 0.56 ABCE 0.54 ABC 0.46 D 0.20 BDE 0.17 CDE 0.17 BCDE 0.13 CD 0.09 BD 0.09 BCD 0.07 E 0.05 BE 0.02 CE 0.02 BCE 0.01
node b.	AD 61.52 A 21.16 D 3.47 ADE 3.17 ABD 3.06 ACD 3.06 AE 0.88 AB 0.82 AC 0.82 ACDE 0.28 ABDE 0.28 ABCD 0.28 DE 0.23 BD 0.20 CD 0.20 ABE 0.08 ACE 0.08 ABC 0.08 E 0.06 C 0.05 B 0.05 ABCDE 0.04 CDE 0.03 BDE 0.03 BCD 0.03 ABCE 0.01 CE 0.01 BE 0.01
node c.	A 60.86 AE 25.36 AD 1.86 ABCDE 1.60 ADE 1.22 AB 1.19 AC 1.19 ABE 1.13 ACE 1.13 ABCE 0.99 ABDE 0.81 ACDE 0.81 ABCD 0.56 ABD 0.42 ACD 0.42 ABC 0.42 E 0.01
node d.	A 64.16 AE 32.72 ADE 0.56 ACE 0.49 ABE 0.49 E 0.39 AD 0.38 AB 0.32 AC 0.32 ABDE 0.03 ACDE 0.03 ABCE 0.03 DE 0.01 ABD 0.01 ACD 0.01 ABC 0.01 BE 0.01 CE 0.01
node e.	A 95.21 AD 1.13 AE 0.59 AB 0.53 AC 0.53 ABCDE 0.30 ABCD 0.20 ABDE 0.17 ACDE 0.17 ABD 0.17 ACD 0.17 ADE 0.16 ABCE 0.16 ABC 0.14 ABE 0.14 ACE 0.14 D 0.05 E 0.01 BCDE 0.00 DE 0.00 BDE 0.00 BCD 0.00 CDE 0.00 CD 0.00 BD 0.00 C 0.00 B 0.00 BCE 0.00 / 0.00 BC 0.00 CE 0.00 BE 0.00
node f.	A 98.61 AE 0.90 AD 0.16 AC 0.15 AB 0.15 ADE 0.01 ACE 0.01 ABE 0.01
node g.	A 76.09 AE 3.46 AB 3.24 AC 3.24 AD 3.15 ABCDE 1.93 ABCE 1.08 ABCD 1.08 ABC 0.93 ABE 0.89 ACE 0.89 ACD 0.82 ABD 0.82 ABDE 0.78 ACDE 0.78 ADE 0.74 E 0.02 D 0.01 BCDE 0.01 C 0.01 B 0.01 BCE 0.00 BCD 0.00 BDE 0.00 CDE 0.00 CE 0.00 BE 0.00 DE 0.00 BD 0.00 CD 0.00 BC 0.00 / 0.00
node h.	A 95.51 AD 2.51 AE 0.62 AB 0.59 AC 0.59 ADE 0.04 ABD 0.04 ACD 0.04 ACE 0.02 ABE 0.02 ABC 0.02 D 0.01 ABCD 0.00 ACDE 0.00 ABDE 0.00 ABCE 0.00 BDE 0.00 BCD 0.00 BC 0.00 CDE 0.00 / 0.00 BCE 0.00 BCDE 0.00 BE 0.00 B 0.00 DE 0.00 E 0.00 C 0.00 ABCDE 0.00 CE 0.00 BD 0.00 CD 0.00
node i.	A 99.51 AE 0.21 AD 0.09 AC 0.09 AB 0.09
node j.	A 90.80 AD 8.76 AE 0.12 AC 0.11 AB 0.11 ADE 0.04 ABD 0.03 ACD 0.03
node k.	A 65.32 AD 34.23 ADE 0.08 ABD 0.08 ACD 0.08 AE 0.07 AB 0.07 AC 0.07
node l.	AD 74.92 A 24.33 D 0.15 ADE 0.13 ACD 0.13 ABD 0.13 AE 0.07 AC 0.07 AB 0.07
node m.	A 99.79 AD 0.06 AE 0.05 AC 0.05 AB 0.05 D 0.00 ABC 0.00 ACDE 0.00 ABCD 0.00 ABDE 0.00 ACE 0.00 ADE 0.00 ACD 0.00 ABE 0.00 ABD 0.00 ABCE 0.00 BDE 0.00 CDE 0.00 BC

	0.00 ABCDE 0.00 BCDE 0.00 / 0.00 BCD 0.00 BCE 0.00 C 0.00 DE 0.00 E 0.00 B 0.00 BE 0.00 CE 0.00 BD 0.00 CD 0.00
node n.	ABCDE 46.27 ABCD 17.30 ABCE 17.19 ADE 5.32 ACDE 3.05 ABDE 3.05 ACD 1.57 ABD 1.57 ABE 1.54 ACE 1.54 CDE 0.28 BDE 0.28 AD 0.26 AE 0.25 BCDE 0.25 BCE 0.09 BCD 0.09 ABC 0.03 A 0.01 AB 0.01 AC 0.01 CE 0.01 BE 0.01 CD 0.01 BD 0.01
node o.	DE 92.39 D 3.63 E 3.62 ADE 0.20 CDE 0.05 BDE 0.05 AE 0.01 AD 0.01

Appendix 3.11. a) Morphological character matrix for ancestral character reconstruction and b) character states delineated in ancestral character reconstruction

a)

character/state	state 0	state 1	state 2	state 3
nodes between pseudobulbs	none	one or more		
pseudobulb shape	ovoid, laterally flattened, lenticular or spheroid	ellipsoid including apically flattened	globose	
pseudobulb surface	rugose	crenulate	smooth, slightly wrinkled	warty
pseudobulb ribs	inconspicuous	prominent		
pseudobulb sheath	membranous	white scareaceous		
leaf surface	glabrous	rugose		
leaf margin	attenuate	crenulate		
leaf petiole	absent	present		
photosynthetic pit at the leaf base	absent	present		
number of flowers per inflorescence	single flower	2-5 flowers	>5 flowers	
peduncle surface	glabrous	verrucate	hirsute	
ovary surface	glabrous	verrucate	hirsute	
tepal surface	glabrous	hirsute		
number of veins in petals	one	three		

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labellum lateral lobes	entire	trilobed with prominent side lobes	obscurely trilobed	
wings along lower margin of stelidia	inconspicuous	prominent		
shape of wings along lower margin of the stelidia	deltoid	tooth shaped		
attachment of the labellum	absent	present		

b)

	nodes between pseudobulbs	pseudobulb shape	pseudobulb surface	pseudobulb ribs	pseudobulb sheath	leaf surface
<i>B. argyropus</i>	1	1	0	0	1	0
<i>B. tuberculatum</i>	1	1	0	0	1	0
<i>B. weinthalii</i>	1	1	0	0	1	0
<i>B. lageniforme</i>	1	0	3	1	0	0
<i>B. lilianae</i>	1	0	2	1	0	0
<i>B. lingulatum</i>	1	0	2	1	0	0
<i>B. exiguum</i>	1	0	2	1	0	0
<i>B. newportii</i>	1	0	2	1	0	0
<i>B. pygmaeum</i>	1	2	1	0	0	1
<i>B. elisae</i>	1	1	1	0	0	0
<i>B. bracteatum</i>	1	1	1	0	0	0
<i>B. boonjee</i>	1	1	1	0	0	0
<i>B. minutissimum</i>	0	2	2	0	0	0
<i>B. globuliforme</i>	0	2	2	0	0	0
<i>B. corythium</i>	1	1	0	0	1	0
<i>B. keekee</i>	1	2	1	0	0	0

