

Phylogeny of *Eremophila* and tribe Myoporeae (Scrophulariaceae)

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A thesis submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

May 2018

School of Biosciences

Faculty of Science

The University of Melbourne



Floral diversity in tribe Myoporeae (left to right, top to bottom): *Eremophila crenulata*, *E. obliquisepala*, *E. latrobei*, *Glycocystis beckerii*, *E. laanii*, *E. gibossa*, *E. fallax*, *E. cuneifolia*, *E. fraseri*, *E. caespitosa*, *Myoporum parvifolium*, *E. maculata*, *E. dempsteri*, *E. duttonii*, *E. calorhabdos*, *E. freelingii*, *E. alternifolia*, *M. bateae*, *E. oppositifolia*, *E. muelleriana*, *E. alternifolia*, *E. subfloccosa*. All photographs taken by Rachael Fowler.

Abstract

Myoporeae is one of eight tribes recognised in the large, cosmopolitan plant family Scrophulariaceae *sensu stricto*. Tribe Myoporeae contains seven genera, four of which are endemic to Australia (*Calamphoreus*, *Diocirea*, *Eremophila*, *Glycocystis*), and the remaining three are distributed in the Caribbean (*Bontia*); Southern China and Japan (*Pentacoelium*); and throughout Australia, islands of the Pacific, Hawaii and Mauritius (*Myoporum*). The largest of these genera, *Eremophila*, contains over 220 species and is a major component of Australia's largest biome, the Eremean (arid) zone. A monograph of the tribe was completed just over a decade ago (Chinnock, 2007), which provided an extensive and comprehensive taxonomic framework from which to explore the relationships and evolutionary history of the group. The first phylogenetic study of the Myoporeae (Kelchner, 2003) used two chloroplast markers to better understand generic and species level relationships in the tribe, however, due to a lack of phylogenetic resolution, the results were inconclusive.

The aim of this thesis was to generate a comprehensive molecular phylogeny of tribe Myoporeae, utilising the capabilities of high throughput sequencing (HTS) technology. A genome skimming approach was implemented using a custom in-house method of library preparation, to allow for inclusion of the large number of samples required for the study. All three plant genomes (chloroplast, nuclear, mitochondrial) were represented using the genome skimming method, allowing for comparisons to be made between phylogenetic analyses of each genomic dataset.

Entire chloroplast genomes (cpDNA) were assembled for 317 taxa, resulting in a well resolved and highly supported phylogeny (see Chapter Three). All allied genera were found to be nested in a paraphyletic *Eremophila*, with high levels of support. Chinnock's (2007) sectional classification of *Eremophila* was only partially supported, with many of the 25 sections scattered throughout numerous clades. For the majority of species included with more than a single sample, a lack of monophyly was observed, which is largely attributed to the effects of introgressive hybridization, incomplete lineage sorting, and/or inappropriate species boundaries.

In Chapter Four the entire nuclear ribosomal cistron (nrDNA) was assembled for 355 taxa, then analysed to produce a moderately supported phylogeny. This phylogeny was largely congruent with the morphology-based taxonomy of the group, though differed markedly from the cpDNA phylogeny of Chapter Three. From a generic perspective, all allied genera were still nested in a polyphyletic *Eremophila*, while Chinnock's (2007) sectional classification was better supported by monophyletic lineages (though still in need of revision). An increase in species rank monophyly was also observed relative to the cpDNA analysis, indicating that at least for some species, introgressive hybridization is likely to impact the chloroplast phylogenetic signal.

In Chapter Five the mitochondrial genome (mtDNA) was explored, and five regions selected for analysis across a subset of 31 taxa in Myoporeae. The size and prevalence of structural rearrangement within the tribe meant assembly of entire mitogenome(s) was not feasible. Regions selected for analysis displayed low levels of variation, allowing for a moderately well-resolved phylogeny, mostly congruent with the nuclear ribosomal phylogeny of Chapter Four.

Overall, construction and comparison of the three genomes in this study allowed for robust interpretation and increased understanding of the complexity in the evolutionary history and phylogenetic relationships of taxa in tribe Myoporeae.

Taxonomic revision is needed at generic and sectional levels; however these changes will not be undertaken until further nuclear sequence data allows the relationships of taxa at the basal nodes of the nuclear phylogeny to be resolved. Aside from future taxonomic work, it is anticipated that this study will inform new research on the tribe Myoporeae, including the chemistry of Myoporeae (for pharmacological application); the study of plant:insect interactions between Myoporeae and members of the insect family Miridae; and biogeographic study of Australia's Eremean zone.

Declaration and statement of authorship

This is to certify that:

1. This thesis comprises only my original work towards the PhD.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is fewer than 100,000 words, exclusive of tables, maps, bibliographies and appendices.

A handwritten signature in black ink, appearing to read 'Rachael Marlow Fowler', with a stylized, cursive script.

Rachael Marlow Fowler

Acknowledgements

This research was funded by the Jim Ross PhD Studentship (Cybec Foundation in conjunction with Royal Botanic Gardens Victoria), Helen McLellan Research Grant and the Australian Conservation Taxonomy Award, and was supported by the Alfred Nicholas Fellowship, The University of Melbourne Botany Foundation, the Australian Biological Resources Study student travel award (STG15-09), International Botanical Congress student travel award and University of Melbourne School of BioSciences – Science Abroad Travelling Scholarship.

I would like to take the opportunity to thank the many people who have contributed to my research both directly and indirectly over the course of my PhD.

First up, to both of my supervisors; Mike Bayly and Dan Murphy. Mike, I started in your lab with little botanic experience and having never heard of *Eremophila*, but the Sys lab now feels like my second home and I can't imagine venturing back to zoology! You have given me incredible freedom in all aspects of my research but always seem available when I pop past your office. I really appreciate the time, advice and guidance that you've given me over these past few years, and am looking forward to the next stages of *Eremophila* research.

Dan, I've known and worked with you for years now, at the RBG, during my Honours and now my PhD, and I can genuinely say I've enjoyed every moment. You are always available when I need guidance (PhD related or otherwise) and I consider you a great role model and mentor. Thank you for all your time and thorough draft edits, and I look forward to continuing working with you on interesting research into the future.

I would like to thank all current and previous members of the Systematics lab for making me feel so at home. To Todd McLay, Erin Batty, Cat Clowes, Tanja Schuster, Steph Conway, Will Neale, Mike Whitehead, Gill Brown, Karen Muscat, Rose Barrett, Alice Crowe, Daniel Ohlsen, Emma Lewis, Jo Birch, Bee Gunn, Javier Aju, Kia Matley, Ai Trann, Heather Merrylees, Beau Picking, Grace Boxshall, Sue Zhang, Megan Rixon, Kathy Vohs, I can honestly say I look forward to coming into work every day and a large part of that has been you guys ☺ Chatting with everyone over tea or in corridors has helped keep me sane and I consider so many of you my good mates.

Todd – I can't imagine how I would have got through my PhD without you. I can't thank you enough for being the best office/PhD mate I could have asked for; chatting through research problems, lab problems, general life problems, you've been my sounding board and best mate. I can't wait to see the awesome research you continue with on Hibisceae and beyond.

Erin and Steph, the first few years wouldn't have been the same without you! I've learnt so much from both of you and hope one day we're all back in the same city to enjoy a regular after work wine.

Karen, Rose, Alice, I've had so many good life chats with you ladies over the years and feel like I have so much in common with all of you. Thank you for your friendship and advice.

Tanja, we started our positions at similar times and have shared so much over the years!! I have found our work together really rewarding and I think you understand some of the most

challenging moments of our time in the lab more than anyone! Thank you for your assistance, guidance, general life advice (and regular egg drop-offs).

Cat, wow lady what would I have done without you?!? I think we probably drove Todd mad in the early days with our regular morning catch up chats! You really have been such an inspiration, your passion, drive and ability to dig in and get work done (all while juggling a million other things, family/work/reno's/etc) is awe inspiring. Can't wait to see where you get with your research, I'm looking forward to working alongside you well into the future.

Will and Jo, you guys are the best! Wish I had gotten to know you so much earlier in the picture. Far out Jo, maybe we could have finished months earlier?! Can't wait to hang once this madness is over.

To Pauline, Gary and Andrew – you have all been incredible role models for myself and all other members of the lab. Thank you for all your advice, and the opportunities that you have helped open up for me.

I would like to thank Heroen Verbruggen and other members of the algae lab (Jo Costa, Chris Jackson, Vanessa Rossetto, Margaret Brookes) for their advice, technical assistance and opening up their lab and equipment for me to use. I really appreciate it, access to Nightowl really was a game changer for my research – thank you for your trust and generosity.

To Josquin Tibbits, thank you for providing us with your genome skimming protocol and being accessible to answer my questions as I set up our lab for the protocol. I couldn't have achieved my results and the sheer amounts of data that ultimately my PhD has rested upon.

I would particularly like to thank Stephen Wilcox from the Walter and Eliza Hall Institute for his ongoing technical support and advice surrounding all things library preparation and sequencing. You went above and beyond in allowing our entire lab to access your equipment and always treat each sequencing run so personally. I cannot thank you enough.

I would like to thank a large number of people from the *Eremophila* community for their practical advice, ideas and help in collecting the large number of species needed for my research. I would particularly like to thank Bevan and Glenda Buirchell for their time in taking me out in remote WA for collections, for talking me through *Eremophila* identification, sharing knowledge of the group and welcoming me into their home. To Ron and Claire Dadd for their hospitality, kindness and access to their incredible *Eremophila* garden (without which many of my collections could not have been made). To Russell Waite, Peter and Rhonda Hall, Ian Tranter and many others who have sent me material and kept me in the loop with the *Eremophila* community.

A number of botanic gardens opened up their collections to me including the Australian National Botanic Garden (Canberra), Royal Botanic Garden of Victoria (Melbourne), Botanic Gardens of South Australia and State Herbarium (Adelaide), Australian Arid Lands Botanic Garden (Port Augusta), Olive Pink Botanic Garden (Alice Springs), Kings Park and Botanic Garden (Perth), Fairchild Tropical Botanic Garden (Florida), Norfolk Island National Park (Natural Resource Manager Melinda Wilson). And to individuals who sent me specimens including Jennifer Tait, Jason Cantley, Timothy Gallaher, Dr Chiou-Rong, Dr Tang and staff of the Joseph F. Rock Herbarium in Hawaii.

Thanks to Bob Chinnock and Scot Kelchner, your seminal work on *Eremophila* provided me with the best of frameworks to extend our knowledge of the group from a molecular perspective. I appreciate your time, advice and openness to help throughout my project.

To Lizzie Nielson, for reaching out to start the fruitful collaboration between our labs focusing on *Eremophila*. I really appreciate your generosity in time, advice, and I'm looking forward to working closely with your Danish lab well into the future.

To my friends in Melbourne and afar for sticking around, checking in and remaining interested as my world gradually shrank to be filled with my research. Thanks for knowing when to ask 'How's it going?' and when to ignore my PhD altogether, for getting me out of the house, for the many rounds, and for just generally being really fun, interesting people that I want to hang out with. I've had to cut short or say no to so many dinners/events/celebrations over the years but you keep asking me along anyway. I can't wait to join you again properly soon.

Finally, the biggest thanks is for my family for their unconditional support, dinners, mini-breaks at the farm and for reminding me that there's more to life than my PhD! Especially thanks to Mum and Dad for emphasising the wonder of nature as I grew up and making me believe I can achieve anything I set my mind to. I promise this is it on the study front.

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Key to abbreviations used in this thesis

°C	Degrees Celsius
µL	microlitre
A	adenine, DNA nucleotide
ACT	Australian Capital Territory
AIC	Akaike information criterion
AUD	Australian dollar
BI	Bayesian inference
BME	B-mercaptoethanol
bp	basepair, nucleotide
BSA	bovine serum albumin
c.	circa, approximately
CDS	coding DNA sequence
cpDNA	chloroplast DNA
CTAB	cetyl trimethylammonium bromide
DNA	deoxynucleic acid
ETS	external transcribed spacer
Gb	giga base pairs
GB	gigabyte
HTS	high throughout sequencing
ICN	The International Code of Nomenclature for Algae, Fungi and Plants
ILS	incomplete lineage sorting
IR	inverted repeat, chloroplast
ITS	internal transcribed spacer
kbp	kilo base pairs
km	kilometre
LSC	large single copy region, chloroplast
m	meters
M	molar
ma	million years ago
Mb	mega base pairs
MCMC	Markov chain Monte Carlo
mg	milligram
mL	millilitre
mM	millimolar
mm	millimetre
MP	maximum parsimony
mtDNA	mitochondrial DNA
NaCl	Sodium Chloride
ng	nanogram
NGS	next generation sequencing
nrDNA	nuclear ribosomal DNA
NSW	New South Wales
NT	Northern Territory
NTS	non-transcribed spacer region
PCR	polymerase chain reaction
PEG	Polyethylene Glycol 8000

PIC	parsimony informative character
pp	posterior probability value
QLD	Queensland
qPCR	quantitative polymerase chain reaction
R1 or R2	Read one or two of an Illumina sequence output
RNA	ribonucleic acid
rpl	revolutions per minute
RRL	reduced representation library
rRNA	ribosomal ribonucleic acid
SA	South Australia
sect.	section, of <i>Eremophila</i>
sp.	species
SSC	small single copy region, chloroplast
STE	Sodium chloride-Tris-EDTA
subsp.; ssp.	sub-species
tRNA	transfer ribonucleic acid
unpub.	unpublished
VIC	Victoria
WA	Western Australia
WEHI	Walter and Eliza Hall Institute

Colour coded key to sections of *Eremophila*

	<i>Platycarpus</i>
	<i>Pholidiopsis</i>
	<i>Crustaceae</i>
	<i>Austraophilae</i>
	<i>Chamaepogonia</i>
	<i>Duttonia</i>
	<i>Arenariae</i>
	<i>Eremaeae</i>
	<i>Carnosae</i>
	<i>Platycalyx</i>
	<i>Eremophila</i>
	<i>Platychilus</i>
	<i>Pholidia</i>
	<i>Sentis</i>
	<i>Decussatae</i>
	<i>Hygrophanae</i>
	<i>Contortae</i>
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	<i>Subscissocarpae</i>
	<i>Amphichilus</i>
	<i>Stenochilus</i>
	<i>Virides</i>
	<i>Scariosepalae</i>
	<i>Pulchrisepalae</i>

Chapter One: Introduction

1.1 Introduction to tribe Myoporeae

Tribe Myoporeae is a member of the predominately southern hemisphere distributed plant family Scrophulariaceae (order Lamiales). Originally circumscribed at the rank of family (Myoporaceae), Myoporeae was recircumscribed as a tribe within Scrophulariaceae *sensu stricto* based upon a number of molecular analyses of the traditionally large family Scrophulariaceae *sensu lato* (Olmstead and Reeves, 1995; Olmstead et al 2001; Oxelman et al., 2005). Further molecular analyses have confirmed the central American tribe Leucophylleae, containing the three genera *Capraria* L., *Leucophyllum* Humb. & Bonpl. and *Eremogeton* Standl. & L.O.Williams, to be the sister group to tribe Myoporeae (Oxelman et al., 2005; Gandara and Sosa 2013). Other core members of Scrophulariaceae *sensu stricto* include tribes Aptosimeae (mostly endemic to Africa includes *Anticharis* Endl., *Aptosimum*, *Peliostomum*), Buddlejaceae (worldwide distribution includes *Buddleja*, *Gomphostigma*, *Nicodemia*), Hemimerideae (mostly South African includes *Alonsoa*, *Diascia*, *Nemesia*), Limoselleae (globally distributed includes *Hebenstretia*, *Limosella*, *Phyllopodium*), Scrophularieae (globally distributed includes *Antherothamnus*, *Scrophularia*, *Verbascum*) and Teedieae (South African distribution includes *Oftia*, *Phygellus*, *Teedia*) (Oxelman et al., 2005; Tank et al., 2006).

Tribe Myoporeae consists of seven genera, distributed largely through Australia and the Pacific region (see Table 1.1). By far the largest of these genera is *Eremophila*, currently consisting of 233 species, 58 subspecies and a large number of putative taxa awaiting formal description (Chinnock, 2007; Brown and Buirchell, 2007, 2011; Chinnock and Doley, 2011; Edgington, 2015; Brown and Davis, 2016; Buirchell and Brown; 2016). Commonly known as emu bush, poverty bush or native fuchsia, *Eremophila* species are endemic to Australia, with distributions spanning the arid and semi-arid zones of the continent. The next largest genus within Myoporeae is *Myoporum*, consisting of 30 species, 17 of which are found in Australia and the other 13 distributed throughout New Zealand, the islands of the Pacific, Hawaii and Mauritius (Chinnock, 2007). In Australia, *Myoporum* species are commonly known as boobialla and are generally distributed in more temperate, coastal habitats. The remaining members of Myoporeae are the small

Western Australian genus *Diocirea* (four species), the monotypic genera *Calamphoreus* and *Glycocystis* (both endemic to Western Australian), *Bontia* (Bahamas and Caribbean), and *Pentacoelium* (Southern China and Japan) (Chinnock, 2007).

Table 1.1 Genera of tribe Myoporeae based on Chinnock (2007), Brown and Buirchell (2007, 2011), Chinnock and Doley (2011), Edgington (2015), Brown and Davis (2016) and Buirchell and Brown (2016).

Genus	Described species - Australia	Described species - outside of Australia	Undescribed species (estimate)	Total taxa
<i>Eremophila</i>	233 (c. 58 subspecies)	0	c. 60	c. 350
<i>Myoporum</i>	17 (2 subspecies)	13 (2 subspecies)	0 known	34
<i>Calamphoreus</i>	1	0	0 known	1
<i>Diocirea</i>	4	0	0 known	4
<i>Glycocystis</i>	1	0	0 known	1
<i>Bontia</i>	0	1	0 known	1
<i>Pentacoelium</i>	0	1	0 known	1
Total				390+

1.2 Distribution of genera

The following is a list detailing species distributions of all genera in tribe Myoporeae.

Eremophila All species of *Eremophila* are endemic to Australia, and for the most part, distributed in the arid and semi-arid regions of the continent (see Figure 1.1). Species are found in all states and territories except Tasmania and the Australian Capital Territory (ACT). The majority of *Eremophila* species are found in Western Australia (i.e. 208 (c. 90%) of the currently described c. 233 species). *Eremophilas* grow in a range of vegetation types (mulga scrub and woodlands, mallee woodlands, hummock grasslands), and on a wide variety of substrates, including; rocky cliffs, stony plains, sandy loams, red loams, cracking clays, silty loams and sand dunes (Kelchner, 2003; Chinnock, 2007). Individual species distributions vary considerably within *Eremophila*, a large proportion of species are considered narrow endemics, found in a single or small number of locations on a particular substrate. Other species are more widely distributed, and a handful (*E. deserti*, *E. gilesii*, *E. bignoniiflora*, *E. latrobei*, *E. longifolia*, *E. maculata*, *E. glabra*) display widespread, cross-continental distributions.

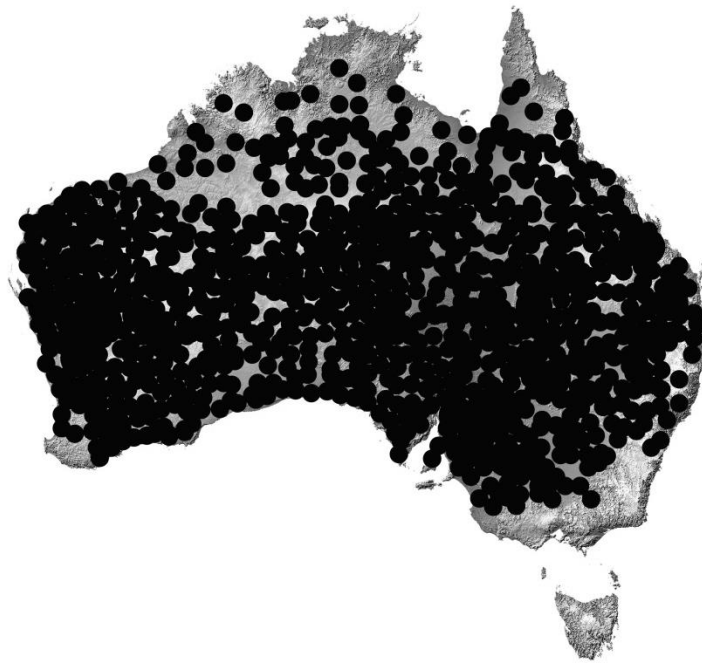


Figure 1.1 Distribution of *Eremophila* adapted from The Australasian Virtual Herbarium (as at 04/01/17).