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	leaf margin	leaf petiole	photosynthetic pit at the leaf base	number of flowers per inflorescence	peduncle surface	ovary surface
<i>B. argyropus</i>	0	1	0	1	0	1
<i>B. tuberculatum</i>	0	1	0	1	0	1
<i>B. weinthalii</i>	0	1	0	0	0	0
<i>B. lageniforme</i>	0	1	0	1	0	1
<i>B. lilianae</i>	0	1	0	1	0	1
<i>B. lingulatum</i>	0	1	0	1	0	0
<i>B. exiguum</i>	0	1	0	1	0	0
<i>B. newportii</i>	0	1	0	1	0	0
<i>B. pygmaeum</i>	1	1	0	0	2	2
<i>B. elisae</i>	0	1	0	2	0	0
<i>B. bracteatum</i>	0	1	0	2	0	0
<i>B. boonjee</i>	0	1	0	1	0	0
<i>B. minutissimum</i>	0	0	1	0	1	1
<i>B. globuliforme</i>	0	0	1	0	1	1
<i>B. corythium</i>	0	1	0	1	0	1
<i>B. keekee</i>	0	1	0	0	0	1

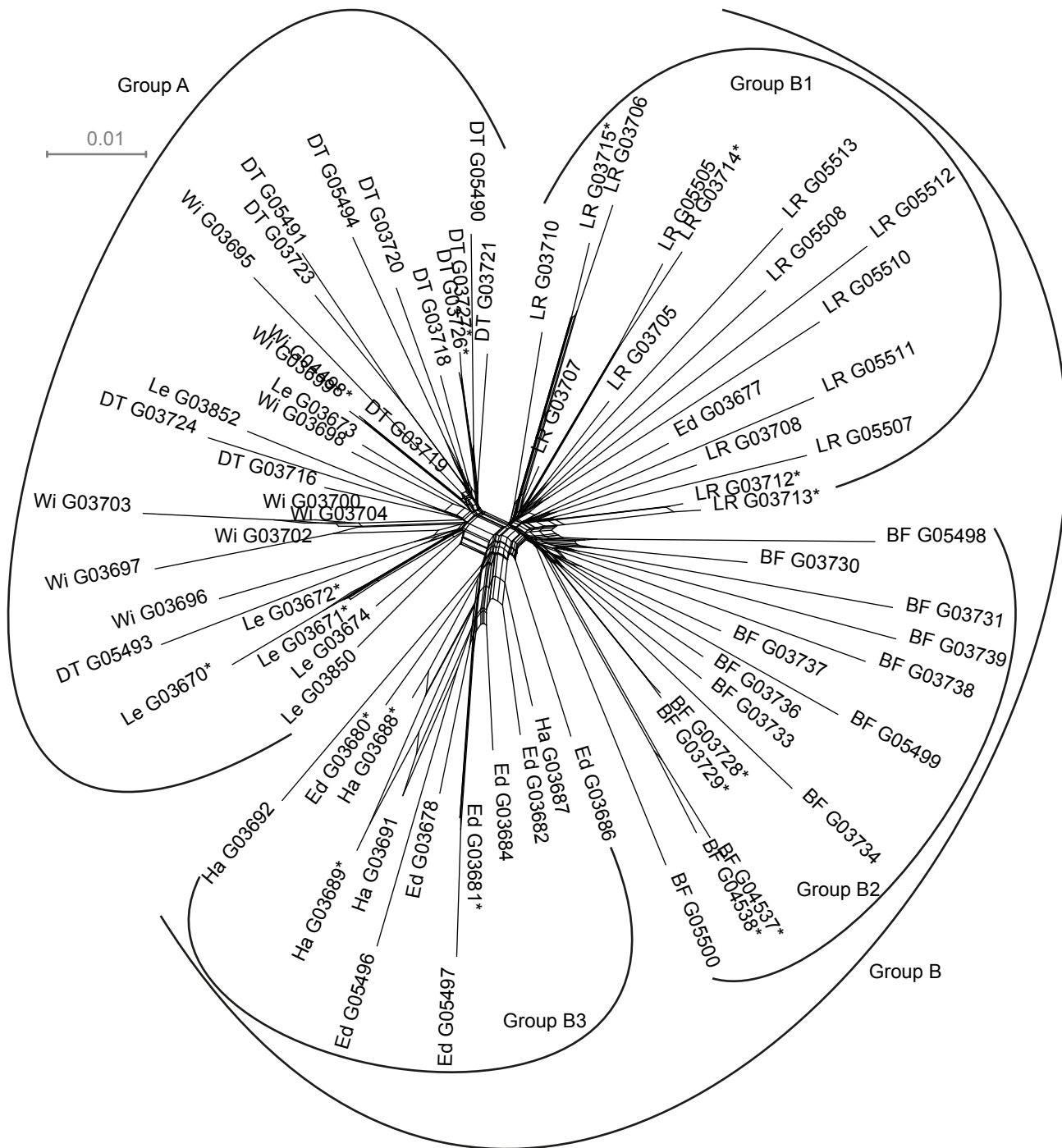
	tepal surface	number of veins in petals	labellum lateral lobes	wings along lower margin of stelidia	shape of wings along lower margin of the stelidia	attachment of the labellum
<i>B. argyropus</i>	0	0	1	0	-	1
<i>B. tuberculatum</i>	0	0	1	0	-	1
<i>B. weinthalii</i>	0	1	1	0	-	1
<i>B. lageniforme</i>	0	1	2	1	0	1
<i>B. lilianae</i>	0	1	2	1	0	1
<i>B. lingulatum</i>	0	1	2	1	1	1
<i>B. exiguum</i>	0	1	2	1	1	1
<i>B. newportii</i>	0	0	2	1	1	1
<i>B. pygmaeum</i>	1	0	0	0	-	0
<i>B. elisae</i>	0	1	1	0	-	1
<i>B. bracteatum</i>	0	0	1	0	-	1
<i>B. boonjee</i>	0	1	1	0	-	1
<i>B. minutissimum</i>	1	0	0	0	-	0
<i>B. globuliforme</i>	1	0	0	0	-	0
<i>B. corythium</i>	0	0	1	0	-	1
<i>B. keekee</i>	0	0	0	0	-	0

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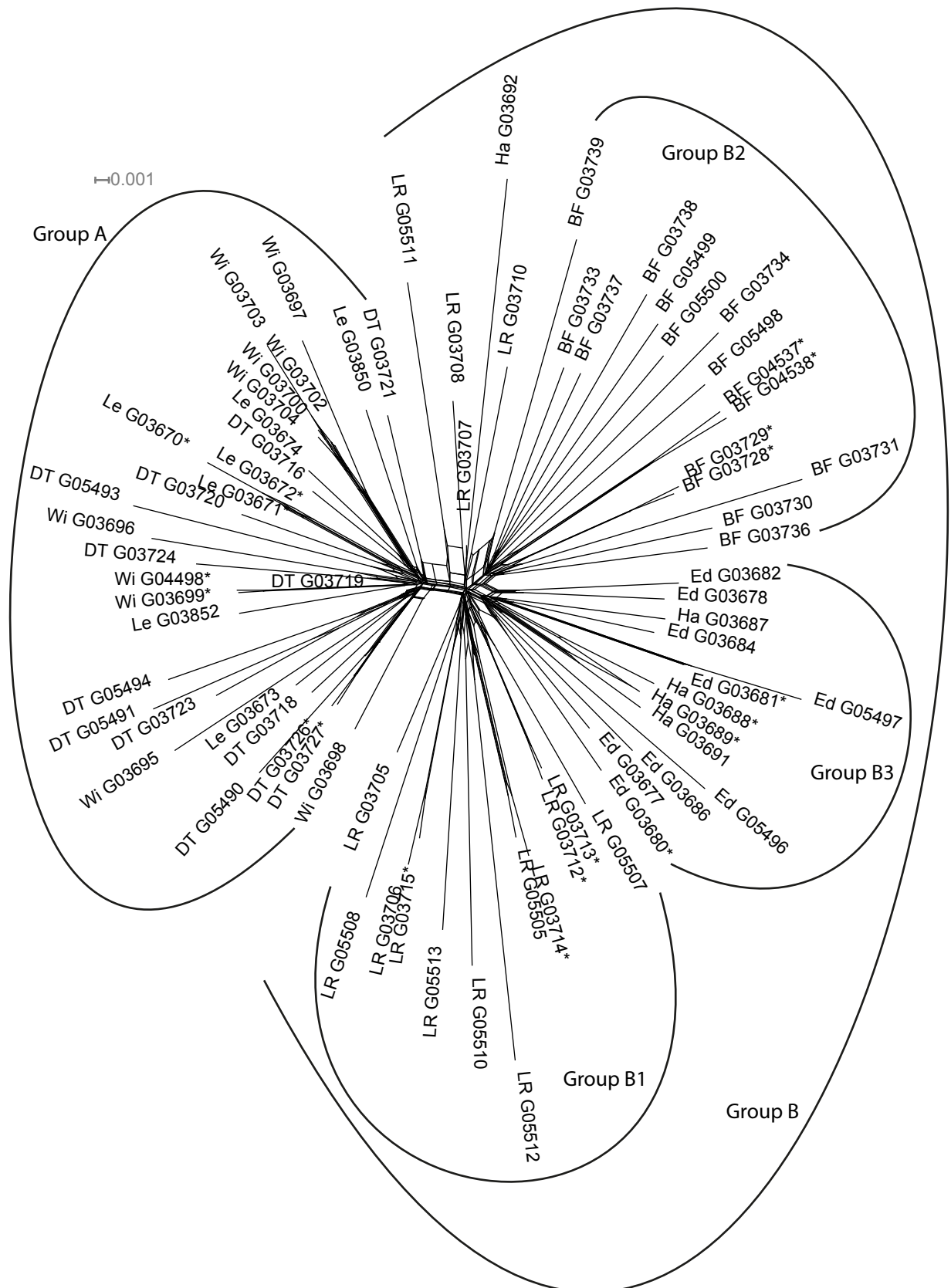
Appendix 3.12. Genome size measurements in *Bulbophyllum* sect. *Adelopetalum* (2C-value [pg]).

Species	Accession	2C value [pg]	±SD	2C values species mean [pg]	±SD	Published chromosome number	Reference
<i>B. argyropus</i>	L.Simpson 505	2.51	0.021	-	-	2n=36	Dawson et al. 2007 (published as <i>Adelopetalum argyropum</i>)
<i>B. bracteatum</i>	L.Simpson 504	2.94	0.009	2.90	0.064	-	-
	L.Simpson 506	2.85	0.026				
<i>B. elisae</i>	L.Simpson 507	2.66	0.014	2.66	0.000	-	-
	L.Simpson 508	2.66	0.015				
<i>B. exiguum</i>	L.Simpson 509	2.62	0.015	2.59	0.042	-	-
	L.Simpson 510	2.56	0.031				
<i>B. lageniforme</i>	L.Simpson 137C	2.90	0.022	2.89	0.006	-	-
	L.Simpson 149C	2.89	0.031				
	L.Simpson 163	2.89	0.028				
<i>B. lilianae</i>	L.Simpson 511	2.63	0.020	2.64	0.064	-	-
	L.Simpson 512	2.60	0.001				
	L.Simpson 513	2.70	0.014				
<i>B. newportii</i>	L.Simpson 514	2.61	0.026	2.65	0.015	-	-
	KSchulte 40	2.65	0.023				
	L.Simpson 153B	2.63	0.006				
	L.Simpson 155A	2.66	0.052				
<i>B. pygmaeum</i>	-	-	-	-	-	2n=38	Dawson et al. 2007 (published as <i>Ichthyostomum pygmaeum</i>)
<i>B. tuberculatum</i>	-	-	-	-	-	2n=38	Dawson et al. 2007 (published as <i>Adelopetalum tuberculatum</i>)