Myoporum As the second largest genus of the tribe, *Myoporum* is by far the most widely distributed (see Figure 1.2). Seventeen of the thirty species occur in Australia, with the remaining species distributed throughout the Pacific Islands, New Zealand, New Caledonia, Norfolk Island, Hawaii and the Indian Ocean islands of Mauritius and Rodrigues (Chinnock, 2007). The majority of *Myoporum* species are salt tolerant (Chinnock, 2007) and grow in coastal, temperate environments on sandy calcareous soils or soils derived from volcanic or sedimentary rock (Kelchner, 2003; Chinnock, 2007).

Bontia The single species in this genus, *Bontia daphnoides*, has a widespread distribution throughout islands of the Caribbean-Bahamas, including Cuba, Turks and Caicos Islands, Hispanola, Puerto Rico, Virgin Islands, Jamaica, Cayman and Swan Islands and the Lesser Antilles (see Figure 1.2). *Bontia* occurs in coastal forest and scrub habitat, along sandy shores and is salt tolerant (Chinnock, 2007).

Pentacoelium The single species in this genus, *Pentacoelium bontioides*, is restricted to coastal areas of southern Japan, southern China, Taiwan and Hong Kong (see Figure 1.2). *Pentacoelium* occurs above the water line in mangrove-like habitats (Chinnock, 2007).

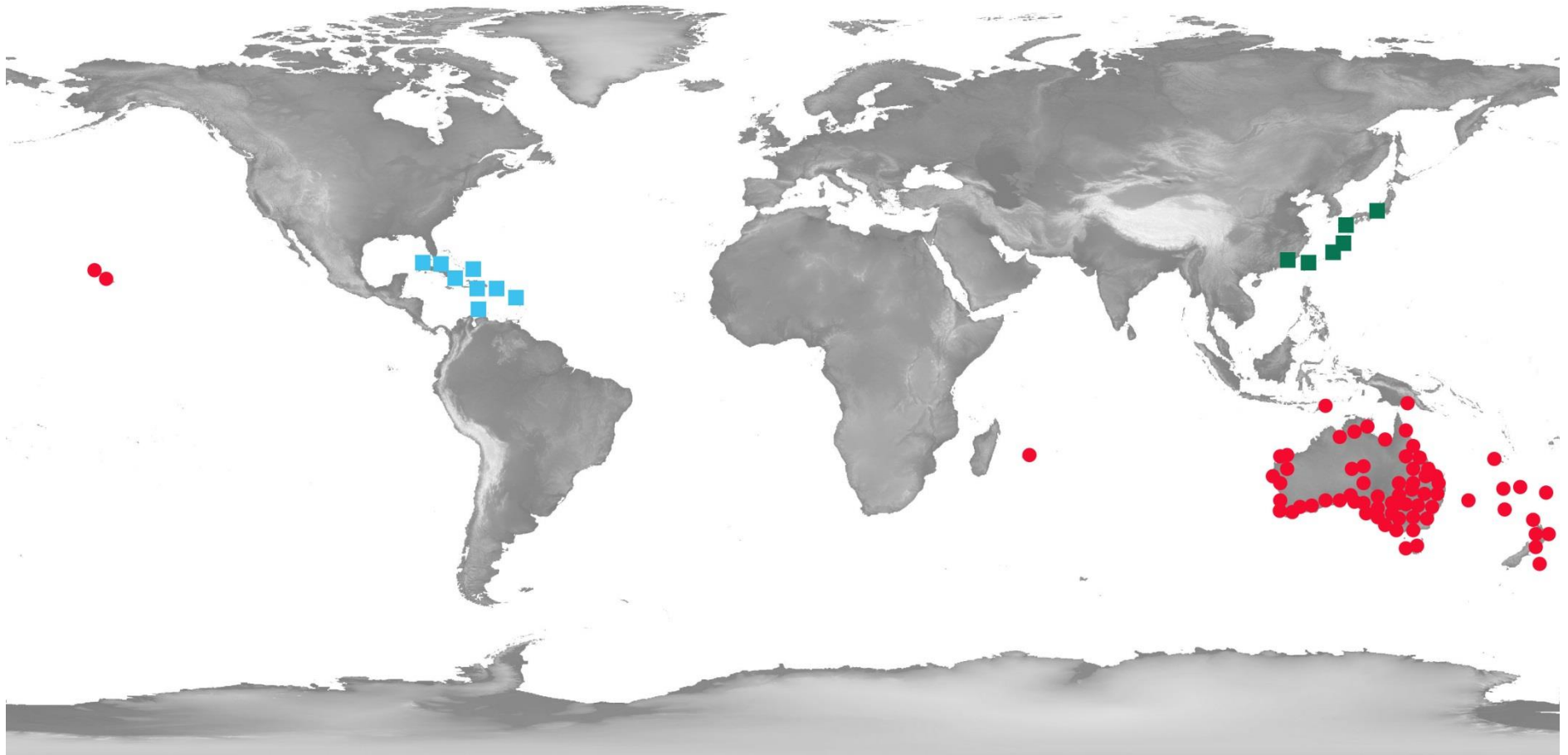


Figure 1.2 Distributions of *Myoporum* ● , *Bontia* ■ and *Pentacoelium* ■ adapted from The Australasian Virtual Herbarium (as at 04/01/17) and Chinnock (2007).

Glycocystis The single species in this genus, *Glycocystis beckeri*, is restricted to south-western Western Australia, particularly surrounding the Ravensthorpe area and a disjunct population south east of Balladonia (see Figure 1.3). *Glycocystis* is found on sandy soils, under low *Eucalyptus* woodland or mallee heath (Chinnock, 2007).

Calamphoreus The single species in this genus, *Calamphoreus inflatus*, is restricted to south-western Western Australia, from Marvel Loch South to Lake King (see Figure 1.3). *Calamphoreus* is thought to be a disturbance opportunist, often found on roadsides adjacent to *Eucalyptus* woodland in clay loams (Chinnock, 2007).

Diocirea The four species in genus *Diocirea* are all restricted to south-western Western Australia, spanning from Coolgardie to Grass Patch and East towards Balladonia (see Figure 1.3). *Diocirea* is found in *Eucalyptus* woodlands in red-brown clay loams, and is often associated with species of *Eremophila* (Chinnock, 2007).

1.3 Features of tribe Myoporeae

Members of tribe Myoporeae display substantial diversity in terms of plant habit and vegetative morphology. Species range from prostrate, creeping shrubs to erect woody shrubs and small trees (see Figures 1.4 and 1.5). A prominent feature of the tribe is the presence of resin-filled vesicles in vegetative and reproductive tissue, (for which the tribe is named, *Myo* – to close/shut, - *poros*, opening/pore), which often results in a viscid or hard resinous surface layer (Kelchner, 2003; Chinnock, 2007). Corolla structure is extremely variable due to pollinator adaptation (Chinnock, 2007), but the number of sepals/corolla lobes is usually 5, and the number of stamens 4 (–5). The corolla ranges from almost actinomorphic to strongly zygomorphic, bilabiate, campanulate or urceolate; and the majority of flowers are bisexual (Chinnock, 2007). Flowers range in colour from white through mauve, purple, blue, red, orange, carmine, yellow and green. The gynoecium in Myoporeae typically consists of 2 carpels, containing 1–3 ovules in parallel rows, and fruit are dry or drupaceous. Members of *Myoporum*, *Pentacoelium*, *Diocirea*, *Glycocystis*, *Calamphoreus* and c. 80% of *Eremophila* species are considered to be insect-pollinated, while *Bontia* and the remaining c. 20% of *Eremophila* species are bird-pollinated (Chinnock, 2007). Chromosome counts for a subset of species in Myoporeae indicate a general trend of $n=18$, however polyploidy ($n=36, 54, 68, 109$) appears to be relatively common, with many species exhibiting

multiple polyploid races associated with distinct geographic patterns (Barlow, 1971; Ey and Barlow, 1972).

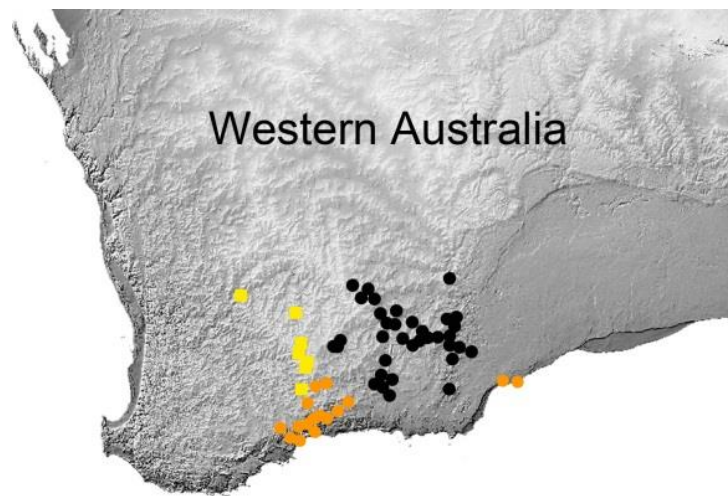


Figure 1.3 Distributions of *Glycocystis* ●, *Calamphoreus* ■ and *Diocirea* ●. Adapted from The Australasian Virtual Herbarium (as at 04/01/2017).



Figure 1.4 Diversity of plant habit (top to bottom, left to right): prostrate creeping shrub *Eremophila biserrata*; erect shrub (1-4 m) *Eremophila fraseri*; large shrub to small tree (up to 10 m) *Myoporum platycarpum* subsp. *platycarpum*.

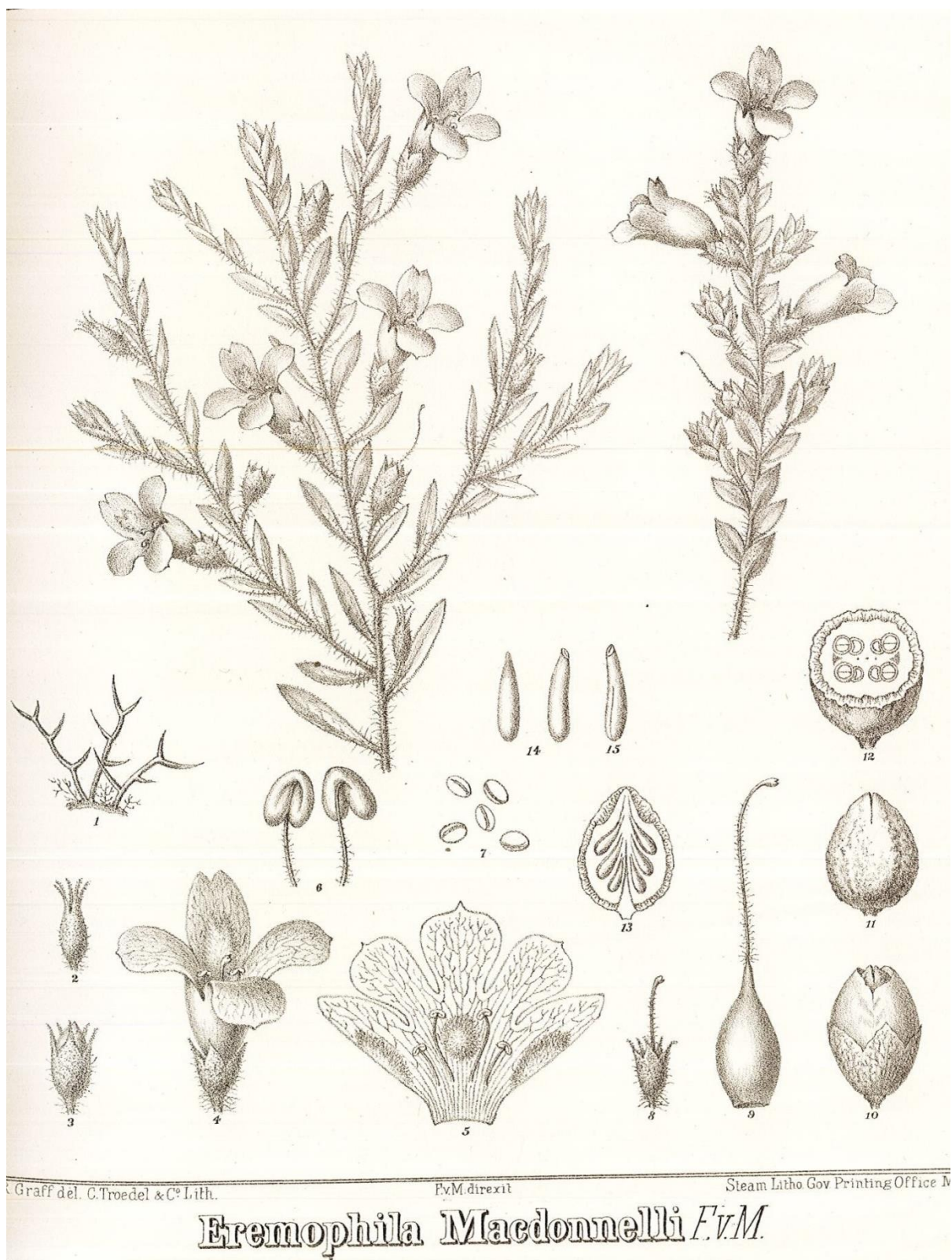


Figure 1.5 Illustration of *Eremophila macdonnellii* originally published in Mueller (1886), including 1. Branched hairs; 2. Flower bud; 3. Unexpanded flower; 4. Expanded flower; 5. Corolla laid open; 6. Stamens (front and back); 7. Pollen grains; 8. Calyx with pistil; 9. Style and ovary; 10. Fruit bearing calyx; 11. Side view of fruit; 12. Transverse section of fruit; 13. Longitudinal section of fruit; 14. Seeds; 15. Embryo.

1.4 Chemistry and traditional uses of tribe Myoporeae

Species of *Eremophila* and *Myoporum* have played important roles for Aboriginal Australians, who have traditionally used them for their medicinal properties, but also during ceremonial and cultural traditions (Richmond and Ghisalberti, 1994; Richmond and Ghisalberti, 1995; Chinnock, 2007). Medicinally, *E. alternifolia* and *E. longifolia* are recorded as being the most significant species of *Eremophila* used by Aboriginal people, and up to 18 species have documented traditional uses (Richmond, 1993; Richmond and Ghisalberti, 1994). Medicinal preparations vary between species and for different ailments, but include smoke inhalation and steeping branches, leaves or roots in water, and mixing with animal fats to produce ointments; and can either be applied topically, or ingested (Richmond and Ghisalberti, 1994; Latz, 1995). In comparison to *Eremophila*, species of *Myoporum* were less utilized for medicinal purposes; however, *M. montanum* is documented as a headache cure, and in New Zealand, *M. laetum* has been used by Maori as a treatment for wounds and toothache (Richmond and Ghisalberti, 1995).

Neither *Eremophila* nor *Myoporum* species are widely used as a source of Aboriginal food, though nectar from flowers of *E. freelingii* and *E. latrobei* is drunk as a delicacy, and the fleshy fruit of some myoporums consumed as famine food (Richmond, 1993). The wood of *Myoporum* is used to craft spears and shields and the sticky resin used for fastening tools. Both *Myoporum* and *Eremophila* species are known to produce significant quantities of essential oils, and both groups have been implicated in stock poisonings (Sadgrove et al., 2013; Sadgrove and Jones, 2014).

More recently, interest has grown in the use of *Eremophila* for modern medicinal purposes, with phytochemical and pharmacological studies revealing *Eremophila* species to contain over 200 secondary metabolites and exhibit antimicrobial, antiviral, anti-inflammatory and immunomodulatory properties (Ndi et al., 2007; Anakok et al., 2011; Beattie et al., 2011; Sadgrove et al., 2013; Singab et al., 2013). A handful of species screened have also been identified to contain diterpenoid compounds recognised for the therapeutic management of type II diabetes (Tahtah et al., 2016; Wubshet et al., 2016). *Eremophila* has been flagged as a medically important genus with significant prospects in conventional medicine, and collaborative research linked to, but not included in, this PhD study is currently being undertaken with the Plant Biochemistry

Laboratory at the University of Copenhagen. It is anticipated that results from the current phylogenetic study of *Eremophila* and tribe Myoporeae will directly inform the inclusion of *Eremophila* species targeted in ongoing chemical screenings and more detailed chemical analyses.

1.5 Conservation status

Three non-Australian species from tribe Myoporeae are listed on the International Union for Conservation of Nature (IUCN) red list of threatened species; *Myoporum rapense*, *M. rimatarense*, *M. stokesii*, with *M. stokesii* considered critically endangered. A number of members of tribe Myoporeae are considered threatened under the Australian Government's *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). According to the Australian Federal Department of Environment and Energy (as at 09/05/2018) four species of Myoporeae are considered vulnerable, 13 endangered and three critically endangered on the Species Profile and Threats Database (see Appendix One). In addition to this federal level threatened species listing, state level legislation in Western Australia, South Australia, Queensland and Victoria also includes taxa from Myoporeae on their own threatened species lists. Under the State Government of Western Australia's *Wildlife Conservation Act 1950*, 22 taxa have been listed under the Wildlife Conservation (Rare Flora) Notice 2018 (Western Australian Department of Parks and Wildlife) as vulnerable, endangered or critically endangered in the state of Western Australia (see Appendix Two). In addition to these taxa, a further 108 taxa have been given a priority listing (see Appendix Three). Priority listing is for taxa that are considered rare, threatened or near threatened by the Western Australian Department of Parks and Wildlife; however, insufficient data exist to make an accurate assessment. These taxa include members from four genera within Myoporeae: *Calamphoreus*, *Diocirea*, *Eremophila* and *Myoporum* and constitutes a significant proportion of species in Myoporeae.

In South Australia, the following ten species are listed as rare under the National Parks and Wildlife Act 1972 (Version 15.3.2017): *Eremophila praecox*, *E. barbata*, *E. gibbifolia*, *E. hillii*, *E. interstans* subsp. *interstans*, *E. parvifolia*, *E. pentaptera*, *E. polyclada*, *E. subfloccosa* subsp. *glandulosa*, and *Myoporum parvifolium*. In Queensland, two species of *Eremophila* (*E. stenophylla* and *E. tetraaptera*) are listed as vulnerable in the Nature

Conservation (Wildlife) Regulation 2006 under the *Nature Conservation Act 1992* (as at 09/05/2018). While in Victoria, 13 taxa are listed as rare or threatened under the *Advisory List of Rare or Threatened Plants in Victoria 2014* (see Appendix Four). A number of these species (highlighted in bold) are also formally listed as threatened under the Victorian state legislation *Flora and Fauna Guarantee Act 1988*.

Overall, a significant proportion of taxa, particularly within *Eremophila*, are listed on federal and state level legislation as being rare and/or threatened. In particular, in Western Australia, where the highest number of species occur (and are often narrow endemics), it is significant that 24 putative species and six putative subspecies of *Eremophila* awaiting formal description have been designated a priority code under the Threatened and Priority Flora List 2018. Given the significant number of species listed as threatened, rare or vulnerable, the current research may provide insight into management of particular (arid zone) regions and species, as well as contribute to a greater understanding of currently undescribed taxa.

1.6 Taxonomic history

The first species formally described within tribe Myoporeae, as currently circumscribed, was *Bontia daphnoides* by Carl Linnaeus in 1753, though earlier descriptions and drawings of *Bontia* are recorded from as far back as 1694 (Chinnock 2007). *Myoporum* was described by Georg Foster in 1786, based on four species collected in New Zealand (*M. laetum*, *M. pubescens*) and New Caledonia (*M. crassifolium*, *M. tenuifolium*), although he attributed the names to the earlier unpublished work, of Joseph Banks and Daniel Solander on Captain Cook's first voyage to New Zealand (Chinnock 2007), i.e., with the author citation for the genus commonly given as "Banks & Sol. ex G.Forst.". It was not until 1810 that Robert Brown first described the order Myoporinae, which included three new genera, *Pholidia*, *Stenochilus* and *Eremophila*, plus existing genera *Myoporum* and *Avicennia*. Over the last 200 years, the number of genera within Myoporeae has fluctuated considerably throughout various iterations and revisions at the generic and sub-generic levels, (for a full review and revision see Chinnock 2007). By 1885, the largest contribution to the understanding and discovery of Myoporaceae was made by Ferdinand von Mueller, describing an additional 53 species and three new genera, *Pholidiopsis*, *Duttonia* and *Sentis*. Prior to the most recent revision of the group by

Chinnock (2007), four genera were recognised (*Eremophila*, *Myoporum*, *Pholidia* and *Stenochilus*) encompassing approximately 130 species.