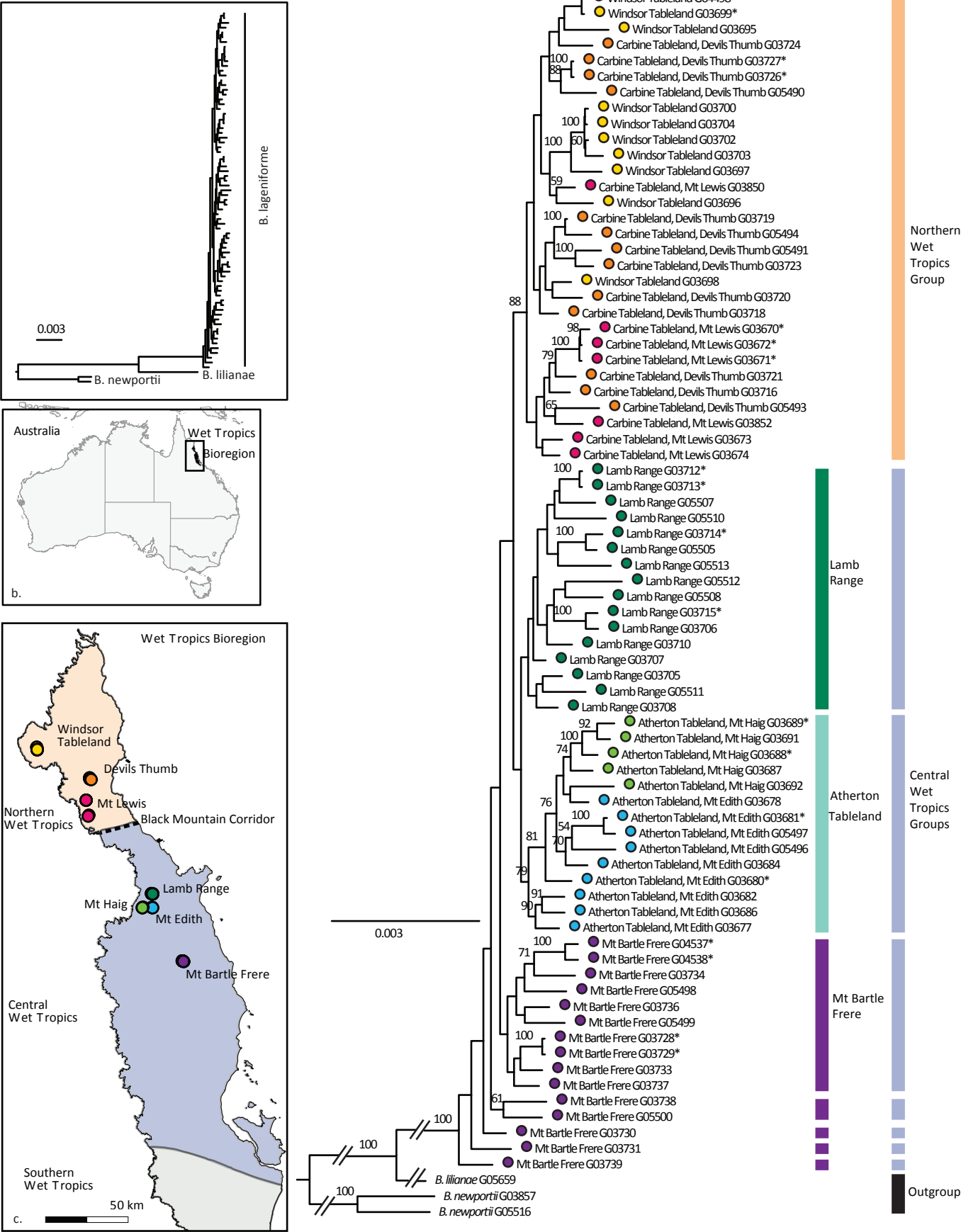
<i>B. weinthalii</i> ssp. <i>weinthalii</i>	L.Simpson 493	3.19	0.007	-	-	-	-
	all species mean	2.74					
	all species median	2.66					



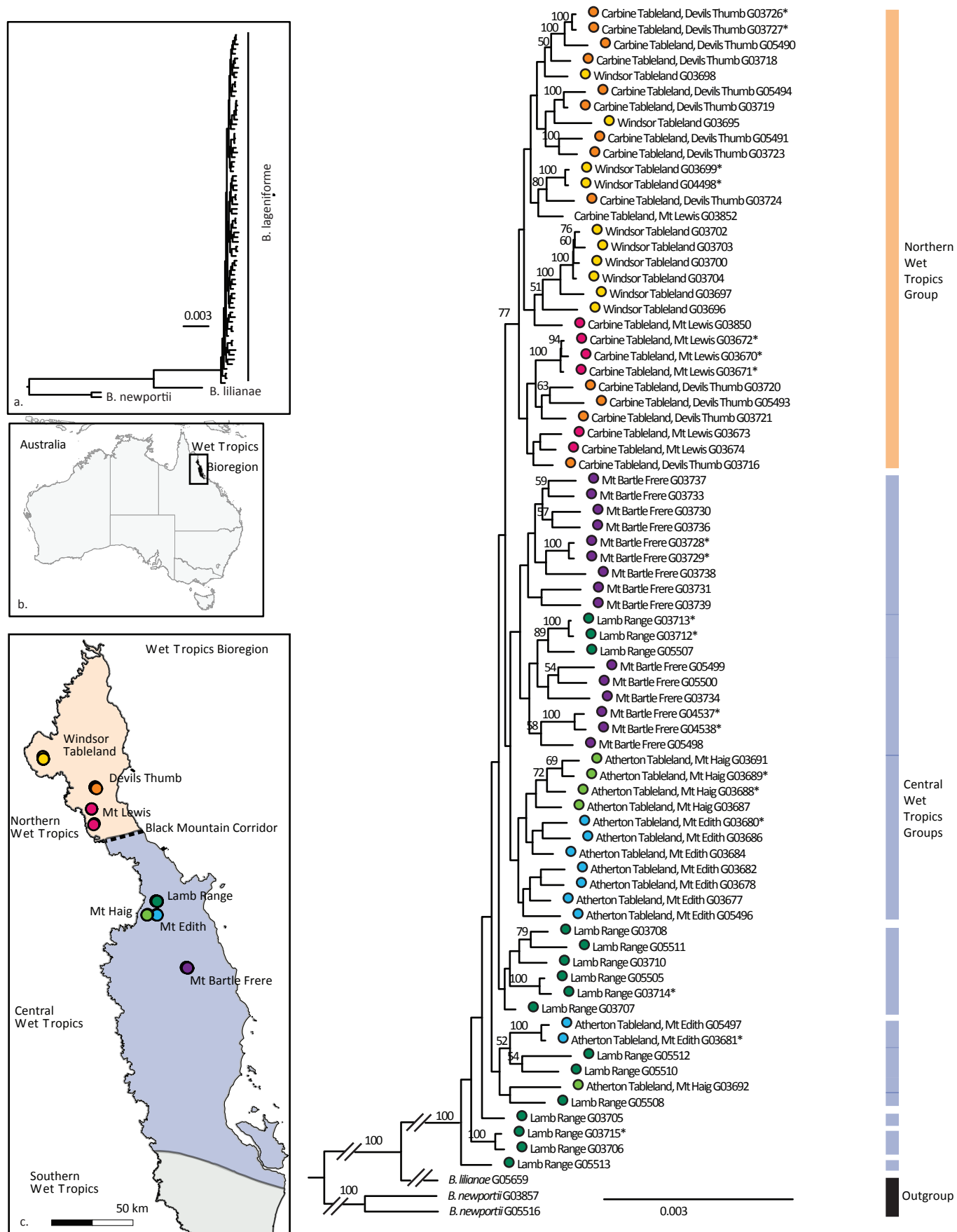
Appendix 4.1 NeighborNet diagram of 65 *B. lageniforme* individuals based on the uncorrected P distances (Nei and Kuma, 2000) derived from 51,531 SNPs contained in dataset MS1 excluding outgroups. Replicate samples are indicated with an asterisk. BF: Mt Bartle Frere, DT: Devils Thumb, Ed: Mt Edith, Ha: Mt Haig, Le: Mt Lewis, LR: Lamb Range, Wi: Windsor Tableland.



Appendix 4.2 NeighborNet diagram of 65 *B. lageniforme* individuals based on the uncorrected P distances (Nei and Kuma, 2000) derived from 17,508 SNPs contained in dataset MS8 excluding outgroups. Replicate samples are indicated with an asterisk. Mt Bartle Frere, DT: Devils Thumb, Ed: Mt Edith, Ha: Mt Haig, Le: Mt Lewis, LR: Lamb Range, Wi: Windsor Tableland.