1.7 Current classification of Myoporeae (Chinnock, 2007)

The most recent and comprehensive taxonomic treatment of Myoporeae was conducted by Bob Chinnock (2007). Chinnock established three new genera (*Calamphoreus*, *Diocirea* and *Glycocystis*), described 95 new species (from within *Calamphoreus*, *Diocirea*, *Glycocystis*, *Eremophila* and *Myoporum*), 46 new subspecies (from within *Eremophila*), and revised the infrageneric classification of *Eremophila* and *Myoporum*. Chinnock proposed that *Eremophila* be divided into 25 sections and *Myoporum* into 4 sections, based on vegetative, floral and fruit morphology (see Appendix Five for all species and sections in tribe Myoporeae). Upon establishing the new genera, Chinnock hypothesised that the four endemic Australian genera (*Eremophila*, *Calamphoreus*, *Diocirea* and *Glycocystis*) and Australian members of *Myoporum* be more closely related to one another than to *Bontia*, *Pentacoelium* and non-Australian Myoporums (see Figure 1.6 for Chinnock's hypothesised generic relationships).

From an infrageneric perspective, Chinnock divided *Eremophila* into sections as 'no well-defined discontinuities could be found to subdivide it into subgenera'. Throughout his monograph Chinnock suggests relationships between what he predicts to be closely related species. However, no formal phylogenetic analysis has been conducted to determine the relationships, both among Chinnock's proposed sections, or among species within these sections, and Chinnock's notions of relationship were not explicitly based on consideration of potentially apomorphic character states. Since Chinnock's revision, 18 additional species and one subspecies of *Eremophila* have been formally described; *Eremophila densifolia* subsp. *erecta*, *E. grandiflora*, *E. koobabbiensis*, *E. woodiae*, *E. buirchellii*, *E. calcicola*, *E. ballythunnensis*, *E. capricornica*, *E. daddii*, *E. ferricola*, *E. hamulata*, *E. jamesiorum*, *E. laccata*, *E. pusilliflora*, *E. regia*, *E. resiliens*, *E. scrobiculata*, *E. victoriae* and *E. yinnetharrensensis* (Brown and Buirchell 2007; Chinnock and Doley 2011; Edgington 2015; Brown and Davis 2016; Buirchell and Brown 2016).

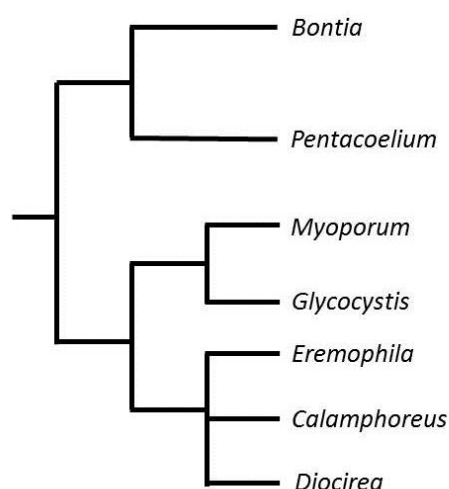


Figure 1.6 Cladogram of generic relationships of Myoporeae inferred from statements made by Chinnock (2007) based on morphology-based estimation of the tribe.

1.8 Revision of family status

In his monograph, Chinnock (2007) maintained Myoporaceae at the rank of family; however, the publication of a number of molecular studies has called this classification into question. A preliminary examination of the monophyly of Scrophulariaceae, using chloroplast genes *rbcL* and *ndhF*, revealed the group to be polyphyletic, with two distinct clades emerging that contained traditional elements of Scrophulariaceae (Olmstead and Reeves, 1995). A single member of Myoporaceae (*Myoporum mauritianum*) was included in this phylogeny, and was placed as sister to one of the core Scrophulariaceae clades identified (which included *Scrophularia*, *Verbascum*, *Buddleja*). A more comprehensive follow-up to this study by Olmstead et al. (2001) used three chloroplast genes (*rbcL*, *ndhF* and *rps2*) to further clarify the major lineages within Scrophulariaceae and relationships between families in Lamiales. In this phylogeny, Myoporaceae is strongly supported as belonging to Scrophulariaceae *sensu stricto*, and forms a well-supported clade with Central American species *Leucophyllum frutescens* (see Figure 1.7). Oxelman et al. (2005) further elaborated on this classification, with the inclusion of additional taxa and chloroplast markers, including the protein coding gene *rps16* in conjunction with *rbcL* and *ndhF* for phylogenetic reconstruction. Oxelman et al., (2005) further supported the classification of Myoporaceae as a tribe (Myoporeae) within Scrophulariaceae, and with the inclusion of multiple members of Myoporeae (*Myoporum*, *Eremophila* and *Bontia*) and

Leucophylleae (*Leucophyllum* and *Capraria*), also supported their monophyly and sister relationship status (see Figure 1.8).

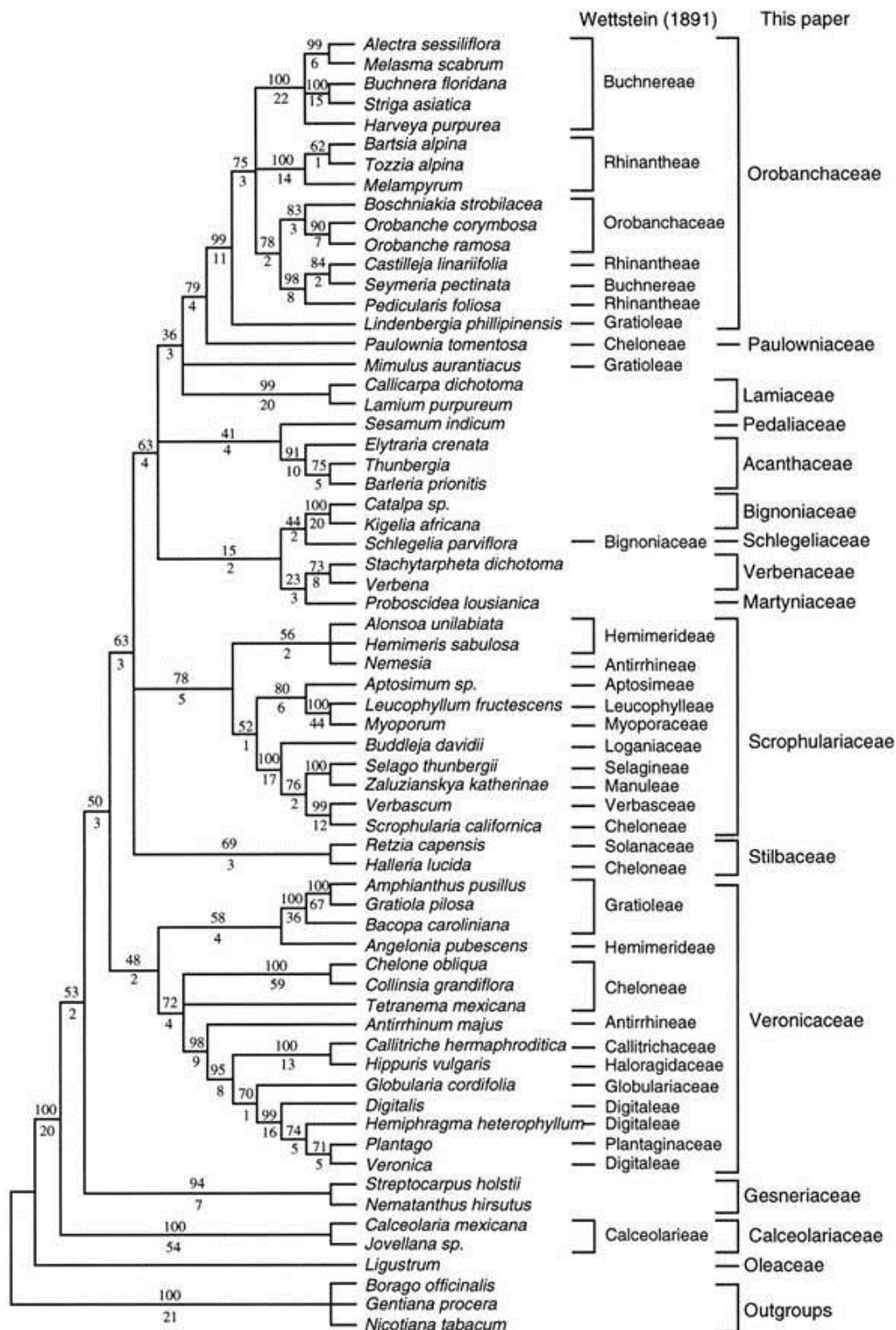


Figure 1.7: Strict consensus of Maximum Parsimony analysis (240 trees) based on three plastid genes (*rbcl*, *ndhF*, *rps2*) as published by Olmstead et al. (2001; their Figure 2). Bootstrap values recorded above branches, decay values recorded below.

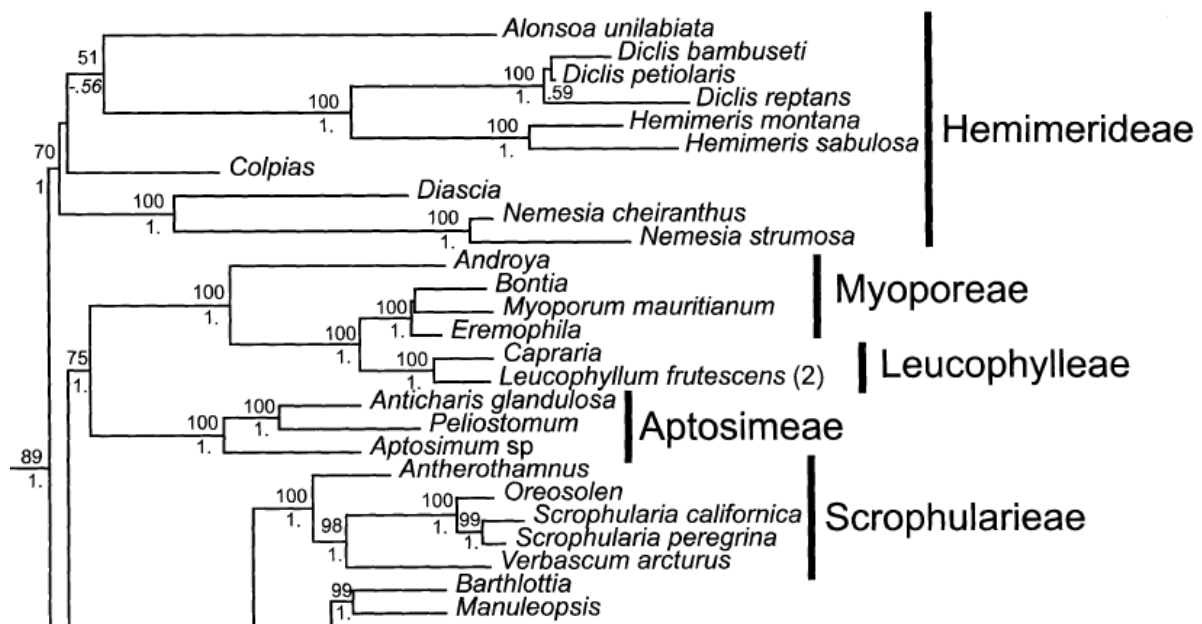


Figure 1.8: Phylogram of one of the most parsimonious trees produced by Oxelman et al. (2005; adapted from their Figure 3), based on plastid markers *rbcL*, *ndhF*, *rps16*. This shows a highly supported sister relationship between tribes Myoporeae and Leucophylleae. Bootstrap values shown above branches, posterior probabilities shown below.

1.9 Sister relationship: tribe Myoporeae and tribe Leucophylleae

Following the publication of the molecular studies outlined above, Chinnock (2007) lent his support to the treatment of Myoporaceae as a tribe within Scrophulariaceae, though questioned the validity of combining tribes Myoporeae and Leucophylleae, as suggested by a number of morphological studies and the phylogeny generated by Olmstead et al. (2001). A recent study by Gandara and Sosa (2013) focused specifically on the monophyly and placement of tribe Leucophylleae in relation to Myoporeae and the wider Scrophulariaceae, and confirmed a distinct and well supported sister clade relationship between Myoporeae and Leucophylleae (posterior probability value =1, bootstrap support =100) using two chloroplast regions (*trnL-F*, *rps16*) and the nuclear internal transcribed spacer (ITS) regions.

1.10 Phylogenetic research within tribe Myoporeae

To date, molecular-based research on relationships within tribe Myoporeae has been limited. While the placement of tribe Myoporeae as a clade within Scrophulariaceae is widely accepted (discussed above), few studies have sought to resolve the relationships between, and within, the seven genera in the tribe. The most significant of these studies

was conducted by Scot Kelchner (2003, PhD thesis unpub.) at The Australian National University. Kelchner's study was the first to assess the evolutionary history of Myoporeae using molecular data to construct a phylogeny. Two non-coding regions from the chloroplast genome (*rpl16* intron and *trnT-trnL* intergenic spacer) and a morphological dataset were collected and analysed from 61 taxa (56 species) representing six of seven genera in Myoporeae. Phylogenetic analysis of the morphological dataset produced a highly unresolved tree with minimal bootstrap support, and when combined with molecular datasets reduced the confidence of subsequent phylogenetic estimation, so was omitted from the final total evidence phylogeny. The two chloroplast DNA datasets were combined in a single data matrix and analysed using Parsimony, Maximum Likelihood and Bayesian Inference. The final dataset included 2143 DNA characters, of which 116 were parsimony informative. Strict consensus trees of the Equal-weight Parsimony analysis (EP) and Weighted Parsimony analysis (WP) were nearly identical, so were combined in a final tree (shown here as Figure 1.9). Overall, this phylogeny supported the grouping of tribe Myoporeae as a well-defined monophyletic lineage; however, the relationships between genera within the tribe remained substantially unresolved or with little bootstrap support. Despite the uncertainty of this phylogeny, the hint of pattern that emerges from this analysis indicates that: 1) tribe Myoporeae is monophyletic; and 2) all genera within the tribe are nested within a paraphyletic *Eremophila*. Three major clades were identified, the first contained a single species, *Eremophila tetraptera*, which appears to be sister to all other Myoporeae sampled. The second major lineage contained members of *Eremophila*, *Bontia* and a large number of *Myoporum* species. The third lineage contained members of *Diocirea*, *Calamphoreus*, *Glycocystis*, *Eremophila* and *Myoporum*. From Kelchner's study it is evident that the inclusion of additional ingroup taxa and additional genetic sequence data are required for a more complete and reliable understanding of the relationships and evolutionary history of this group.

As a follow up to this initial molecular study and under the supervision of Scot Kelchner, Masters student Liu Chang from Idaho State University completed a study focussing specifically on *Myoporum* (2008, thesis unpub.). Chang included 22 of the 30 formally recognised species of *Myoporum* from throughout Australia, New Zealand and islands of the Pacific and Indian Oceans, in addition to eight species representative of *Eremophila*,

Bontia daphnoides, *Pentacoelium bontioides* and two outgroup taxa from *Leucophyllum*. Chang sequenced and analysed six chloroplast loci (*rpl16*, *petD*, *ndhA*, *trnT-trnL*, *trnS-trnG* and *trnD-trnT*) and a single low copy nuclear gene (*waxy*) in order to construct a better resolved phylogeny for the genus and answer questions surrounding the phylogeny and biogeography of *Myoporum*, including how *Myoporum* came to exhibit its current wide distribution. Maximum Parsimony, Maximum Likelihood and Bayesian Inference analyses were conducted separately on the chloroplast and nuclear datasets. The chloroplast phylogeny was relatively well resolved and showed good support values (see Figure 1.10). *Myoporum* was shown to be non-monophyletic, with taxa split across two separate clades. In comparison, the nuclear phylogeny was more problematic to interpret. Multiple copies of the nuclear gene, *waxy*, were recovered for the majority of taxa included in the study. When analysed, different copies from the same individual were often placed in two separate and incongruent clades of the nuclear phylogeny, while variation in copy number was also found between species. Such incongruence between multiple copies of *waxy* within an individual could be the result of gene duplication (i.e. via polyploidy or duplication of a segment of a chromosome), incomplete lineage sorting or hybridization, while variation in the number of copies of *waxy* between taxa suggests that hybridization and/or incomplete lineage sorting may be evident in *Myoporum*, resulting in issues when trying to infer evolutionary history of the group. Chang recommends caution in inferring phylogeny based solely on chloroplast DNA, which due to its uniparental inheritance, may reflect a chloroplast gene tree rather than a potentially more complex species tree.

In combination, the two studies of Kelchner (2003) and Chang (2008) represent the total molecular phylogenetic knowledge of tribe Myoporeae to date. Kelchner's findings (2003) show *Eremophila* to be non-monophyletic, with all other genera nested within at least two of three *Eremophila* dominated clades; however, the phylogeny lacked statistical support. Chang (2008) focused solely on *Myoporum*, her results indicating the genus to be non-monophyletic with likely incongruence between chloroplast and nuclear phylogenies. In light of these studies, our understanding of the evolutionary history of tribe Myoporeae is still uncertain, and relationships between genera within the tribe and between species within genera remained substantially unresolved.

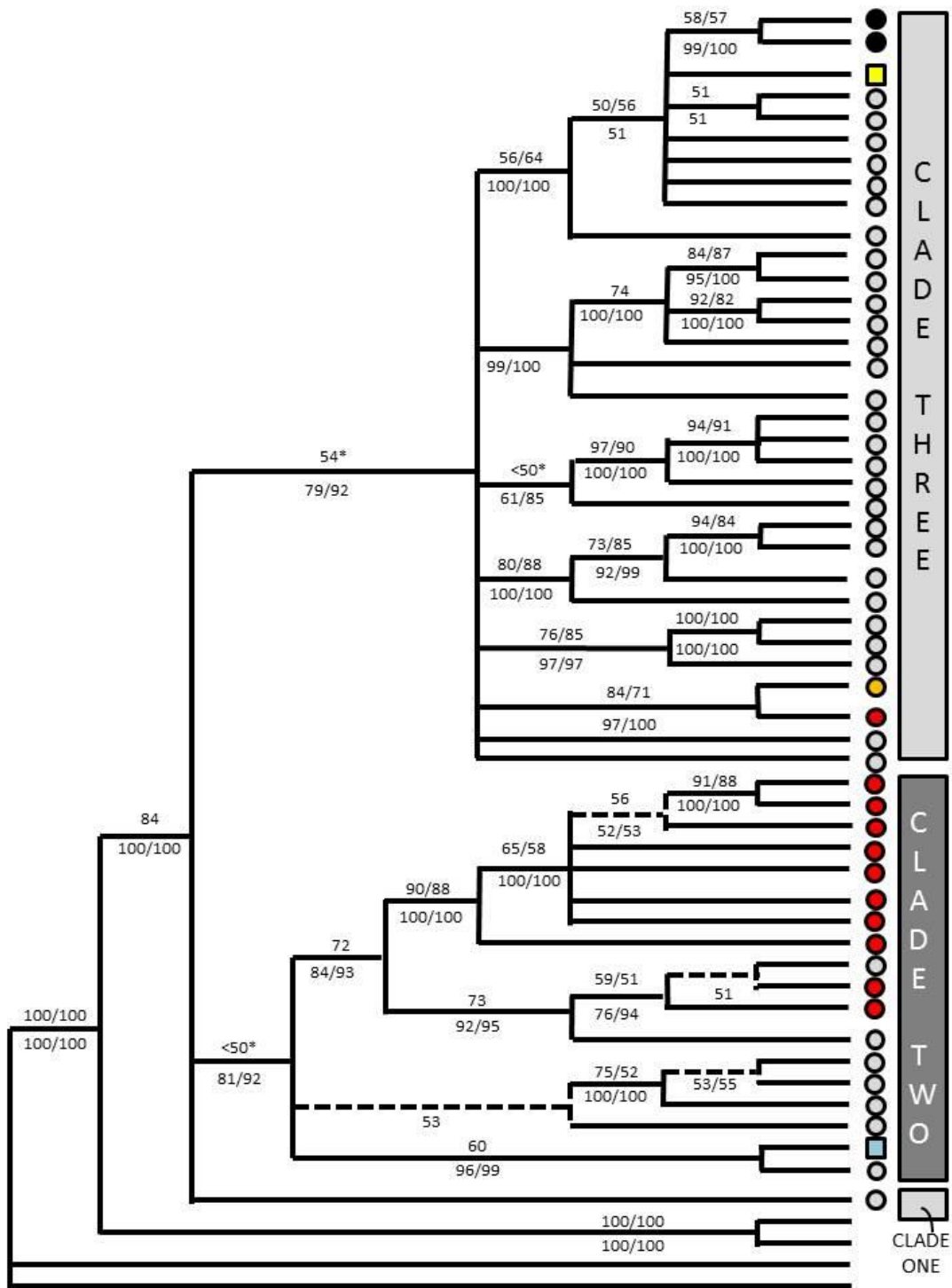


Figure 1.9 Strict consensus from equal-weight parsimony analysis of two chloroplast loci (*rpl16*, *trnT-trnL*) adapted from Figure 3.8 of Kelchner (2003). Values above each branch represent equal-weight parsimony and weighted parsimony bootstrap values respectively, while values below each branch represent 5× and 10× matrix equal-weight bootstrap values respectively. Symbols at terminals represent *Eremophila* ○, *Myoporum* ●, *Glycocystis* ●, *Diocirea* ●, *Bontia* ■ and *Calamphoreus* ■.

Tree length = 257
 Consistency index (CI) = 0.9027
 Retention index (RI) = 0.9464

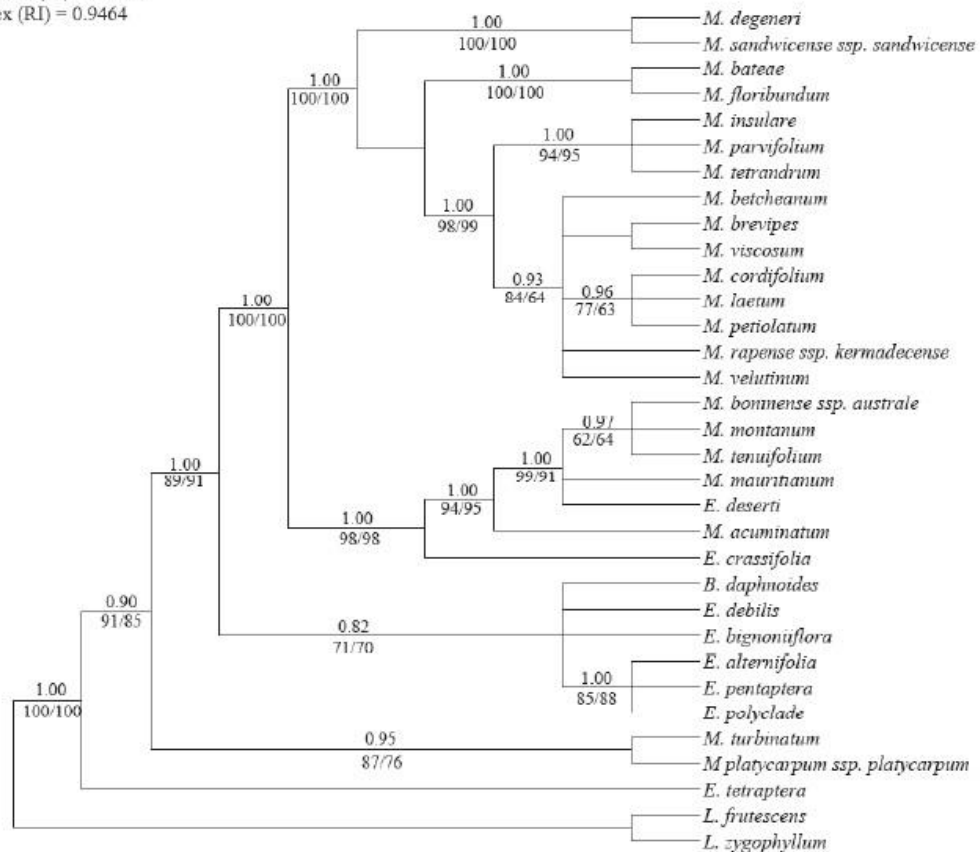


Figure 1.10 Consensus tree of equal weighted maximum parsimony analysis of combined chloroplast loci (*rpl16*, *petD*, *ndhA*, *trnT-trnL*, *trnS-trnG* and *trnD-trnT*) from Chang's thesis (Figure two, 2008). Values above branches represent Bayesian posterior probabilities, values below branches represent Maximum Likelihood and Maximum Parsimony bootstrap values, respectively.

1.11 Application of high throughput sequencing

Until recently, one, to a small handful of molecular markers (totalling hundreds, to thousands of nucleotides) were typically sequenced for use in phylogenetic analyses. In plant systematics, a few molecular markers from the chloroplast genome (i.e. *rbcl*, *matK*, *trnL-F*), have often been used individually, in combination, or in conjunction with the nuclear ribosomal internal transcribed spacer regions (ITS), to infer phylogeny. This type of sequencing was traditionally completed using capillary-based, Sanger biochemistry (Sanger et al., 1977; Shendure and Ji, 2008; Wang et al., 2012). Sanger sequencing was well suited to the synthesis of these kinds of data, because it achieves read lengths of c. 1000 bases, meaning target regions can be sequenced in a single run, and with very high (99.999%) base call accuracy (Shendure and Ji, 2008). It is also

relatively affordable, and the preparation of samples easily implemented in even small scale systematics laboratories. However, in the past 10–15 years, new sequencing technology has advanced rapidly, resulting in the era of High Throughput Sequencing (HTS).

Also known as Next Generation Sequencing (NGS), HTS refers to a wide range of sequencing platforms that produce vast quantities of sequence data for a relatively low per base pair cost. The suite of sequencing platforms that fall under the HTS umbrella include 454 (Roche Sequencing), Illumina (Illumina Inc.), SOLiD (Applied Biosystems), Ion Torrent (ThermoFisher), PacBio (Pacific Biosciences of California), Nanopore (Oxford Nanopore Technologies), GeneReader (Qiagen), and True Single Molecule Sequencing (SeqLL). These platforms differ in specific sequencing strategies and vary widely in the amount of sequence data generated, the length of the fragments of DNA generated, time taken to run, error types and rates, and sequencing costs (Goodwin et al., 2016). These differences in platform specifications make some technologies better suited to specific sequencing applications than others, though what unites them is their ability to sequence multiple samples in parallel, producing hundreds of millions, or in the case of latest Illumina platform HiSeq X, close to a trillion base pairs (900 Gb) of data per sequencing run (Goodwin et al., 2016). For a fraction of the cost of Sanger sequencing, HTS has revolutionised the field of phylogenetics, allowing for the sequencing and assembly of whole plastid genomes, whole mitochondrial genomes, transcriptomes, targeted nuclear regions and even complete nuclear genomes (Rohland and Reich, 2012). Many approaches have been devised to use these data, including targeted sequencing, reduced representation libraries, and as implemented in the current study, genome skimming.

Genome skimming (also known as shotgun sequencing), refers to the sequencing of total genomic DNA (untargeted, unenriched) to a low depth of coverage (Dodsworth, 2015). The concept of genome skimming is relatively recent (Straub et al., 2012) and is reliant on the vast amounts of data generated through HTS technology. In plants, due to the relative proportions of chloroplast, nuclear and mitochondrial genomes, and differences in their size, a genome skimming approach results in variable coverage of each genomic component. High copy regions (i.e. plastid DNA, mitochondrial DNA and the nuclear ribosomal cistron), are all recovered in relatively high numbers, allowing for

complete, or near complete reconstruction, while the majority of the nuclear genome will receive little to no coverage (Straub et al., 2012; Malé et al., 2014). Advantages of genome skimming include:

- 1) Sample (library) preparation is relatively straightforward; during library preparation fragments of DNA are selected at random, eliminating the need to target specific regions (absence of primer/bait development), meaning the process can be easily applied to previously unstudied/non-model organisms. A number of commercial kits are available to complete library preparation, but are significantly more expensive than in-house protocols.
- 2) Samples are sequenced to a shallow depth; due to the size and ratio of each (chloroplast, nuclear, mitochondrial) genome, an average low depth of coverage for the bulk of the nuclear genome (1x or less) still results in high coverage of high copy regions in the genome such as the chloroplast genome, mitochondrial genome and nuclear ribosomal cistron (Straub et al., 2012; Ripma et al., 2014). An average low depth of coverage means multiple samples can be combined (multiplexed) in a single sequencing run, significantly reducing per sample/base sequencing costs.
- 3) Regions of DNA previously targeted for use in plant systematics are automatically captured; traditionally a handful of chloroplast markers in combination with the internal transcribed spacer (ITS) have been used for phylogenetic reconstruction in plants (Olmstead and Palmer, 1994; Schaal et al., 1998; Feliner and Rosselló, 2007; Shaw et al., 2014). As all of these markers occur in high copy numbers in the cell/genome, these regions are captured by default (along with many others), meaning genome skimming data are directly comparable with previous studies, and old datasets can be added to or improved (Williams et al., 2016).
- 4) Ability to access previously unstudied regions of the plant genome; for instance, the mitochondrial genome has historically been understudied in plants, but its occurrence in high copy numbers within the cell mean it is now more easily explored through a skimming approach (Duminil, 2014; Smith, 2016). Other understudied regions such as 5S nuclear ribosomal DNA and the nuclear

ribosomal non-transcribed spacer region (NTS) can also be included in phylogenetic study through genome skimming.

A number of limitations are also recognised in using an HTS genome skimming approach, including;

- 1) Shorter DNA fragment lengths compared to traditional sequencing; while Sanger sequencing produces sequences up to c. 1000 bases in length (depending on the sequencing platform used) a genome skimming approach produces reads of 75–400 base pairs in length. Shorter reads can be problematic when trying to *de-novo* assemble stretches of DNA without a close reference sequence, and/or when assembling stretches of repetitive/poly base DNA sequence (Reuter et al., 2015).
- 2) Lower sequence accuracy; while Sanger sequencing exhibits per base error rates as low as 0.001%, error rates of the latest HTS technology are magnitudes higher (Shendure and Ji, 2008). Illumina sequencing platforms (which hold the largest share of the HTS market) have error rates ranging from 0.1-1% (Reuter et al., 2015; Goodwin et al., 2016).
- 3) Highly informative, low copy nuclear regions remain largely unsequenced; by its very nature, genome skimming excludes the majority of the nuclear genome, in favour of high copy regions. In order to access low copy nuclear genes, a genome skimming approach must be paired with other techniques, i.e. target enrichment (Weitemier et al., 2014).
- 4) Downstream processing of data can be laborious; while the millions (mega base pairs, Mb) to hundreds of millions (giga base pairs, Gb) of base pairs of data are advantageous for phylogenetic reconstruction, storage, processing and analysis become far more complex than dealing with traditional sequencing outputs.

Genome skimming approaches have been successfully applied to growing number of plant systematic studies (Malé et al., 2014; Weitemier et al., 2014; Zhang et al., 2015; Turner et al., 2016; Berger et al., 2017), with the advantages of increased data output and reduced costs outweighing any current limitations of the process.

1.12 Research objectives

As outlined above, Myoporeae is a significant component of Australia's flora, particularly in the arid and semi-arid biomes, yet one that is still relatively unknown from a phylogenetic perspective. Chinnock's (2007) monograph of the tribe is comprehensive and illuminating; however, the evolutionary history of tribe Myoporeae is still uncertain, and the group's classification remains unresolved. In order to determine an evolutionarily meaningful classification of the group, based on both morphological and molecular data, a novel research approach is required to discover phylogenetic relationships. The main aim of my PhD research is to explore the relationships between the species and genera currently classified within tribe Myoporeae using the latest high throughput sequencing technology. Chinnock's (2007) morphological classification of the group provides a solid framework from which phylogenetic hypotheses can be interpreted and tested, particularly surrounding the monophyly of each genus, the infrageneric classification of sections within *Eremophila*, and the exploration of congruence between molecular and morphological datasets. My specific research aims are to:

1. Produce a comprehensive molecular phylogeny for tribe Myoporeae, using both chloroplast and nuclear DNA datasets.
2. Explore the relationships between genera within tribe Myoporeae, testing the monophyly of each genus.
3. Test the limits of Chinnock's (2007) classification of sections within *Eremophila*.
4. Explore the utility of mitochondrial DNA for use in phylogenetic estimation.
5. Investigate the monophyly of species, particularly those with widespread and disjunct distributions.
6. Explore biogeographic patterns of tribe Myoporeae, particularly in the context of *Eremophila* and the aridification of Australia.

Chapter Two: General Methods

This chapter outlines the general methods used across this study. More specific methods relevant to Chapters Three, Four and Five (i.e. genome assembly, statistical analyses) will be elaborated upon in the respective chapters.

2.1 Sampling – collection of plant material

To address the core aims of my PhD study, extensive collection of fresh plant material for members of Myoporeae was required. The high throughput shotgun sequencing approach (details below) requires large quantities of high quality DNA, meaning fresh leaves from sample taxa were critical for DNA extractions. As such, plant material was collected in the field for as many species of *Eremophila*, and other members of Myoporeae, as possible (total individuals sampled = 457). For species with large distributions, multiple accessions were collected from across their range to assess intraspecific diversity. Where possible plant material was collected from natural populations, but due to the large number of species needed, and because of the rarity and conservation status of some species, specimens were also collected from botanic gardens and private plant collections (for a full list of specimens collected and sequenced see Appendix Five).

Botanic Gardens accessed for this research were: Royal Botanic Gardens Victoria; Australian National Botanic Gardens, Canberra; Kings Park and Western Australian Botanic Garden; Adelaide Botanic Garden; Australian Arid Lands Botanic Garden, Port Augusta; Olive Pink Botanic Garden, Alice Springs; and Fairchild Tropical Botanic Garden, Florida. Plant collection permits for public lands were granted through: Western Australian Department of Parks and Wildlife (CE004550, SW015904); South Australian Department of Environment, Water and Natural Resources (Q26312-2); New South Wales Office of Environment and Heritage (SL101210); Forestry Corporation of NSW; Victorian Department of Environment, Land, Water and Planning (10006072, 10007218); Parks Australia and the Australian Federal Director of National Parks (NINP-2016-160817).

Sampling involved collecting fresh leaf tissue (5–10 leaves) which was dried using silica beads and then used subsequently for DNA extraction. For each sample, 1–3 herbarium voucher specimens were collected and are in the process of being accessioned for lodgement at the University of Melbourne Herbarium (MELU). Duplicate specimens will be forwarded to the National Herbarium of Victoria (MEL) and/or the State Herbarium of South Australia (AD). For field collections, additional environmental information was recorded, including; latitude, longitude, elevation and a general site description (soil, other plant species present, vegetation type etc.). All species were identified on site then later verified by *Eremophila* experts Bevan Buirchell or Bob Chinnock.

2.2 DNA extraction

Whole genomic DNA was extracted for all 457 individuals from silica dried leaf tissue, using a modified version of Shepherd and McLay's (2011) cetyl trimethylammonium bromide (CTAB) protocol. This protocol was derived from Doyle and Doyle's (1990) original protocol, and was adapted further for optimal DNA yield and quality for taxa of Myoporeae. Approximately 60 mg of dried leaf tissue was ground manually using a mortar and pestle with a small amount of laboratory grade sand to mechanically lyse cells. The Sodium chloride-Tris-EDTA (STE) buffer was added to each sample (1 mL), vortexed thoroughly, then centrifuged at 5000 rpm for 10 minutes. The supernatant was removed and 600 µL of pre-warmed (65°C) CTAB solution, 2 µL (0.2% v/v) of B-mercaptoethanol (BME), 4 µL (0.4% v/v) of Proteinase K (20 mg/mL) and 110 µL of a 10:1 NaCl (5 M):Bovine serum albumin (BSA, 4%) solution were added to each sample, vortexed, then incubated at 65°C for 1.5–3 hours (quality and yield were suboptimal both sides of this time range). Samples were split equally into two tubes, 650 µL of chloroform added to each tube, then each sample centrifuged at maximum speed for 5 minutes. The top aqueous phase was removed from both tubes of each sample and added to a fresh tube (~400 µL total). The addition of chloroform step was repeated, then 110µL Sodium acetate (pH 5.2, 3 M) added to each sample prior to the addition of 500 µL of room temperature isopropanol. The addition of this Sodium acetate reduced the need for a final clean up by increasing DNA quality while reducing the amount of DNA lost in a traditional isopropanol clean up. Samples were incubated at room temperature for 25 minutes then centrifuged at maximum speed for 15 minutes. The resulting DNA pellet was washed in 500 µL of 70% ethanol, and centrifuged a further 5

minutes at maximum speed. Ethanol was removed and the DNA pellet left to dry overnight. Final DNA was eluted into 50–100 μ L TE (pH8) and stored at -20°C prior to library preparation. Each sample was quantified and quality checked using a Nanodrop 2000 spectrophotometer and Qubit 2.0 Fluorometer. Qubit concentrations were used for subsequent calculations of DNA input for library preparation of samples.

2.3 Library preparation for high throughput sequencing

One of the large costs associated with genomic sequencing is the preparation of samples (known as library preparation). A number of commercial kits including Nextera DNA library preparation kit (Illumina), NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs), JetSeq Flex DNA Library Preparation Kit (Bioline), or alternatively outsourcing services, are available for this task; however, these are often expensive (c. \$60 – \$130AUD per sample) and are limited in the number of samples that can be combined (multiplexed) into a sequencing run, due to the limited number of identifying index combinations (tags) available to attach to samples. In order to reduce the costs associated with the library preparation process, and allow the large number of samples that I required to be included per sequencing run, an ‘in-house’ library preparation protocol, initially developed by Josquin Tibbits (2013) (briefly outlined in Bayly et al., 2013), was adapted for use in Myoporeae (for summary of process see Figure 2.1). A significant amount of troubleshooting and optimization was required to make this protocol functional across tribe Myoporeae, which range in genome size, ploidy level, chemistry, and extracted DNA yield and quality. I conducted tests at each step of the protocol, resulting in a method that works consistently, and in large sample batches across Myoporeae (as well as a number of other plant families I tested, including Brassicaceae, Myrtaceae, Orchidaceae, Poaceae, Proteaceae, Rhamnaceae, Rutaceae, Xanthorrhoeaceae and the green algal family Codiaceae; data unpublished).