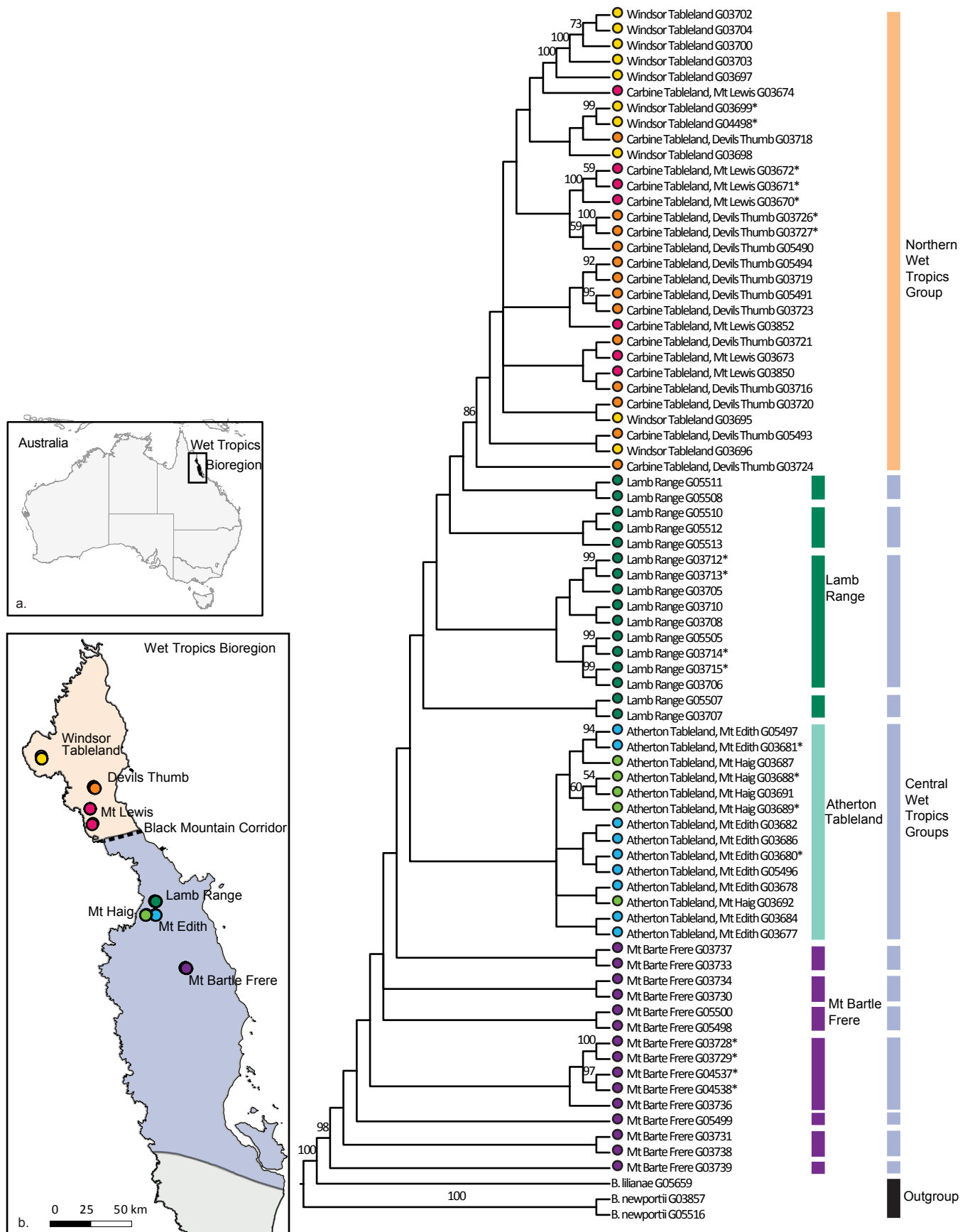


Appendix 4.3 Maximum likelihood reconstruction of 65 *B. lageniforme* individuals and two outgroup species based on 17,289 concatenated RAD loci from dataset MS1 including outgroups. Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset c. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Location of the Wet Tropics Bioregion. Inset c: Map of Wet Tropics Bioregion showing sampling locations.



Appendix 4.4 Maximum likelihood reconstruction of 65 *B. lageniforme* individuals and two outgroup species based on 4,128 concatenated RAD loci from dataset MS8 including outgroups. Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset c. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Location of the Wet Tropics Bioregion. Inset c: Map of Wet Tropics Bioregion showing sampling locations.

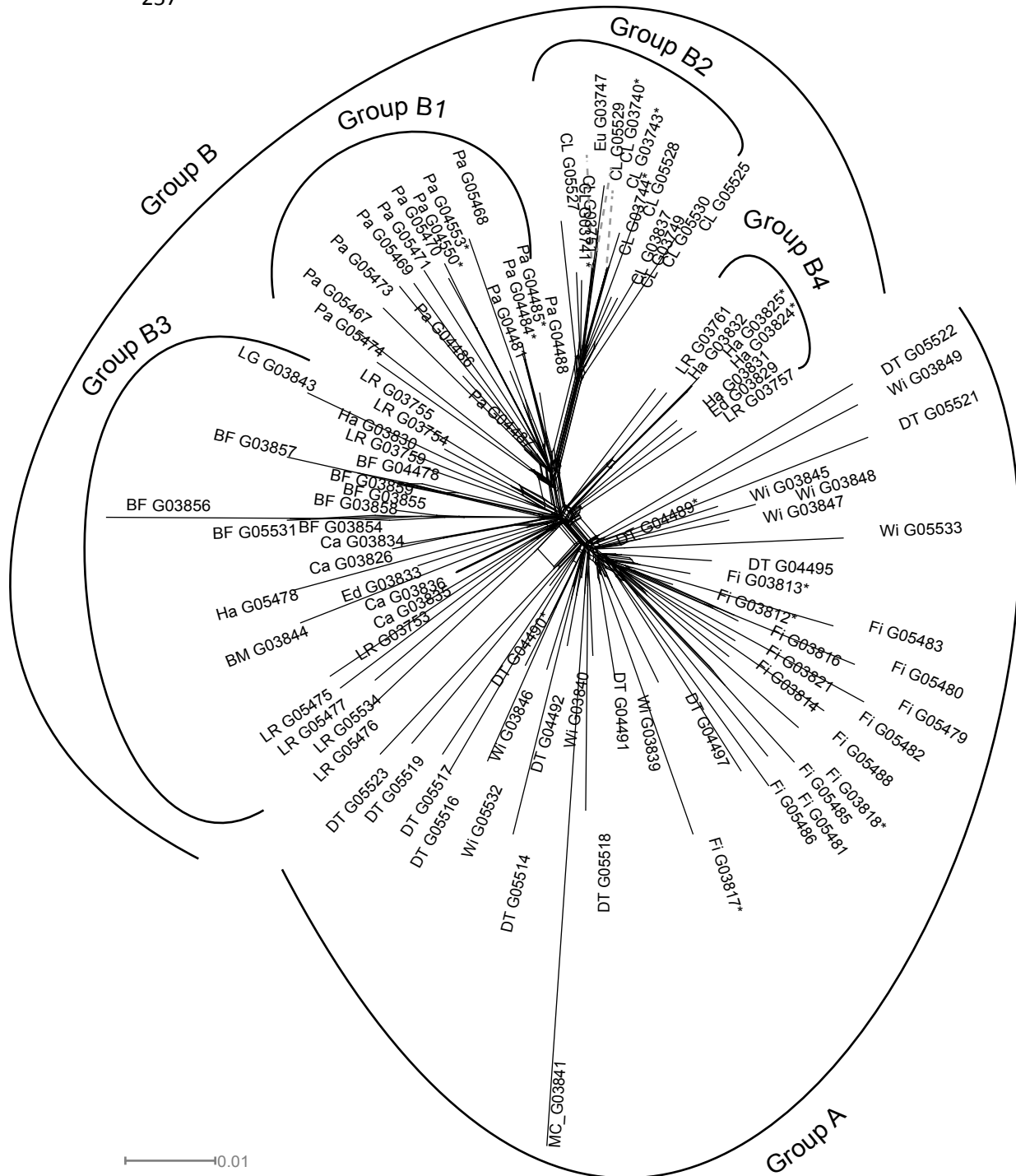
Appendix 4.5 SVD quartet tree showing relationships among 65 individuals of *B. lageniforme* and two outgroup species, based on 17,289 SNPs derived from dataset MS1 including outgroups (one SNP per RAD locus). Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset b. Insert a: Location of the Wet Tropics Bioregion. Insert b: Map of Wet Tropics Bioregion showing sampling locations.