Starting DNA ranged from 500–3000 ng, made up to a volume of 110 μ L in water. Loss of DNA can occur at each of the bead clean up steps (outlined below), meaning a higher starting DNA concentration generally results in a higher yield of library prepared product. A Covaris S2 Sonicator was used to shear DNA to an average fragment length of 600–800 bases with the following settings (Peak incident power: 105w, Duty cycle: 5%, Cycles per burst: 200, Time: 50–60 seconds). Once sheared, 100 μ L of DNA was

transferred to a 200 μ L PCR tube or plate, and 60–90 μ L of custom-prepared Serapure beads (Rohland and Reich, 2012, further details in section below) was added to each sample (bead:sample ratio depended on cutting capacity of each batch of beads prepared). Each sample was vortexed gently then incubated at room temperature for 20 minutes before being placed on an Alpaqua 96 well super magnet for 40 minutes. While on the magnet, DNA (which is size selected and attached to the beads) was captured by the magnetised beads and remained in the tube while the supernatant was removed. The beads should form a tight brown ring around the tube if the magnetization step is working correctly. While the tubes remain on the magnet the supernatant was removed carefully via pipette, then 170 μ L of freshly prepared 80% ethanol was added to each tube for 2 minutes, and then removed via pipette. Beads were left to air dry for approximately 2 minutes, then eluted with End Repair Mix (2 μ L New England Biolabs End Repair Reaction Buffer, 0.4 μ L End Repair Enzyme Mix, 17.6 μ L water). This step blunts the ends of sheared fragments of DNA, ensuring both strands are of equal length, while incubating in a thermal cycler for 60 minutes at 20°C. Following incubation, 50 μ L of 20% PEG/2.5M NaCl and 50 μ L of 100% isopropanol was added to each sample, mixed via vortex, then incubated on the bench for 15 minutes. Following incubation, beads were trapped again on the magnet for 40 minutes then the supernatant removed via pipette. Samples were then washed in 170 μ L of 80% ethanol, air dried for 2 minutes, before the addition of dA Tailing Mix (2 μ L New England Biolabs Buffer Two, 0.5 μ L 10 mM dATP, 0.4 μ L Klenow Fragment Exo, 17.1 μ L water). The dA Tailing Mix should be made on, and added to the sample, when on ice, to prevent premature enzyme function. Once mixed, samples were incubated in a thermal cycler at 37°C for 60 minutes, then at 65°C for a further 20 minutes. At this stage a single 'A' base is added to one strand of DNA at each end of every fragment. Following incubation, samples were placed on ice and 2 μ L of custom made Y-adapters added to each tube along with T4 DNA Ligase Mix (0.8 μ L T4 DNA Ligase Buffer, 0.25 μ L 10 mM ATP, 0.4 μ L T4 DNA Ligase, 6.55 μ L water). Samples were mixed via vortex then incubated overnight at 12°C. At this stage Y-adapters are attached to both ends of each fragment of DNA. These Y-adapters are specially designed to allow for the attachment of specific indexes to the ends of each sample, allowing for both sample identification and attachment to an Illumina flow cell for genetic sequencing. The following day, the reaction was deactivated by increasing the incubation temperature to 65°C for

10 minutes. Exonuclease Mix was added to each sample (0.5 μ L New England Biolabs Buffer One, 0.25 μ L Lambda Exonuclease, 0.25 μ L Exonuclease One, 4 μ L water), mixed via vortex, and incubated at 37°C for 2 hours and then 80°C for 20 minutes. This exonuclease step uses two enzymes to digest any non-ligated pieces of DNA or waste products. Following incubation, samples were further cleaned with the addition of 80 μ L 20% PEG/2.5M NaCl and 80 μ L 100% isopropanol. Samples were mixed, incubated at room temperature for 15 minutes then beads trapped on the magnet for 40 minutes. The supernatant was removed with a pipette and the sample washed with 180 μ L of 80% ethanol. Each sample was air dried for 2 minutes then eluted into final solution (60 μ L of 10mM TrisHCl pH8).

The addition of sample-identifying indexes and fragment duplication is achieved through real time quantitative PCR (qPCR). Polymerase Chain Reaction (PCR) Mastermix was added to a 5 μ L aliquot of each sample (0.5 μ L SYBR Green diluted to 1/1000, 10 μ L Kapa HiFi Readymix, 2.5 μ L water) along with 1 μ L of two identifying indexes. A test qPCR was run for 20–25 cycles with the following profile: 98°C for 30 seconds then (98°C for 10 seconds, 67°C for 30 seconds, 72°C for 30 seconds) $\times$ 20–25 cycles. The qPCR curves obtained from this process were then used to determine the optimal number of cycles required for each sample. A second qPCR was then run using double the PCR Mastermix (1 μ L SYBR Green diluted to 1/1000, 20 μ L Kapa HiFi Readymix, 5 μ L water) with 10 μ L sample and 2 μ L of each index combination, with the qPCR set for the appropriate cycle number for each sample. Once all samples were run to their optimal qPCR cycle number, the final fluorescence values corresponding to each samples qPCR curve was used to quantify the volume of each sample to be pooled for sequencing. Samples were pooled accordingly and a final bead clean up administered to remove any small fragments of DNA or primer dimer. This process was achieved by adding a 0.9:1 ratio of beads to pool volume, incubating at room temperature for 15 minutes, trapping beads on magnet for 15 minutes, removing the supernatant, then washing in 170 μ L of 80% ethanol for 2 minutes, air drying, then repeating the process. Resulting DNA was resuspended in 25 μ L 10 mM TrisHCl pH8 and ready to be sequenced.

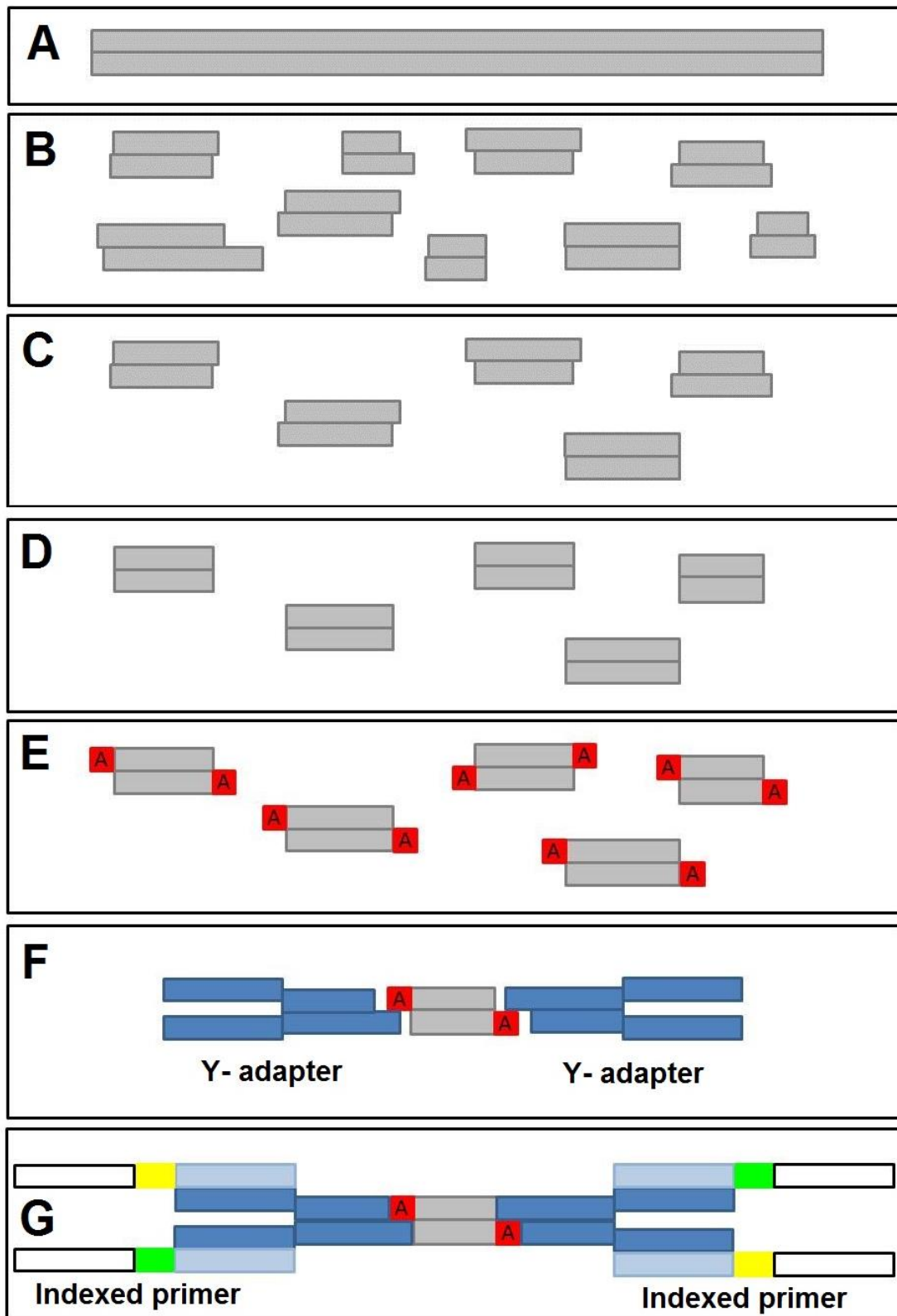


Figure 2.1 Summary diagram of library preparation process; A) total genomic DNA included as input, B) total genomic DNA sheared to 600–800 bp fragments, C) any fragments not in optimal size range are removed using bead clean-up, D) fragment ends are enzymatically blunted, E) single 'A' base added to each end of each fragment, F) custom 'Y-adapters' are ligated to each end of each fragment, G) sample specific primer pairs ligated on each arm of Y-adapter, allowing for sample identification when multiple samples multiplexed in a single sequencing run.

2.4 Preparation of Serapure beads

A large cost component of library preparation is the application of Ampure SPRI beads in fragment size selection and clean-up steps through out the process. These beads work by attracting and binding specific sized fragments of DNA to their surface depending on the ratio of Polyethylene Glycol 8000 (PEG) that is added to the sample. The higher the PEG:bead ratio, the smaller the DNA fragment size attracted. Because these beads are also magnetic, when held in place by a magnet, all other sized DNA fragments and waste can be cleaned from a sample by removing the eluent. An alternative to Ampure SPRI beads are custom-prepared Serapure beads (Rohland and Reich, 2012). For a fraction of the cost (\$0.5 vs \$15.0 per mL), Serapure beads can be made 'in-house' and with comparable results. It must be noted that where testing of Serapure beads showed differences in size selection compared to Ampure beads, bead to sample ratios were adjusted accordingly to ensure optimally sized fragments would still be selected.

2.5 High throughput shotgun sequencing (genome skimming)

I chose to implement a high throughput, 'genome skimming' approach to gather genomic data for this study. This technique allows for the input of untargeted, whole genomic DNA and results in the random sequencing of the entire genome to a low depth of coverage (Straub et al., 2012). In plants, three genomic datasets can be reliably recovered using this technique; high copy regions of the nuclear genome (for example nuclear ribosomal DNA found in tandem repeats, such as the 18S–5.8S–28S cistron repeat unit), the chloroplast genome (which occur in high copy number due to the presence of multiple chloroplasts per cell each of which contain hundreds to thousands of copies of the plastid genome (Rauwolf et al., 2010), meaning the entire genome can often be recovered), and the mitochondrial genome (again, due to the high copy number of mitochondria in each cell often large sections or entire genomes can be recovered) (Straub et al., 2012; Malé et al., 2014; Turner et al., 2016). Nuclear genome size and the number of reads generated for each sample will directly affect the sequence data that can be recovered (Straub et al., 2012; Dodsworth, 2015).

For this study, samples were sequenced using both Illumina HiSeq2000 and Illumina NextSeq500 sequencing platforms, based at AgriBio, Centre for AgriBioscience (LaTrobe University) and the Walter and Eliza Hall Institute (WEHI) in Melbourne, Australia. A

single lane of the HiSeq2000 was shared for this study with three other datasets, with 125 bp paired end reads and 31 GB of data recovered. Three additional Illumina NextSeq500 runs were completed, two utilising a mid-output 150 bp paired end kits resulting in 11 GB and 30 GB of data, and a high-output 150 bp paired end kit resulting in 62 GB of data.

2.6 Contig assembly

Illumina sequence output reads were obtained in forward and reverse (R1 and R2) fastq file format. Initially, *de-novo* assembly of raw Illumina reads was trialled using a number of tools including Geneious version 9.1.8 (<http://www.geneious.com>, Kearse et al., 2012), ORGanelle ASseMbler version 0.1.3 (<https://pythonhosted.org/ORG.asm/>), MegaHit version 1.0.3 (Li et al., 2015, 2016) and CLC Genomics Workbench version 8.5.1 (<https://www.qiagenbioinformatics.com/>). The Geneious *de-novo* assembler generated large and accurate contigs but was too slow for the large number of samples to be processed (approximately 24 hours per sample on a desktop PC). Once installed, ORGanelle ASseMbler was quick to generate contigs however required an average depth of c. 100 reads, which was not always available. MegaHit worked very well, and was probably the fastest tool at producing large, high quality contigs – particularly with optimised system settings, however, an update to this software disrupted its utility, leaving it unable to generate large contigs in late 2016. The majority of samples were then processed using CLC Workbench, which was relatively fast at generating contigs under default settings however did not always accurately assemble contigs, with repetitive indels often omitted from final contigs. This issue was addressed, with any missing indels identified and included through a final checking process in which raw reads were mapped over final consensus sequences (see further details below).

2.7 General sequence/genome construction

Once *de-novo* contigs of total genomic DNA had been generated they were imported into Geneious along with raw read data. Contigs were mapped to a reference using Custom Sensitivity settings (gaps allowed at a maximum of 30% per read, maximum gap size 3000) and 3–5 iterations (see Figure 2.2A). It is notable that these settings required manual adjustment depending on differences in data between samples and the similarity of a sample to the reference sequence available. As an interim step, any gaps

in contig coverage of the reference sequence were assigned base calls that matched the reference, and messy ends of contigs were trimmed to points where they matched the reference. Sections of the reference were then iteratively updated to reflect the sample sequence using the map to reference step of raw reads outlined below. A consensus sequence based on a 50% strict consensus threshold was generated and extracted with reference sequence annotations. This was used as a 'draft' consensus reference for all raw (paired) reads to be mapped to using Custom Sensitivity settings (gaps allowed at 20% per read, maximum gap size 1000) and 3–5 iterations (see Figure 2.2B). Once mapped, it was relatively easy to identify issues in sequence assembly visually in Geneious. Sequence drop-off or mismatched reads were explored further and inaccurate sections of sequence remapped or generated from scratch using boundaries of the region in question as a reference to map all reads. Once I was confident in the accurate alignment of reads mapped to a draft consensus, a final consensus sequence was generated (see Figure 2.2C); again based on a 50% strict consensus threshold and with reference annotations mapped on to the new sequence. This process was repeated for all samples to assemble complete chloroplast genome sequences, the nuclear ribosomal cistron and regions of mitochondrial genome (genome specific methods described in each chapter).

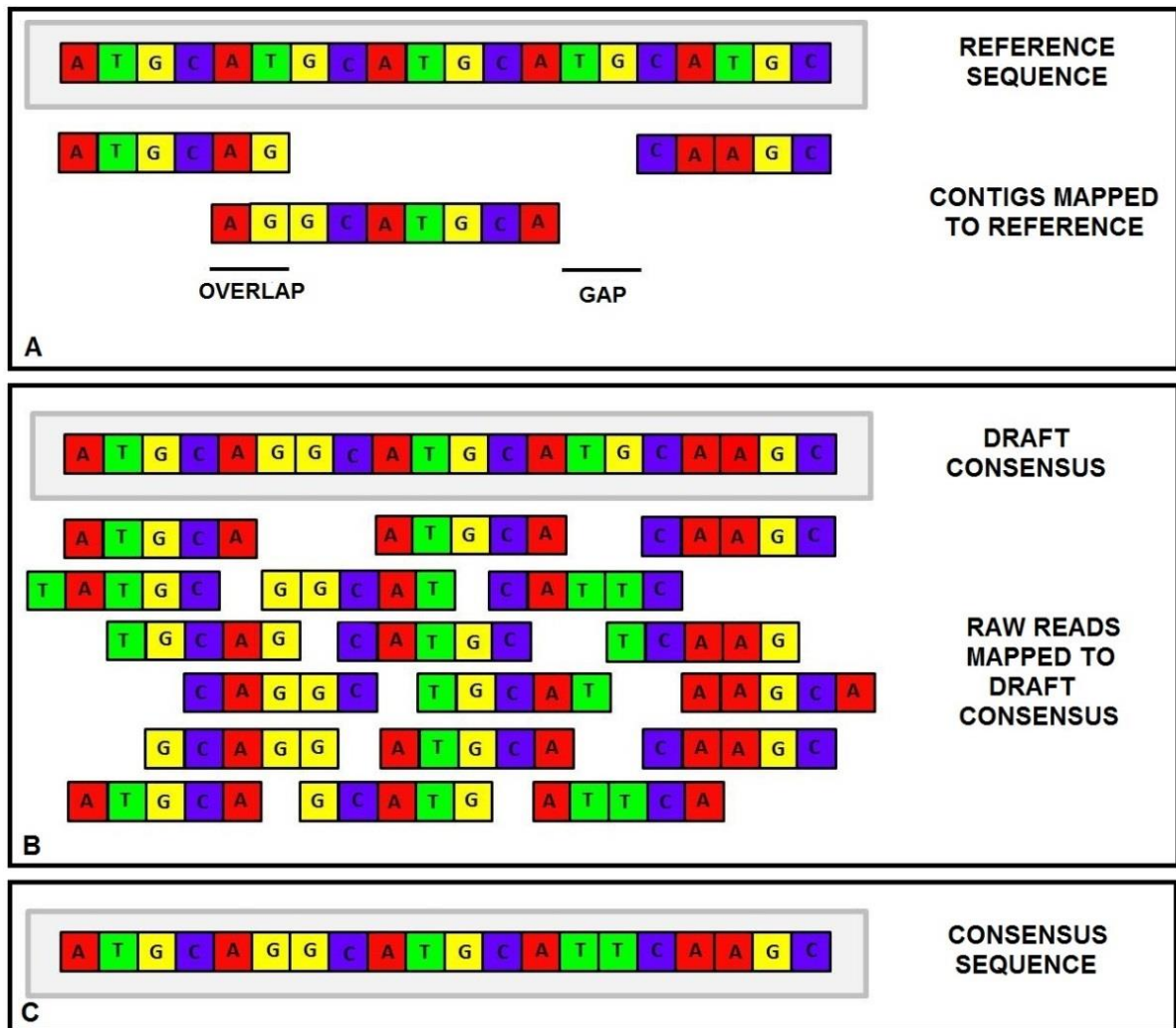


Figure 2.2 Summary of sequence/genome assembly process, A) *de-novo* assembled contigs were mapped to a reference using 'relaxed' settings to allow for variation in sample (relative to the reference) to be captured, then a draft consensus sequence was generated, B) the sample raw reads were mapped to the draft consensus, then, C) a final consensus sequence generated based on the raw read alignment to draft consensus.

Chapter Three: Whole chloroplast genome phylogeny of tribe Myoporeae

3.1 Introduction

Regions of chloroplast DNA have long been used as a tool for phylogenetic inference in plants (Rieseberg & Soltis, 1991; Olmstead & Palmer, 1994; Shaw et al., 2005, 2014). Many features of the chloroplast genome contribute to its utility in phylogenetic reconstruction at a range of taxonomic levels. The plastome is non-recombining and is most commonly inherited maternally (Reboud & Zeyl, 1994; Birky, 1995), which eliminates the need to differentiate between multiple allelic copies or gene families with high levels of sequence similarity (as found in much of the nuclear genome). The structure and order of genes in the chloroplast genome are relatively conserved; most plants have a single, circular plastome of quadripartite structure, with a large single copy region (LSC) and small single copy region (SSC) separated on either side by two identical inverted repeat regions (IR) (Olmstead and Palmer, 1994; Sugiura et al., 1998). Gene (coding) regions are often highly conserved, and have traditionally been important in providing ‘universal’ priming sites that allow amplification of DNA regions across a broad range of flowering plants. These have proven to be informative for phylogenetic reconstruction at deeper evolutionary timescales (i.e. in studying the evolution of angiosperms (Jansen *et al.*, 2007; Foster *et al.*, 2017)). In contrast, chloroplast DNA non-coding spacer regions and introns are more highly variable than the exons – rendering them of particular use in reconstructing relationships at specific and intraspecific taxonomic levels (Jansen et al., 2006; Barrett et al., 2014; Ohlsen et al., 2014). While the actual size of the chloroplast genome can vary greatly between plant species (between 75–250 kilobase pairs), on average plastomes range from 120–160 kilobase pairs and are small in size compared to their nuclear and mitochondrial counterparts (Palmer, 1985; Xiao-Ming et al., 2017). This relatively small size combined with the large number of copies of the chloroplast genome in each plant cell (up to 50,000; Morley and Nielsen, 2016) means the plastome is comparatively easy to extract and amplify in sections, or in its entirety.

Previously, in an era of PCR and direct Sanger sequencing, emphasis was placed on using the most variable regions of the chloroplast genome for a given taxonomic level, with most studies choosing to use a handful of coding and non-coding regions of the plastome to infer phylogeny (Shaw et al., 2005, 2014). While financially and logistically feasible, these studies were often unable to fully resolve the relationships in question, due to a lack of phylogenetic signal (not enough variable characters per DNA sequence for taxa sampled). With the rapid advancement of high throughput sequencing technology (HTS) over the past 5–10 years, the viability of sequencing complete chloroplast genomes has become a reality. Costs of sample preparation and sequencing have greatly reduced, and computationally, access to computer clusters and development of software to deal with large quantities of sequence data have seen a surge in studies focusing on the whole chloroplast genome (discussed further in Chapter One). In many plant groups complete chloroplast genomes are providing near complete resolution of relationships previously unresolved when using a small number of chloroplast loci (i.e. bamboo Zhang et al., 2011; eucalypts Bayly et al., 2013; *Cymbidium* Yang et al., 2013; *Camellia* Huang et al., 2014).

In the case of Myoporeae, Kelchner (2003) used two non-coding regions of the chloroplast genome (*rpl16* intron and *trnT-trnL* intergenic spacer) to explore evolutionary relationships for a subset of the tribe, resulting in a largely unresolved phylogeny with many nodes displaying low bootstrap support values (see Figure 1.9). Kelchner was able to confirm the monophyly of the tribe, and suggested *Eremophila* to be paraphyletic, with members of all allied genera nested within three core *Eremophila* clades, though relationships between the seven genera of Myoporeae and between many species of the largest genus, *Eremophila*, remained uncertain. The lack of resolution in these data can be largely attributed to the quantum of sequence data that was used, and particularly the small number of informative characters relative to the number of taxa included (116 parsimony informative characters from a 2143 base pair alignment). Despite this, the relatively high percentage of parsimony informative characters in the dataset (c. 5.4%) indicated that increasing the size of the dataset would likely increase resolution of relationships in question.

3.1.1 Aims

The aim of this component of research is to generate a comprehensive molecular phylogeny for tribe Myoporeae using complete chloroplast genomes, in an attempt to better understand the relationships at generic, specific and intraspecific levels, and extend the work of Kelchner (2003). A number of more specific research questions to be addressed using cpDNA include:

- 1) What are the relationships between the seven genera within Myoporeae, and are all genera monophyletic?
- 2) Are the morphology-based sections of *Eremophila* proposed by Chinnock (2007) supported by cpDNA?
- 3) Are species monophyletic, particularly large species complexes and those with widespread or disjunct distributions?

3.2 Methods

Details of specimen collection, DNA extraction, library preparation, contig assembly and sequence alignment are outlined in Chapter Two: General methods. The methods specific to the chloroplast sequencing component of this study are detailed below.

3.2.1 Taxon sampling

A total of 317 individuals, representing 195 species of *Eremophila* (including 6 putatively undescribed species), 20 species of *Myoporum*, all 4 species of *Diocirea* and representatives for allied monotypic genera *Calamphoreus*, *Glycocystis*, *Bontia* and *Pentacoelium*, were included in this study (see Appendix Five). These samples represent all 25 of Chinnock's described sections of *Eremophila* (c. 85% of described *Eremophila* species) and all 4 sections of *Myoporum* (c. 66% of described *Myoporum* species). Also included were 4 outgroup taxa; two species of sister group to Myoporeae, *Leucophyllum* (*L. frutescens* and *L. zygothallum*), which were sourced and sequenced for this study, in addition to two complete chloroplast genome sequences downloaded from GenBank for members of more distantly related outgroups in *Scrophularia* (*S. takesimensis* and *S. buergeriana*).

3.2.2 Chloroplast genome construction

Once *de-novo* contigs of total genomic DNA had been generated, they were imported as FASTQ files into Geneious version 9.1.8 (<http://www.geneious.com>, Kearse et al., 2012) along with raw paired read data for each sample. Initially, no chloroplast genome reference sequence was available within tribe Myoporeae, so the closest reference genome available on GenBank (*Scrophularia takesimensis* NC_026202, Scrophulariaceae) was downloaded for use. The sample from the first sequencing run that was most complete and had the largest number of paired reads and longest constructed contigs (RMF159 *E. mackinlayi* subsp. *mackinlayi*) was chosen to build a reference sequence for Myoporeae. Contigs from this sample were mapped to the *S. takesimensis* reference genome using custom sensitivity settings (gaps allowed set to a maximum of 30% per read, maximum gap size 3000) with 3-5 iterations. Once mapped, the contig(s) spanning the Inverted Repeat (IR) region of the chloroplast genome were replicated, and the contig mapping process repeated to include both IR copies in the genome. Any gaps in contig coverage of the genome were called the same as the reference, and unreadable or uncertain ends of contigs trimmed to where the contig matched the reference. Sections of the new reference were subsequently updated to reflect the sample sequence using the map to reference step of raw reads as outlined in Chapter Two (see Figure 2.2). A consensus sequence based on a 50% strict consensus threshold was generated and extracted with reference sequence annotations. This was used as a 'draft' consensus reference for all raw reads (paired) to be mapped to, using custom sensitivity settings (gaps allowed at 20% per read, maximum gap size 1000) and 3-5 iterations. Once mapped, it was relatively easy to identify issues in sequence construction visually in Geneious, and any gaps previously called as the reference replaced with the samples own sequence reads. Sequence drop-off or mismatched reads were explored further and inaccurate sections of sequence remapped or generated from scratch using boundaries of the area in question as a reference to map all reads. Once confident in the accurate alignment of reads mapped to a draft consensus, a final consensus sequence was generated, again based on a 50% strict consensus threshold and with reference annotations mapped across. This process was repeated for all samples using the closest member of Myoporeae already constructed (i.e. most morphologically similar/from same section) to generate complete chloroplast genomes.

3.2.3 Sequence alignment and annotation of whole chloroplast genomes

All 317 chloroplast genome sequences were aligned in Geneious v9.8.1 using the MAFFT pairwise alignment plug-in (MAFFT version 7.222, Katoh and Standley, 2013) with default settings. Once aligned, annotations for each sequence were compared to published annotations of the outgroups obtained from GenBank (*Scrophularia takesimensis* and *Scrophularia buergeriana*). Genes were checked individually in Geneious for correct start/stop codons, erroneous stop codons within genes, and movement of gene boundaries. Boundaries of tRNA and rRNA genes were highly conserved and were thus based entirely on published outgroup annotations. Alignments were checked by eye and adjustments made manually. Sections of alignment unable to be resolved (often due to long stretches of mononucleotide base repeats or repetitive indels) were recorded and excluded from further phylogenetic analyses.

3.2.4 Bayesian inference analysis

Model testing of the chloroplast genome alignment was conducted in MrModeltest version 2.3 (Nylander 2004). The alignment was partitioned in four character sets representing protein coding DNA sequence (CDS), genes coding for ribosomal ribonucleic acid (rRNA), transfer ribonucleic acid (tRNA) and intergenic spacers/introns (SPCR). These partitions were selected as it was assumed that rates of evolution would more likely be similar within these groups than between them. Models selected for each partition of the BI analysis were based on the Akaike information criterion (AIC). The suggested model of evolution for CDS, tRNA and SPCR was GTR+I+G, while a HKY model was suggested for rRNA.

The BI analysis for the chloroplast genome alignment was run in MrBayes version 3.2 (Ronquist et al., 2012) using the default settings. The MCMC analysis was run for 3 million generations with a tree sampling frequency of 500. The average standard deviation of split frequencies reached a value lower than 0.01 over this analysis, with convergence of MCMC chains checked in Tracer version 1.6 (Rambaut et al., 2014). After examination of convergence it was found that deletion of 25% of the trees was appropriate for the analysis burn-in, Bayesian posterior probabilities were estimated for nodes from the consensus of these remaining trees.

3.3 Results

3.3.1 Chloroplast genome structure

From across four Illumina sequencing runs, DNA sequence data used to construct complete chloroplast genomes ranged from 10879 to 918033 reads per sample (representing a mean depth of chloroplast coverage ranging from 9–900 reads per sample genome). This variation in read number can be largely attributed to differences in total read numbers between samples (due to pooling discrepancies) and differences in size between nuclear genomes (the larger the nuclear genome, the higher the proportion of reads allocated to it, and the fewer reads skimmed for high copy regions of DNA such as the chloroplast genome). The total number of reads associated with the chloroplast genome per sample varied greatly between individuals; from 0.4% in *E. granitica* (MJB2459) to 19.5% in *E. tetraptera* (RMF175). This large difference in proportion of reads assigned to the chloroplast genome may reflect the differing ploidy levels known to be associated with species of *Eremophila* (Chinnock, 2007). While this hypothesis cannot be tested directly without chromosome counts of the specific individuals included, this is a subject that will be worth exploring in future research.

The total length of chloroplast genomes ranged in size across tribe Myoporeae from 142340bp (RMF166 *Eremophila maculata* subsp. *filifolia*) to 155526bp (MJB2409 *Eremophila foliosissima*) (for genome size ranges for all genera of Myoporeae see Table 3.1). The arrangement of the chloroplast genome follows a regular quadripartite structure composed of a Large Single Copy (LSC) region, Small Single Copy (SSC) region and two identical Inverted Repeat regions (IR(a),IR(b)) that fall between each of the single copy sections. The length of each region is variable between individuals, though as an example, *Eremophila magnifica* subsp. *magnifica* (MJB2398) is composed of an 87963bp LSC, 18032bp SSC and two identical IR's of 22972bp in length.

While the gene order of Myoporeae remains consistent (both within the tribe and between tribe members and outgroup species of *Leucophyllum* and *Scrophularia*), all members of Myoporeae exhibit significant movement in the boundary between the large single copy (LSC) and Inverted Repeat (IR) regions of the genome relative to the outgroups (both *Scrophularia* and *Leucophyllum*) (see Figure 3.1). Rather than containing two copies of *rpl2* and *rpl23* (one in each IR region) all members of

Myoporeae contain just a single copy as a result of a boundary shift in the limits of the IR regions. The extent of this boundary shift varies between individuals of Myoporeae, though for example, *E. maculata* subsp. *filifolia* exhibits movement of the boundary to include all of *ycf2*, *ycf15*, *trnL_{CAA}* and part of *ndhB* in the LSC region (see Figure 3.1E). Overall, the most variable (and thus informative) regions of the chloroplast genome for tribe Myoporeae are *ycf1*, *ycf2*, *rpl32-trnL*, *rpoC2*, *ndhF*, *trnK-rps16*, *matK* and *rps16-trnQ*, each of which exhibit more than 150 parsimony informative characters (see Figure 3.2).

Table 3.1: Maximum and minimum chloroplast genome size (in base pairs, bp) for each genus within Myoporeae. Genera with a single value are monotypic and were represented by a single individual.

Genus	Individual	Minimum genome size (bp)	Maximum genome size (bp)
<i>Bontia</i>	<i>Bontia dahnoides</i> (FTBG64271)	n/a	148057
<i>Calamphoreus</i>	<i>Calamphoreus inflatus</i> (RMF220)	n/a	151631
<i>Diocirea</i>	<i>Diocirea microphylla</i> (MJB2383)	151656	
	<i>Diocirea ternata</i> (RMF111)		152100
<i>Eremophila</i>	<i>Eremophila maculata</i> (RMF166)	142340	
	<i>Eremophila foliosissima</i> (MJB2409)		155526
<i>Glycocystis</i>	<i>Glycocystis beckeri</i> (RMF214)	n/a	151764
<i>Myoporum</i>	<i>Myoporum bateae</i> (RMF42)	151155	
	<i>Myoporum tetrandrum</i> (RMF209)		152304
<i>Pentacoelium</i>	<i>Pentacoelium bontioides</i> (MSTANG)	n/a	151748
Outgroup:	<i>Leucophyllum zygophyllum</i> (RMF286)	154157	
<i>Leucophyllum</i>	<i>Leucophyllum frutescens</i> (RMF275)		154300
Outgroup:	<i>Scrophularia takesimensis</i>	152425	
<i>Scrophularia</i>	<i>Scrophularia buergeriana</i>		153631

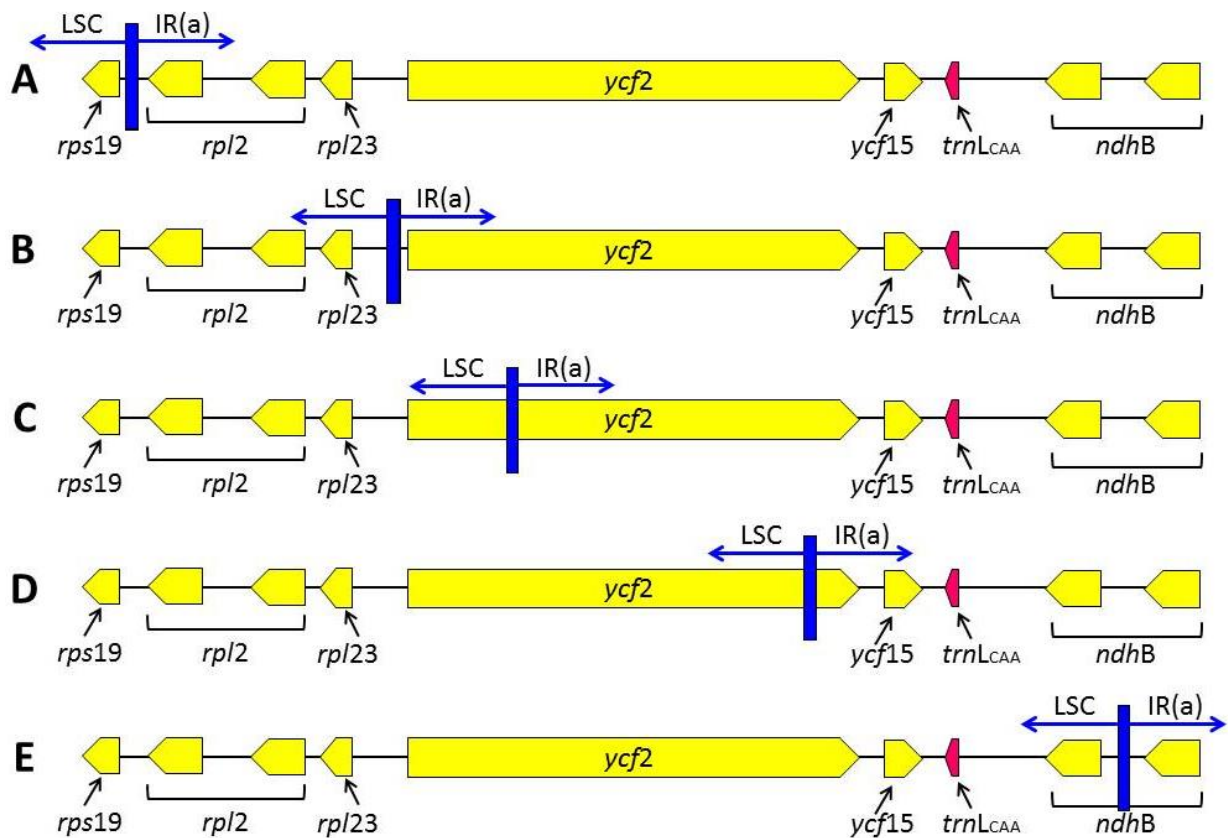


Figure 3.1 Movement of the boundary between the Large Single Copy (LSC) and Inverted Repeat (IR(a)) regions of the chloroplast genome for members of tribe Myoporeae relative to outgroup species; A) outgroup *Leucophyllum frutescens* exhibits two IR copies of *rpl2*, *rpl23*, *ycf2*, *ycf15*, *trnLCAA* and *ndhB*, B) *Eremophila tetraptera* (RMF175) showing movement of the IR boundary to include *rpl2* and *rpl23* in the LSC region, C) *Eremophila debilis* (RMF290) showing movement of the IR boundary to include *rpl2*, *rpl23* and part of *ycf2* in the LSC region, D) *Eremophila ballythunensis* (BB48) showing movement of the IR boundary to include *rpl2*, *rpl23* and part of *ycf2* in the LSC region, E) *Eremophila maculata* subsp. *filifolia* (RMF166) showing movement of the IR boundary to include all of *rpl2*, *rpl23*, *ycf2*, *ycf15*, *trnLCAA* and part of *ndhB* in the LSC region.

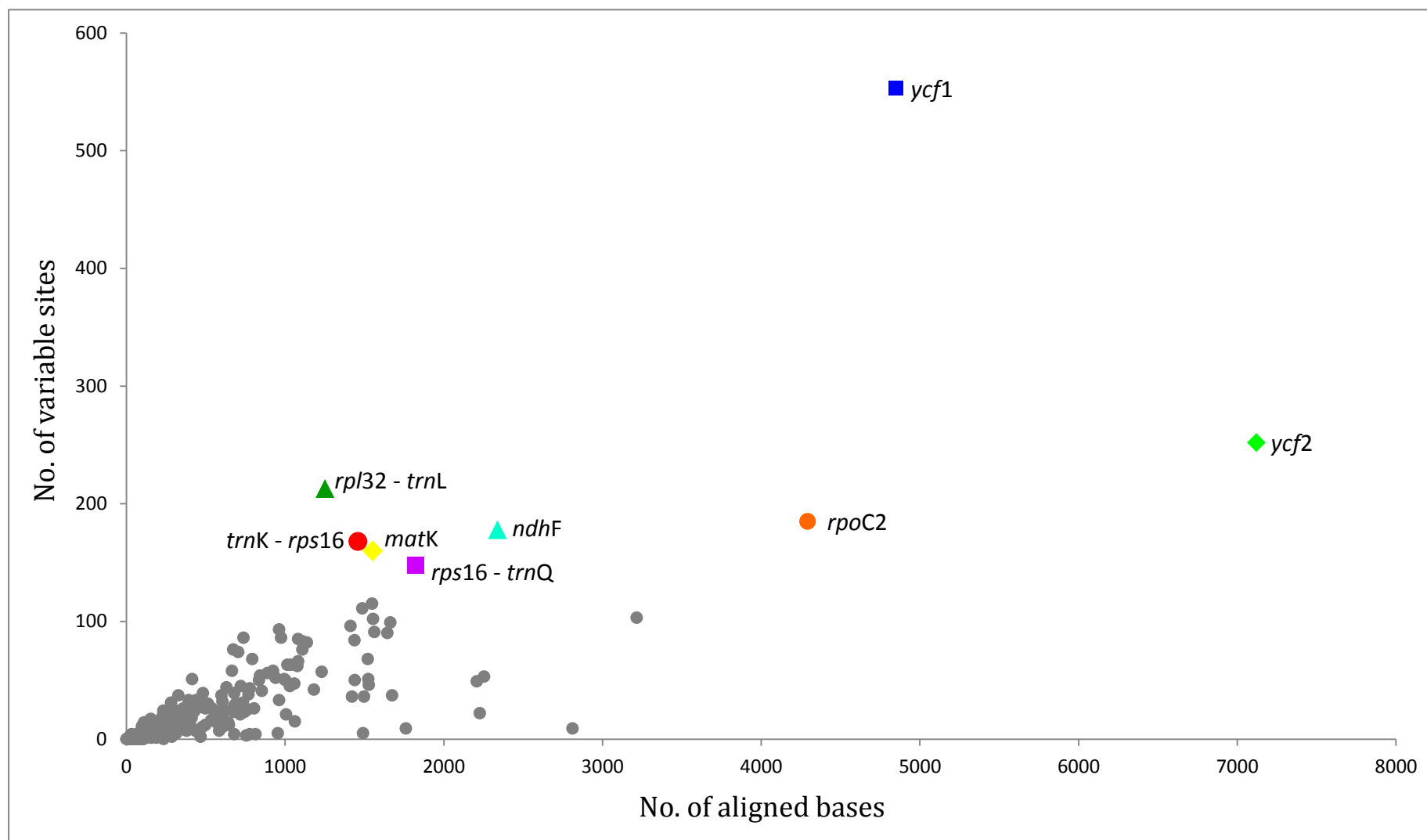


Figure 3.2 Sequence variation across chloroplast genes and spacer regions for tribe Myoporeae. Each data point represents a particular gene or intergenic spacer. Identities are shown for the eight most variable regions; other regions are represented by the grey circles.

3.3.2 Phylogenetic reconstruction

The Bayesian Inference analysis resulted in well supported phylogenetic reconstruction of tribe Myoporeae. Due to the large number of terminal taxa and thus large size of the BI tree, a summary tree with major clades collapsed is presented in Figure 3.3. Each clade is expanded to show details of terminal taxa in Figures 3.4 – 3.8.