Appendix 4.6 SVD quartet tree showing relationships among 65 individuals of *B. lageniforme* and two outgroup species, based on 4,128 SNPs derived from dataset MS8 including outgroups (one SNP per RAD locus). Replicate samples are indicated with an asterisk. Bootstrap support values greater than 50% are shown above branches. Clades are colour coded according to sampling locations shown in inset b. Insert a: Location of the Wet Tropics Bioregion. Insert b: Map of Wet Tropics Bioregion showing sampling locations.

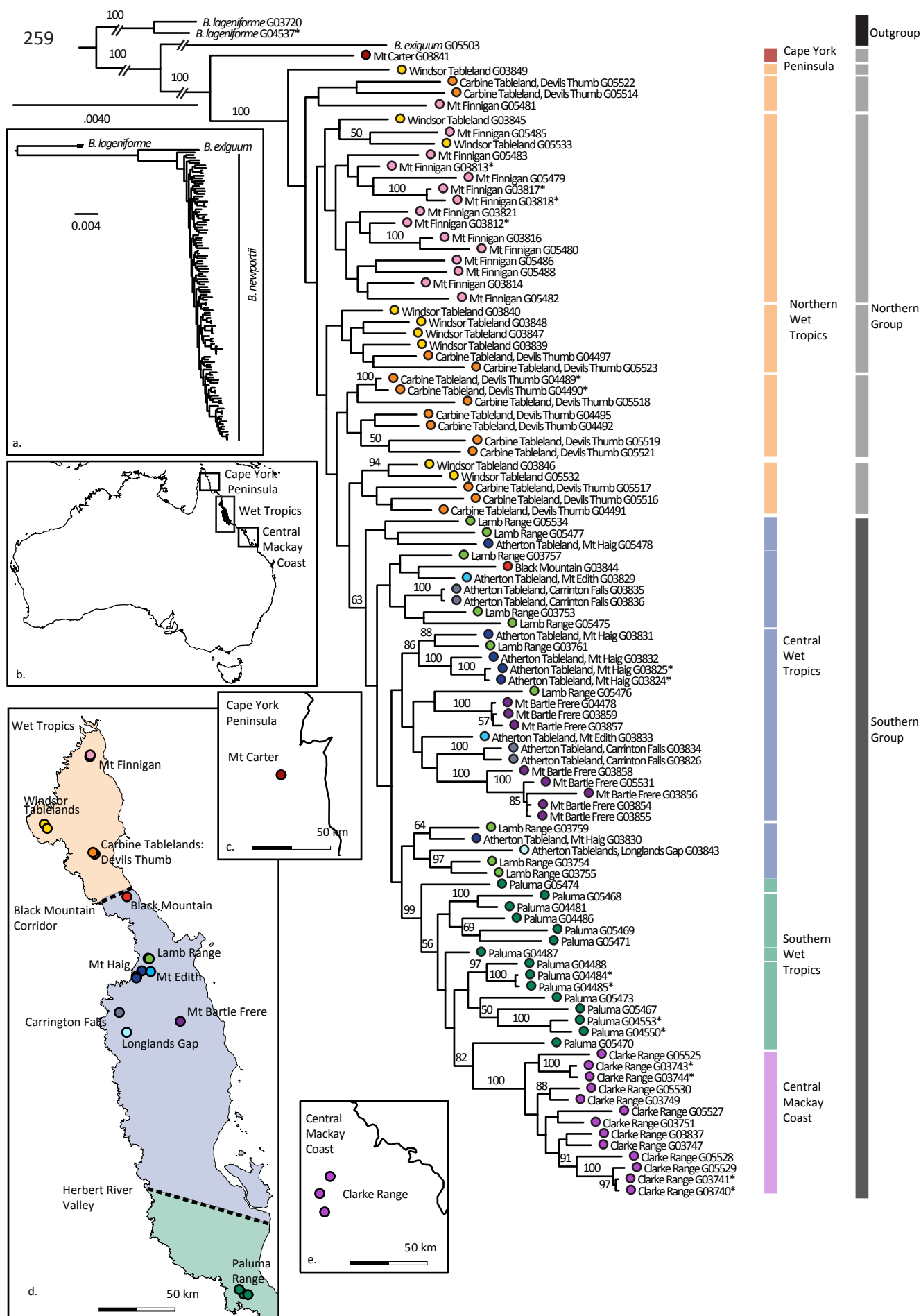
Appendix 4.7 ΔK values determined for the number of genetic groups (K) based on the second order rate of change of the likelihood function with respect to K.

K	Nreps	deltaK	estLnProbMean	estLnProbStdev	lnPK	lnPPK
2	10	0	-163861.03	1250.7	0	0
3	10	1.599	-162401.74	1472.327	1459.29	2354.217
4	9	6.914	-163296.67	2427.657	-894.927	16783.787
5	10	1.4	-180975.38	47777.999	- 17678.713	66908.564
6	7	0	-265562.66	161742.759	- 84587.277	0