Overall the cpDNA phylogeny is highly supported, with all but one of the deeper nodes showing pp=1, and the majority of nodes within clade and terminal branches resolved with high support (265/283 internal branches with pp>0.95). Broadly, the phylogeny can be divided into nine well supported clades (see Figure 3.3). *Myoporum* was found to be non-monophyletic, split across two major lineages (clades three and four) and nested with members of *Eremophila*.

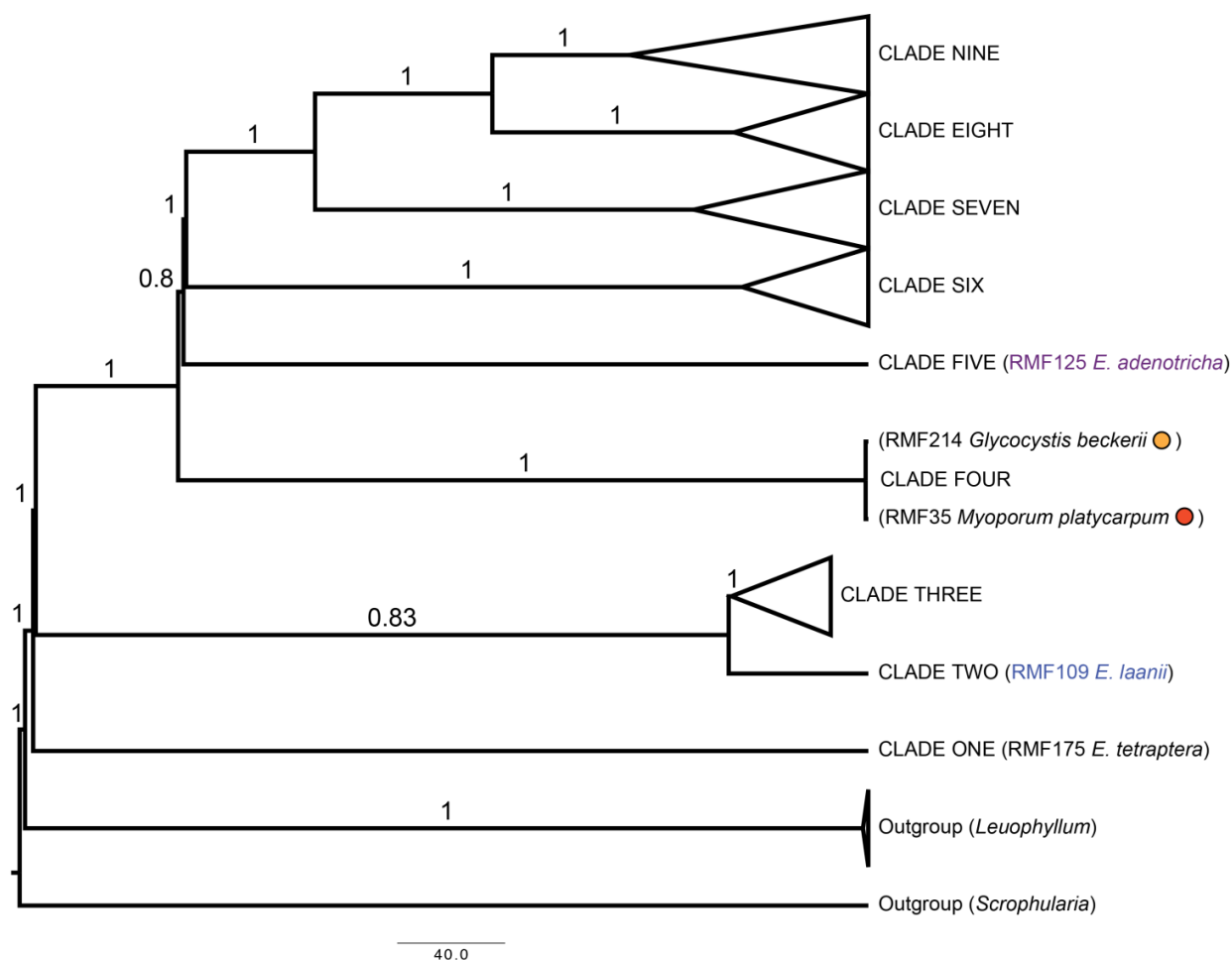


Figure 3.3 Majority-rule consensus tree from the Bayesian Inference (BI) analysis of complete chloroplast genomes, with clades collapsed in summary. Posterior probability (pp) values are shown for each branch.

The small Western Australian genus, *Diocirea*, was found to be non-monophyletic, with all four species scattered amongst species of *Eremophila* in clade nine, while the monotypic genera *Bontia*, *Calamphoreus*, *Glycocystis* and *Pentacoelium* were all found to be nested amongst species of either *Eremophila* or *Myoporum* dominated clades. The largest genus within Myoporeae, *Eremophila*, is represented in every major clade of the phylogeny, except clade four.

Clade one (n=1, see Figure 3.3) contains a single species, *E. tetraptera*, and is highly supported (pp=1) as sister to the remainder of tribe Myoporeae. This placement is consistent with the findings of Kelchner (2003), who also recovered *E. tetraptera* as a distinct lineage.

Clade two (n=1, see Figure 3.4) also contains just a single species, *E. laanii*, from sect. *Amphichilus*. Both other members of sect. *Amphichilus* are placed separately in clade seven of the phylogeny. Support is low (pp=0.8) for the sister relationship between *E. laanii* and clade three.

Clade three (n=52, see Figure 3.4) contains members from four of the seven genera classified in tribe Myoporeae; *Eremophila*, *Myoporum*, *Bontia* and *Pentacoelium*. All six sections of *Eremophila* represented in clade three are found uniquely within this lineage, including sections *Pholidiopsis* (*E. deserti*, *E. fallax*, *E. santalina*), *Chamaepogonia* (*E. debilis*), *Duttonia* (*E. gibbifolia*, *E. crassifolia*, *E. behreiana*, *E. barbata*), *Platyichilus* (*E. bignoniiflora*, *E. polyclada*), *Scariosepalae* (*E. alternifolia*, *E. purpurescens*) and *Carnosae* (*E. pentaptera*). All but a single species of *Myoporum* (*M. platycarpum*) are placed in clade three, along with monotypic genera *Bontia* and *Pentacoelium*. The Caribbean genus *Bontia* is resolved as sister to the widespread eastern Australian species *E. debilis*, while the Asian genus *Pentacoelium* is placed sister to a lineage of non-Australian species of *Myoporum*, including; *M. sandwicense*, *M. degeneri* (Hawaii), *M. obscurum* (Norfolk Island), *M. stokesii* (Austral Islands), and *M. wilderi* (Cook Islands). All but one internal branch in clade three are highly supported (pp=1). All sections of *Eremophila* with two or more species are non-monophyletic, and many species also show a lack of monophyly, with species duplicates resolved in separate parts of the lineage (i.e. *E. desertii*, *E. alternifolia*, *E. fallax*, *E. gibbifolia*, *M. petiolatum*, *M. laetum*, *M. sandwicense*).

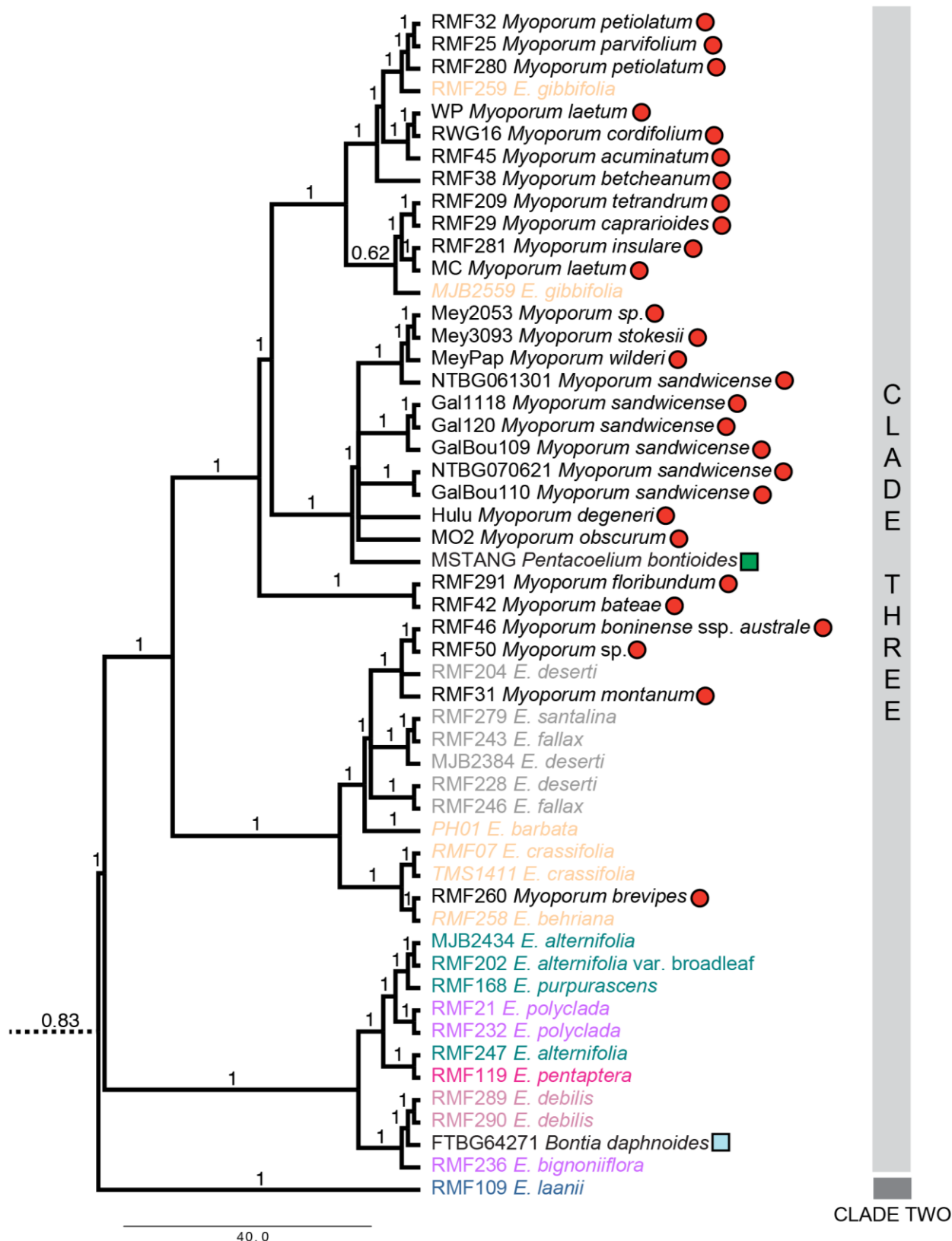


Figure 3.4 Clades two to three of the Bayesian Inference (BI) majority-rule consensus tree. Posterior probabilities are shown for each branch. *Eremophila* species are coloured according to sectional classification (*Amphichilus*, *Platytilus*, *Chamaepogonia*, *Carnosae*, *Scariosepalae*, *Duttonia*, *Pholidiopsis*), and other genera are distinguished with coloured symbols.

Clade four (n=2, see Figure 3.3) contains two species from two genera; a single species of *Myoporum* (*M. platycarpum*) and monotypic genus *Glycocystis*. This is the only clade of the cpDNA phylogeny that does not contain species of *Eremophila*.

Clade five (n=1, see Figure 3.3) contains just a single species, *E. adenotricha*, from sect. *Australophilae*.

Clade six (n=48, see Figure 3.5) is a well-supported lineage (pp=1) containing members from five sections of *Eremophila*. Internal branch support within the clade is high (35/39 branches assigned pp>0.95). The largest component of this clade are species from sect. *Stenochilus*, which, aside from *E. maculata* and *E. racemosa* (found in clade nine), are unique to this lineage. No species within sect. *Stenochilus* are reconstructed to be monophyletic, including the ten subspecies of the large '*E. glabra*' complex. Other sections represented in clade six include *Virides* (4 species), *Australophilae* (9 species), *Eremaeae* (1 species) and *Crustaceae* (1 species). Section *Virides* is the only section found uniquely in this clade.

Clade seven (n=66, see Figure 3.6) contains taxa from across six sections of *Eremophila*. The clade is dominated by members from sects. *Eriocalyx* and *Eremaeae*, however, a small number of individuals from sections *Campanulatae*, *Australophilae*, *Crustaceae* and *Arenaria* are also present. Section *Eriocalyx* is largely restricted to this clade, with 37 of a total of 40 taxa placed within the lineage. Approximately one third of taxa from sect. *Eremaeae* are also placed in this clade (n=20), with the remaining samples resolved in clade eight. The relationships within this clade are generally highly supported, with 53 of the 58 nodes displaying pp values > 0.95.

Clade eight (n=51, see Figure 3.7) is made up of taxa representing four sections of *Eremophila*. The clade is characterised by the dominance of sect. *Eremaeae* (also found in clade seven). Other sections represented in clade eight include *Pulcherisepalae* (3 species), *Eremophila* (6 species) and *Eriocalyx* (2 species). The vast majority of relationships are well resolved within the clade, with 43 of the 45 branches displaying pp values > 0.95, though only a single species represented with multiple accessions (*E. abietina*) is resolved as monophyletic, with *E. phyllopoda*, *E. flaccida*, *E. fraseri*, *E. tietkensii*, *E. platycalyx*, *E. cuneifolia*, *E. freelingii*, *E. gilesii* and *E. simulans* all found to be non-monophyletic.

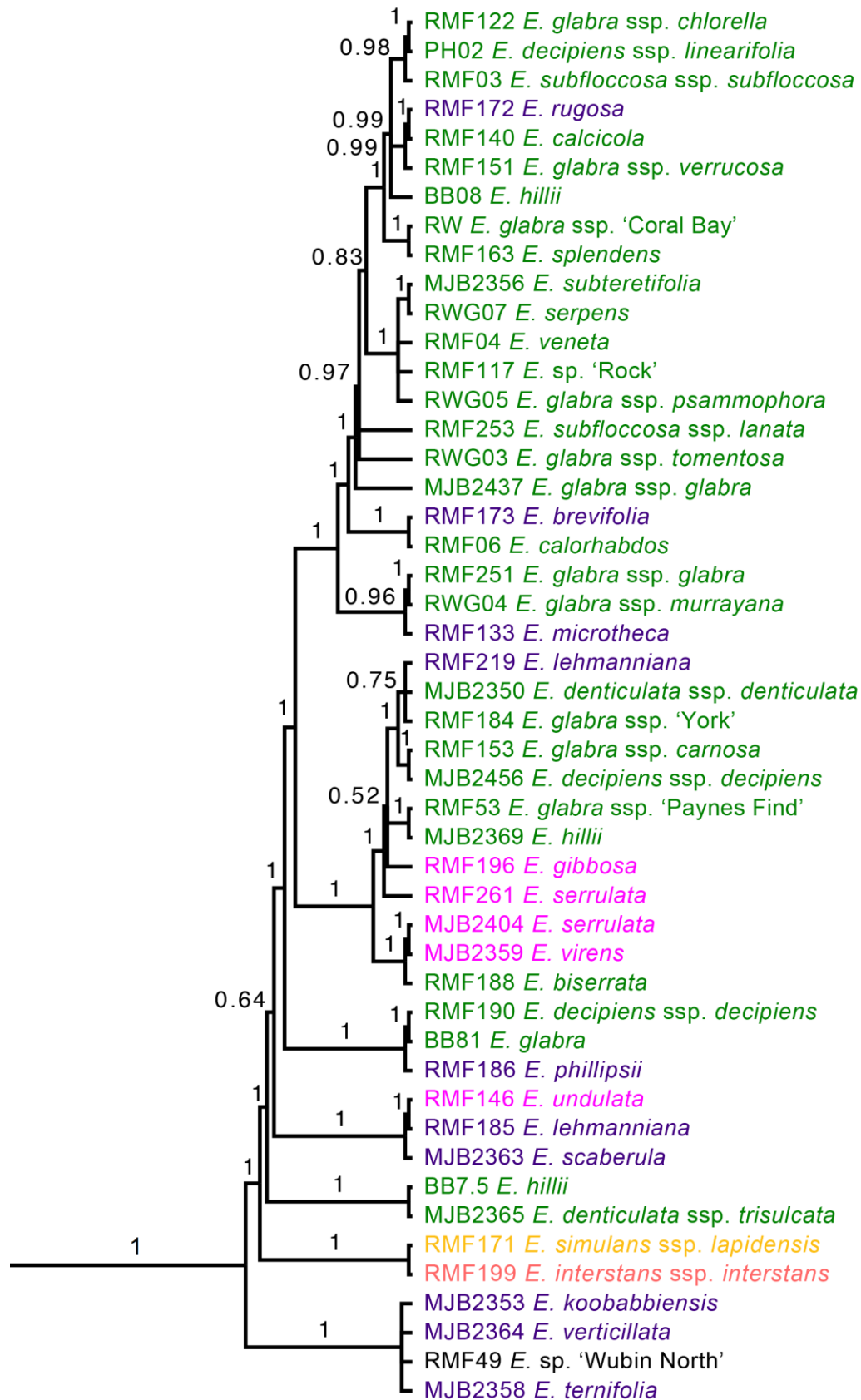


Figure 3.5 Clade six of the Bayesian Inference (BI) majority-rule consensus tree. Posterior probabilities are included for each branch, and taxa are coloured according to sectional classification (*Australophilae*, *Crustaceae*, *Eremaeae*, *Stenochilus*, *Virides*).

Clade nine (n=90, see Figure 3.8) is the largest lineage of the cpDNA phylogeny. Branch support for the clade as a whole is high (pp=1), as is internal branch support, with 81 of the 85 branches showing pp>0.95. Clade nine is composed of 90 terminal taxa, including all four species of the small Western Australian genus, *Diocirea*, and monotypic Western Australian genus, *Calamphoreus*. *Diocirea* is non-monophyletic, with all four species nested within multiple lineages across the clade, and sister to different species of *Eremophila*. *Calamphoreus inflatus* was placed sister to *E. succinea* and nested among species of *Eremophila* from sect. *Crustcaeeae*. In total, 16 out of the 25 sections of *Eremophila* are represented in this clade. A number of these sections are unique to the lineage, including *Decussatae*, *Pholidia*, *Platycarpus*, *Platycalyx*, *Sentis*, *Hygrophanae* and *Contortae*. Other sections that are largely represented in clade nine include *Crustaceae*, *Pulchrisepalae*, *Arenariae*, *Amphichilus* and *Australophilae*. Of all of the sections placed in clade nine, only species within sect. *Hyrophanae* are monophyletic (members of all other sections are largely scattered throughout the clade). At the level of species, of the 14 species in clade nine represented with multiple accessions, six were found to be monophyletic (*E. arachnoides*, *longifolia*, *E. divaricata*, *E. miniata*, *E. platythamnos*, *E. macdonnellii*) and the remainder non-monophyletic (*E. scoparia*, *E. parvifolia*, *E. weldii*, *E. youngii*, *E. mackinlayi*, *E. christophorii*, *E. maculata*, *E. oldfieldii*).