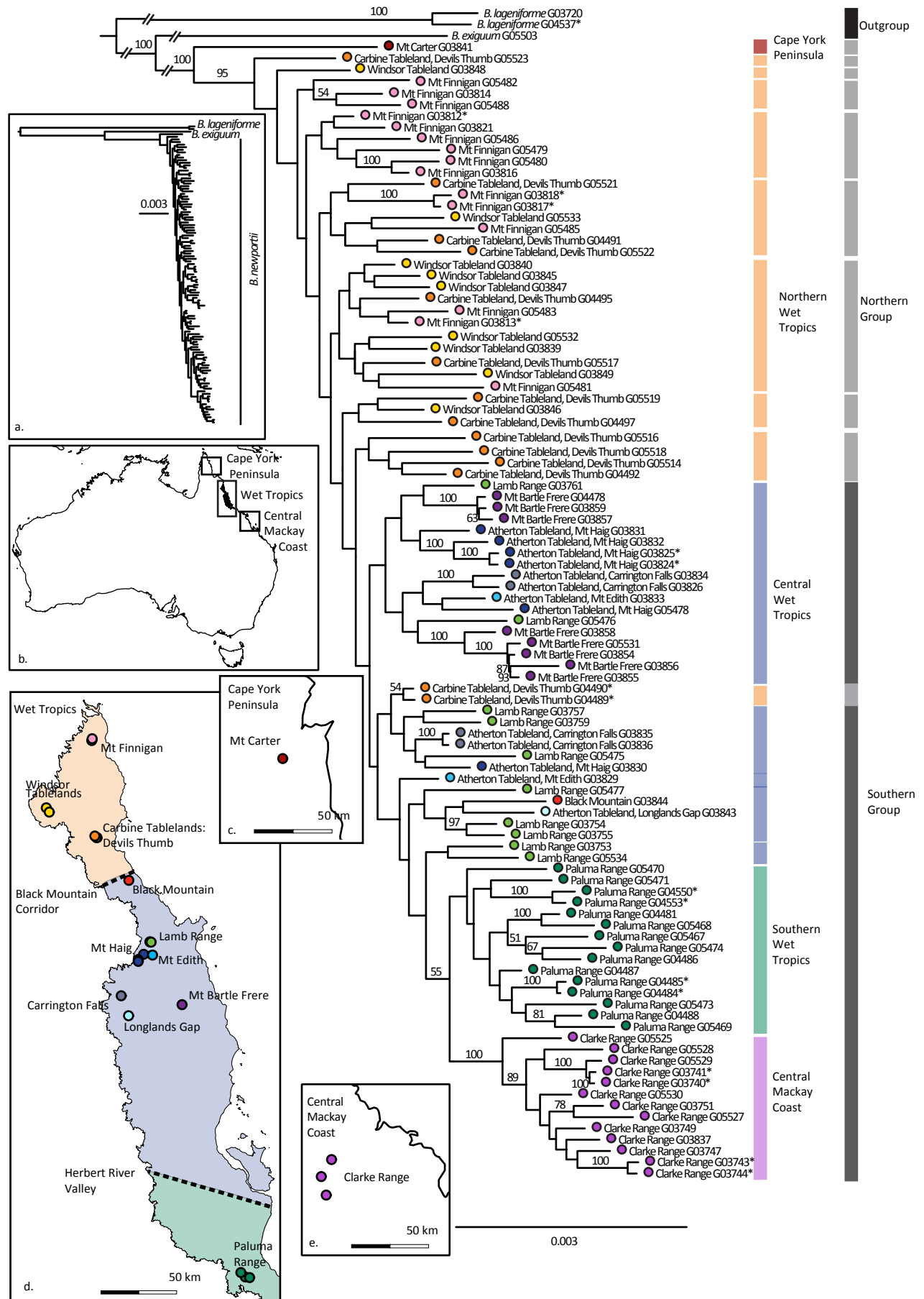


Appendix 5.1 NeighborNet phylogenetic network of 91 *B. newportii* individuals. These results are based on uncorrected P distances derived from 42,897 SNPs contained in dataset MS1 excluding outgroups. Replicate samples are indicated with an asterisk. BF: Mt Bartle Frere, BM: Black Mountain, Ca: Carrington Falls, CR: Clarke Range, DT: Devils Thumb, Ed: Mt Edith, Fi: Mt Finnigan, Ha: Mt Haig, LR: Lamb Range, MC: Mt Carter, LG: Longlands Gap, Pa: Paluma Range, Wi: Windsor Tableland.

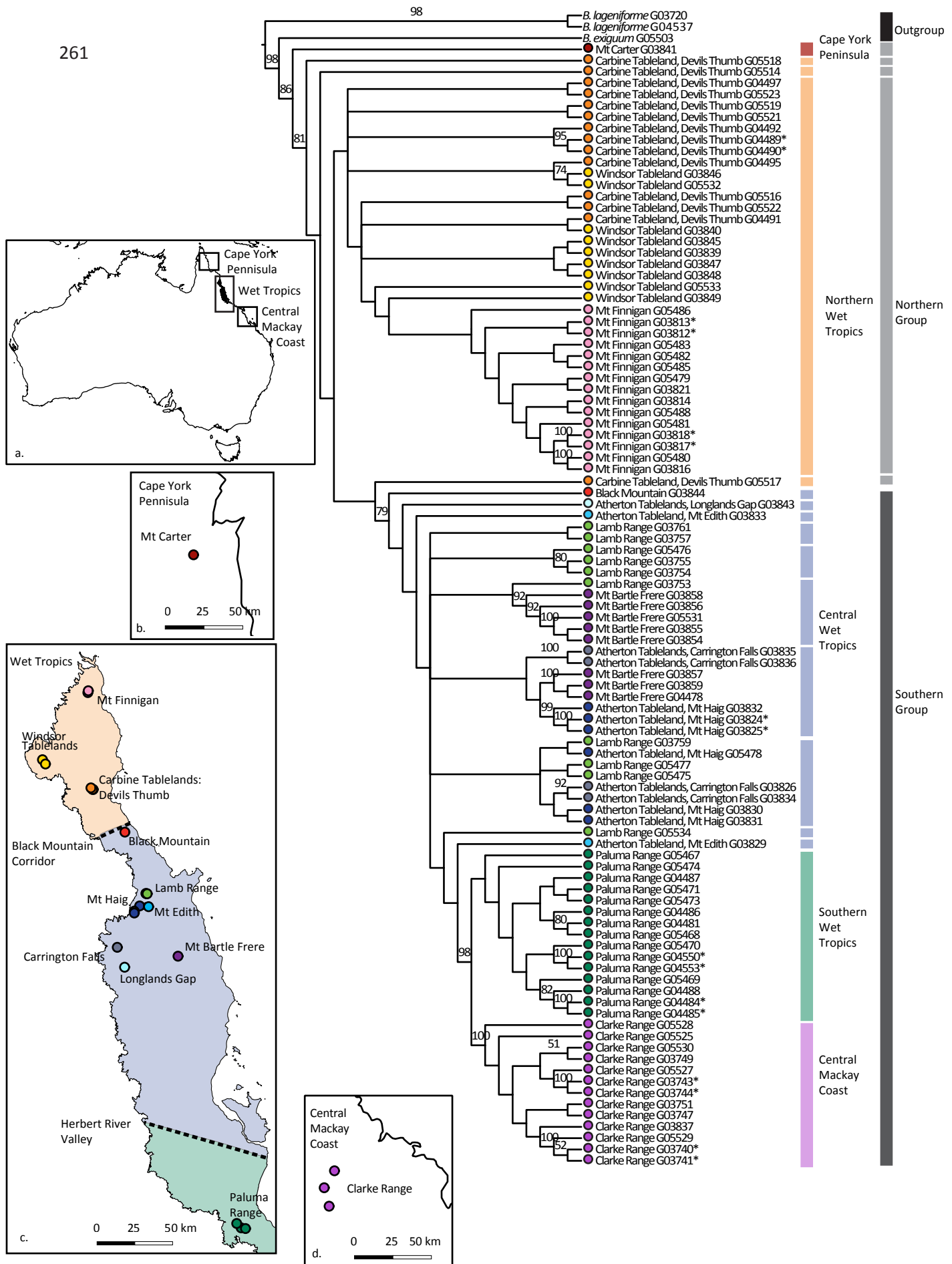
Appendix 5.2 NeighborNet phylogenetic network of 91 *B. newportii* individuals. These results are based on uncorrected P distances derived from 13,077 SNPs contained in dataset MS8 excluding outgroups. Replicate samples are indicated with an asterisk. BF: Mt Bartle Frere, BM: Black Mountain, Ca: Carrington Falls, CR: Clarke Range, DT: Devils Thumb, Ed: Mt Edith, Fi: Mt Finnigan, Ha: Mt Haig, LR: Lamb Range, MC: Mt Carter, LG: Longlands Gap, Pa: Paluma Range, Wi: Windsor Tableland.



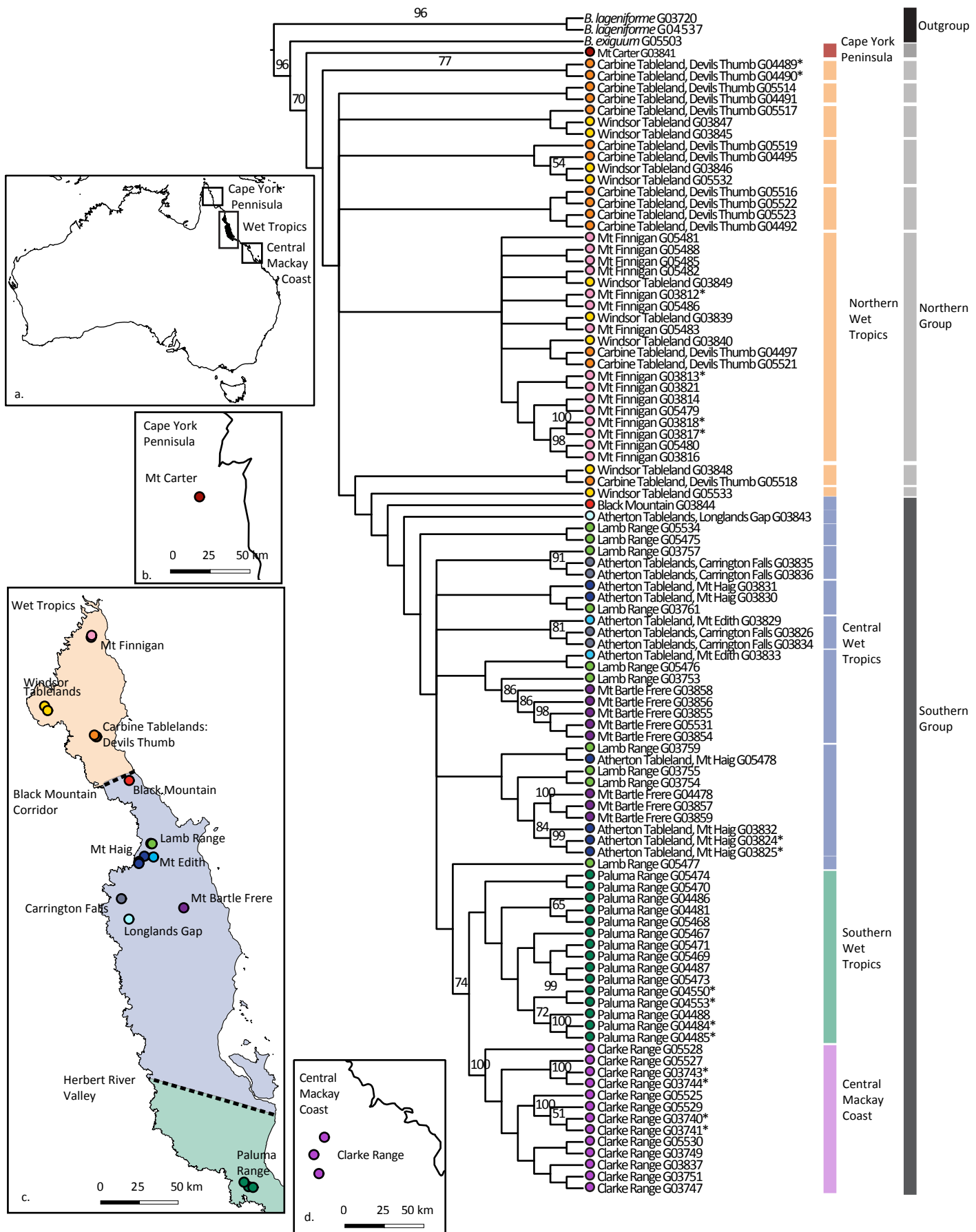
Appendix 5.3 Maximum likelihood phylogenetic reconstruction of 91 *B. newportii* individuals based on 7,010 concatenated RAD loci from dataset MS1 including outgroups. Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Map of Australia showing location of the Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset c: Map showing Mt Carter in the Cape York Peninsula bioregion. Inset d: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset e: Map showing Clarke Range in the Central Mackay Coast Bioregion.



Appendix 5.4 Maximum likelihood phylogenetic reconstruction of 91 *B. newportii* individuals based on 1, 476 concatenated RAD loci from dataset MS8 including outgroups. Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Maximum likelihood phylogenetic reconstruction showing divergence between species. Inset b: Map of Australia showing location of the Cape York Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset c: Map showing Mt Carter in the Cape York Cape York Peninsula bioregion. Inset d: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset e: Map showing Clarke Range in the Central Mackay Coast Bioregion.



Appendix 5.5 SVD quartet tree of 91 *B. newportii* individuals based on 7,010 SNPs derived from dataset MS1 including outgroups (one SNP per locus). Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Map of Australia showing location of the Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset b: Map showing Mt Carter in the Cape York Peninsula bioregion. Inset c: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset d: Map showing Clarke Range in the Central Mackay Coast Bioregion.



Appendix 5.6 SVD quartet tree of 91 *B. newportii* individuals based on 1,476 SNPs derived from dataset MS8 including outgroups (one SNP per locus). Replicate samples are indicated with an asterisk. Bootstrap values greater than 50% are shown above branches. Inset a: Map of Australia showing location of the Cape York Peninsula, Wet Tropics and Central Mackay Coast Bioregions. Inset b: Map showing Mt Carter in the Cape York Peninsula bioregion. Inset c: Map showing mountaintops in the northern, central and southern Wet Tropics regions. Inset d: Map showing Clarke Range in the Central Mackay Coast Bioregion.

Appendix 5.7 ΔK values determined for the number of genetic groups (K) based on the second order rate of change of the likelihood function with respect to K.

K	Nreps	deltaK	estLnProbMean	estLnProbStdev	lnPK	lnPPK
2	10	0	-111863.65	2051.815	0	0
3	10	0.717	-111431	2082.21	432.65	1493.72
4	10	2.888	-109504.63	2847.069	1926.37	8221.53
5	10	0	-115799.79	21020.276	-6295.16	0