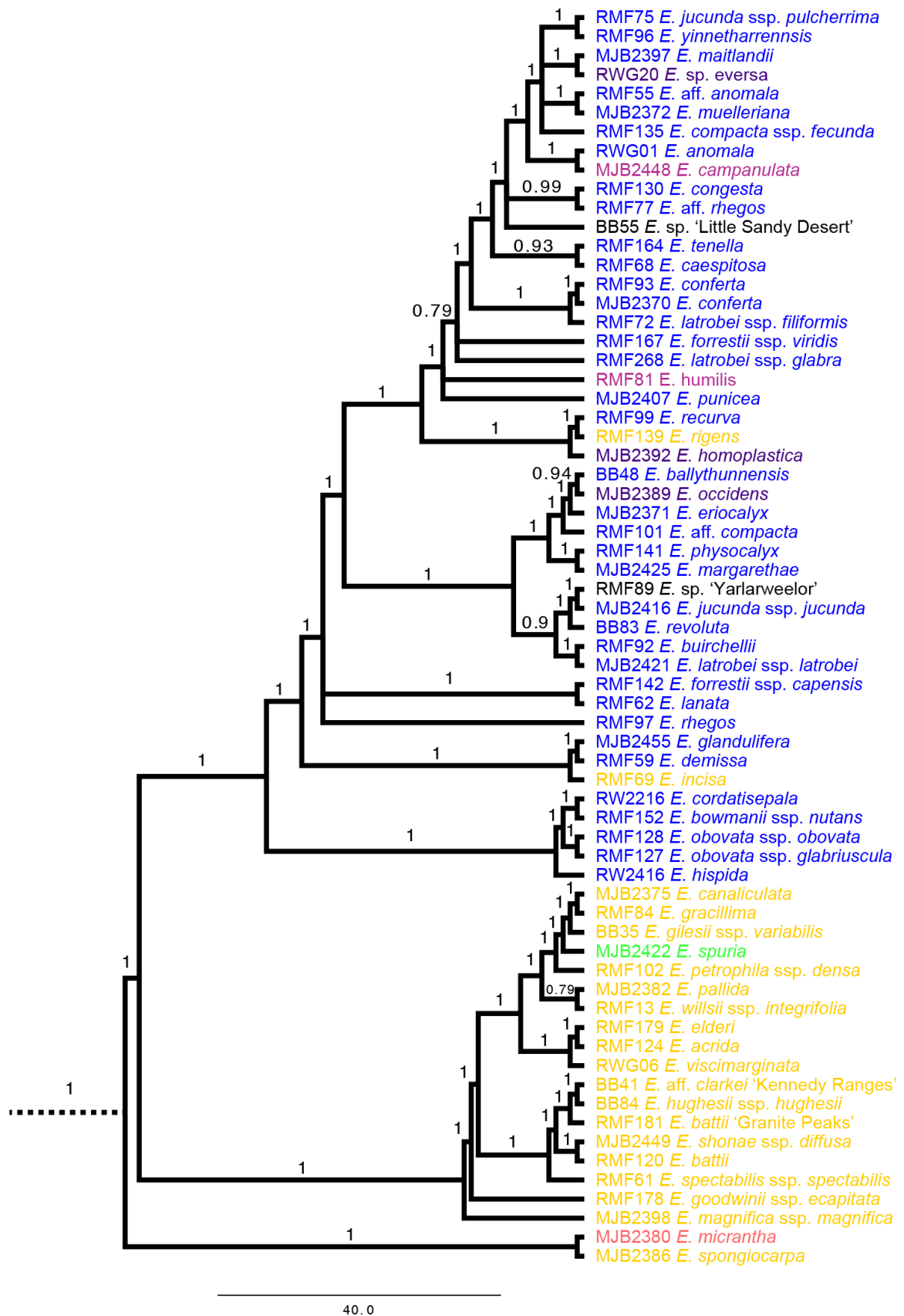


Figure 3.6 Clade seven of the Bayesian Inference (BI) majority-rule consensus tree. Posterior probabilities are included for each branch, and taxa are coloured according to sectional classification (*Eremaeae*, *Crustaceae*, *Arenariae*, *Eriocalyx*, *Australophilae*, *Campanulatae*).

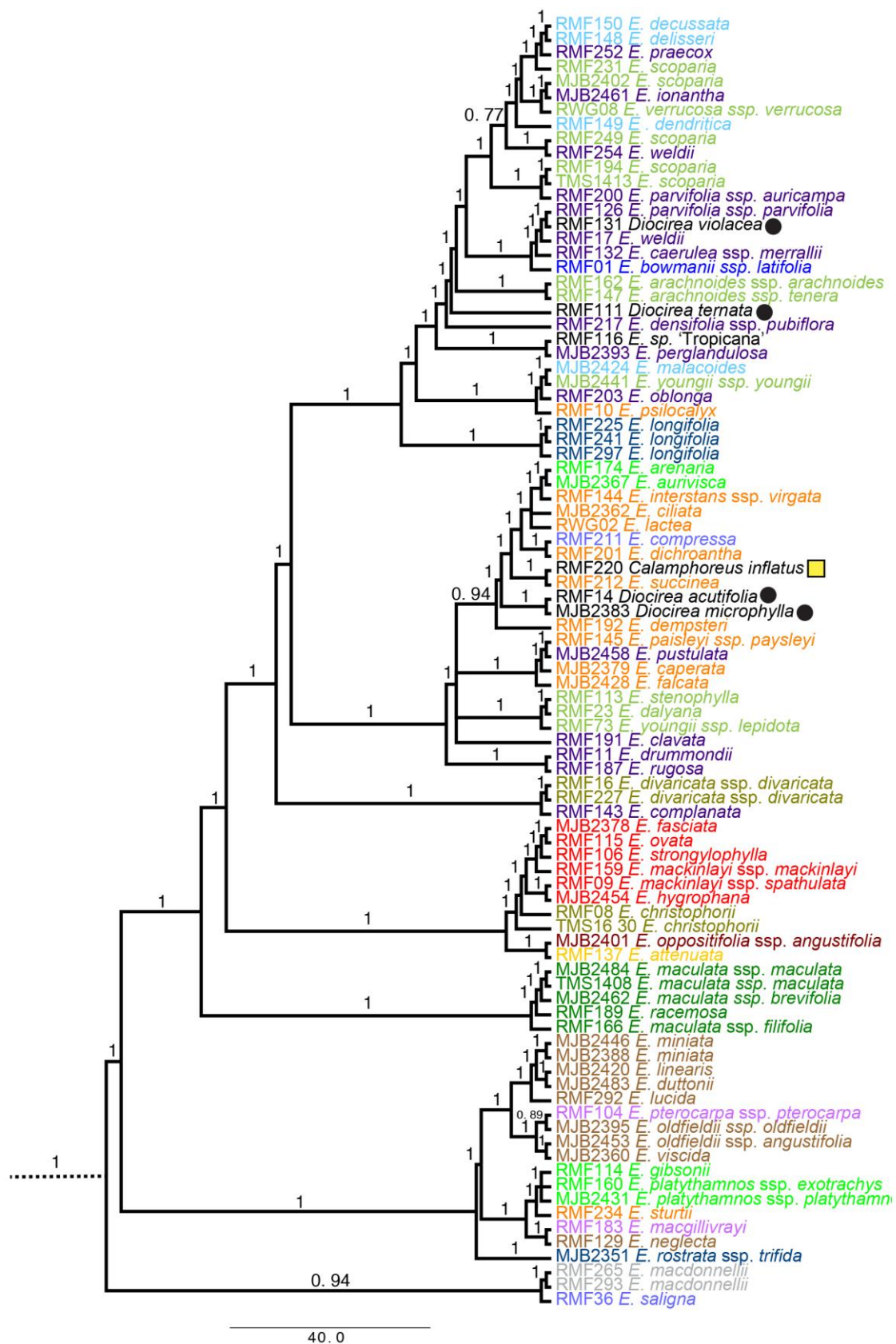


Figure 3.8 Clade nine of the Bayesian Inference (BI) majority-rule consensus tree. Posterior probabilities are included for each branch, and taxa are coloured according to sectional classification (*Platycaurus*, *Platycalyx*, *Amphichilus*, *Pulchrisepalae*, *Contortae*, *Crustaceae*, *Arenariae*, *Stenochilus*, *Eremaeae*, *Eremophila*, *Sentis*, *Hygrophanae*, *Australophilae*, *Pholidia*, *Decussatae*).

3.4 Discussion

Although the technology for large scale determination of DNA sequences for entire chloroplast genomes is now available, most plant phylogenetic studies have continued to focus on sequencing a small handful to tens of specimens of a given taxonomic level (i.e. 44 eucalypts, Bayly et al., 2013; 22 red algae, Costa et al., 2016; 5 *Epimedium* species, Zhang et al., 2016; 14 species of Cornales, Fu et al., 2017). Even large and economically significant plant groups such as the tribe of wheat grasses, Triticeae, have limited complete chloroplast sequences (183 individuals representing 53 species, Bernhardt et al., 2017). The chloroplast genome phylogeny generated in this study is the largest, and most comprehensive dataset of tribe Myoporeae yet, and may also represent the largest complete plastome phylogeny generated (for an Australian plant group) to date. Both MP and BI analyses are very well supported, with 94% of branches in the BI phylogeny displaying posterior probability values of ≥ 0.95 . Such a well resolved and well supported phylogeny allows for sound interpretation of chloroplast relationships in the group and for comparison with Kelchner's (2003) preliminary phylogenetic analysis of the tribe.

3.4.1 Generic relationships and monophyly in tribe Myoporeae based on cpDNA

Prior to the current research, the relationships between each of the seven genera in tribe Myoporeae remained speculative only. Chinnock (2007) estimated some generic relationships based on his morphological understanding of each genus and the tribe as a whole; for example, that Australian genera (*Eremophila*, *Myoporum*, *Diocirea*, *Glycocystis* and *Calamphoreus*) are more closely related to one another than to non-Australian genera *Bontia* and *Pentacoelium* (see Figure 1.6), that *Glycocystis* is most closely related to *Myoporum* sect. *Disoon*, that *Diocirea* is related to *Eremophila* via sect. *Australophilae* and that *Calamphoreus* is closely allied to *Eremophila*, though with some *Diocirea*-like features. Kelchner (2003) sought to test some of these hypotheses with his phylogenetic analysis of Myoporeae, and though being somewhat limited in his interpretation due to a general lack of resolution and support, found both *Eremophila* and *Myoporum* to be non-monophyletic and all other allied genera to be nested amongst species of *Eremophila* (see Figure 1.9).

As in Kelchner's study, the cpDNA phylogeny of this current study does not support the monophyly of *Eremophila* nor *Myoporum*. *Eremophila* is resolved with strong support to be paraphyletic; all six allied genera are nested within *Eremophila* dominated clades throughout the phylogeny. *Myoporum* is split across both clades three and four of the cpDNA phylogeny with high statistical support, and *Glycocystis* is resolved to be most closely related to *Myoporum platycarpum* in clade four. This placement of *Glycocystis* supports Chinnock's morphology-based prediction that *Glycocystis* would be most closely related to a *Myoporum* of sect. *Disoon*, and the preliminary findings of Kelchner (2003), though it calls into question the generic limits of *Myoporum*.

All four species of Western Australian genus *Diocirea* are spread throughout the large and geographically widespread clade nine, which is composed of morphologically diverse species of *Eremophila* from across Australia. *Diocirea microphylla* and *D. acutifolia* are strongly supported as sister to one another, and most closely related to species of *Eremophila* in sections *Crustaceae* (*E. interstans*, *E. ciliata*, *E. lactea*, *E. dichroantha*, *E. succinea*), *Arenariae* (*E. arenaria*, *E. aurivisca*) and *Platycarpus* (*E. compressa*). *Diocirea violacea* is sister to *E. parvifolia* (sect. *Australophilae*) while *D. ternata* is resolved as sister to species from sections *Australophilae*, *Pholidia* and *Decussatae*. The sections of *Eremophila* with which species of *Diocirea* are placed (*Arenariae*, *Australophilae*, *Crustaceae*, *Decussatae*, *Pholidia*, *Platycarpus*) are morphologically quite distinct from *Diocirea*. *Diocirea* is unique in exhibiting a persistent corolla, shallow corolla tube and zygomorphic flower with two fused lobes of the upper lip (Chinnock, 2007), which contrasts to the deciduous, tubular and variable lobe configurations of the corollas of *Eremophila*. Though these relationships between *Diocirea* and *Eremophila* are inconsistent with our morphological understanding of these genera and sections, the species grouping in the gene tree do share similarities in geographic distribution (all species are located in south Western Australia). This overlap in distribution may have allowed for historic or contemporary hybridization amongst species, resulting in the introgression of maternally inherited chloroplast genomes. Alternatively, the effects of incomplete lineage sorting between these closely related species may also be impacting the phylogenetic signal of the chloroplast genome. Both of these alternative hypotheses will be considered further in the context of the nuclear ribosomal dataset of Chapter Four.

Placement of monotypic genera *Bontia*, *Calamphoreus* and *Pentacoelium* are all somewhat unexpected based on Chinnock's generic classification, though both *Bontia* and *Calamphoreus* show similar relationships to those recovered by Kelchner (2003). The Caribbean distributed genus *Bontia* is resolved with strong support as sister to Eastern Australian species, *E. debilis*, a relationship revealed previously by Kelchner. Western Australian genus *Calamphoreus* is well supported in clade nine as sister to *E. succinea*, in a lineage composed predominately of species from sect. *Crustaceae* and members of *Diocirea*. Asian distributed genus *Pentacoelium* has not been included in any prior work, though aligns morphologically with species of *Myoporum*. In the current analysis *Pentacoelium* is nested amongst species of *Myoporum* in clade three, sister to Hawaiian species (*M. sandwicense*, *M. stokesii*, *M. degnerii*, *M. wilderii*) and Norfolk Island endemic *M. obscurum*.

Although not entirely unexpected, these results do not support the morphology based generic classification of tribe Myoporeae. *Eremophila* species are scattered throughout the phylogeny, and the boundaries between all genera in Myoporeae are not clear-cut. Based on this chloroplast genome phylogeny the classification of genera in the tribe will likely need significant revision, and this will be explored further in the context of analyses of the nuclear ribosomal DNA dataset of Chapter Four (with taxonomic implications based on total data discussed in Chapter Six).

3.4.2 Validity of Chinnock's (2007) sectional classification of *Eremophila* based on cpDNA

In his treatment of Myoporeae, Chinnock (2007) divided the c. 230 species of *Eremophila* into 25 sections defined by combinations of vegetative, floral and fruit morphological characters. Sections range in size from those that contain a single species (i.e. sects. *Chamaepogonia*, *Carnosae*, *Platycalyx* and *Subscissocarpae*), to the large species assemblages of sections *Australophilae* (32 sp.), *Eremaeae* (54 sp.) and *Eriocalyx* (36 sp.). Chinnock chose this sectional classification as "no well-defined discontinuities could be found to subdivide (*Eremophila*) into subgenera". While Chinnock did hypothesise the "direction" of evolution in character states for the tribe as a whole (relative to sister tribe Leucophylleae), it is unclear whether the characters used to delimit sections were differentiated by Chinnock into plesiomorphies (ancestral characters) or synapomorphies (shared, derived characters). Despite this uncertainty in

evolutionary context, Chinnock's morphology based sections still provide a good framework to which species relationships (inferred via molecular phylogeny) can now be compared.

Overall, the sectional classification of *Eremophila* is only partially supported by the topology of the cpDNA phylogeny, with just one section (*Hygrophanae*) displaying all species resolved as monophyletic. All other sections are not monophyletic. Ten of the 25 sections contain only one or two species, and were characterised by unique morphological features and were of uncertain affinity to other species/sections of *Eremophila* (Chinnock, 2007). Interestingly, five of these ten small sections (*Subscissocarpae*, *Chamaepogonia*, *Platychilus*, *Carnosae* and *Scariosepalae*) resolve at nodes towards the base of the cpDNA phylogeny (in clades one and three). Sister to all members of Myoporeae (clade one) is *E. tetraptera*, of the monotypic section *Subscissocarpae*. *Eremophila tetraptera* has large, red/orange tubular flowers and unique four winged fruit not seen in the rest of the tribe. This placement is supported by Kelchner's (2003) analysis, which also placed *E. tetraptera* in its own lineage distinct from all other Myoporeae. Sections *Chamaepogonia*, *Platychilus*, *Carnosae* and *Scariosepalae* are all closely related to one another and a number of other small sections in clade three with high support.

3.4.2.1 Sections of *Eremophila* supported by the cpDNA phylogeny

Section *Hygrophanae* is the only monophyletic section of *Eremophila* in the cpDNA phylogeny, with all five species well supported as a distinct lineage of clade nine. Other sections, though non-monophyletic, do include some clades with large numbers of species, and with some minor revisions may still represent evolutionarily meaningful groups in a sectional classification. For example, 37 of the 40 taxa (92%) from sect. *Eriocalyx* are placed in clade seven of the cpDNA phylogeny. A number of other species are also present in this *Eriocalyx* dominated lineage, including both species from sect. *Campanulatae* (*E. humilis* and *E. campanulata*), three species from sect. *Australophilae* (*E. sp. 'eversa'*, *E. homoplastica*, *E. occidentis*), and two species from sect. *Eremaeae* (*E. rigens* and *E. incisa*). Chinnock (2007) hypothesised a close relationship between sections *Campanulatae* and *Eriocalyx* based on shared vegetative characters (compact, shrubby habit, presence of stellate pubescent hairs on vegetative and floral parts), in

which case, the nested placement of sect. *Campanulatae* in sect. *Eriocalyx* would support the reconsideration of their combination. Species from sect. *Australophilae*, also nested within sect. *Eriocalyx* (*E. homoplastica*, *E. sp. 'eversa'* and *E. occidens*), were all noted by Chinnock as unusual for sect. *Australophilae*, with morphological characters that tie them to multiple sections, and distributions outside of the range commonly seen in other members of the section. This uncertainty in morphology is an indicator that the placement of these taxa may also be better with sect. *Eriocalyx*.

Species within sect. *Stenochilus* predominantly fall together in clade six, though also with the inclusion of species from sections *Australophilae* and *Virides*. Chinnock hypothesised a sister relationship between sections *Stenochilus* and *Virides*, based on a number of shared morphological features (narrow corolla tube with an aggregated upper lip of four small lobes and a slender recurved lower lip), though the affiliation between sect. *Stenochilus* and species from sect. *Australophilae* is unclear. One exception to the monophyly of members of sect. *Stenochilus* in this cpDNA phylogeny is the disjunct placement of *E. maculata* and *E. racemosa* as a separate lineage (clade nine). All samples of *E. maculata* are strongly supported as monophyletic with *E. racemosa* but distinct from all other species in sect. *Stenochilus*. It is likely that future classification of this section should maintain this split between core *Stenochilus* and *E. maculata* and *E. racemosa*.

3.4.2.2 Sections of *Eremophila* not supported by the cpDNA phylogeny

A number of Chinnock's morphology-based sections of *Eremophila* do not hold up in a phylogenetic context; the most significant examples being sections *Australophilae*, *Eremaeae*, *Pholidia*, *Decussatae* and *Amphichilus*. *Australophilae* is one of the largest sections delimited by Chinnock, containing 32 species, of which 25 were included in the current cpDNA analysis. Species of sect. *Australophilae* were grouped morphologically based on their enclosed stamens, mauve/purple corolla, small fruit, and the presence of hair on leaves and branches, and their geographic distribution in the South-West botanical province of Western Australia (Chinnock 2007). However, the cpDNA analysis reveals a significant lack of monophyly in sect. *Australophilae*, with species scattered throughout multiple clades (five, six seven and nine) of the phylogeny, and often, most closely related to species from other sections of *Eremophila* than they are to one

another. The characters used to delimit sect. *Australophilae* are almost certainly homoplasious rather than synapomorphic. *Australophilae* and a number of the other sections resolved as non-monophyletic in the current analysis are likely to need significant revision and further morphological study, this will be discussed in further detail in the context of the nuclear ribosomal dataset in Chapter Four.

3.4.3 Species level monophyly in tribe Myoporeae based on cpDNA

Similarly to the lack of monophyly recovered at generic and sectional ranks, the chloroplast phylogeny of Myoporeae also revealed widespread non-monophyly with the current morphology-based species taxonomy. In total, 57 of the 223 species (26%) included in the cpDNA analysis were sampled with multiple specimens. Species with particularly widespread distributions, disjunct distributions, and those identified to exhibit intraspecific morphological variation (i.e. presence of subspecies, varieties) were specifically targeted with multiple collections to confirm species boundaries and assess degrees of within species variation. As such, a lack of monophyly in some of these species was not necessarily unexpected. However, non-monophyly of the samples was unexpected for some species and of the 57 species targeted, 12 (21%) were strongly supported as monophyletic, while the remaining 45 species (79%) were supported as para- or polyphyletic.

3.4.3.1 Monophyletic species based on cpDNA

The 12 species strongly supported as monophyletic ($pp \geq 0.95$) in the current analysis include: *Eremophila debilis* (2), *E. polyclada* (2), *E. crassifolia* (2), *E. obovata* (2), *E. conferta* (2), *E. abietina* (2), *E. macdonnellii* (2), *E. platythamnus* (2), *E. miniata* (2), *E. divaricata* (2), *E. longifolia* (3) and *E. arachnoides* (2). Each of these species were sampled from widely across their distribution, and where possible, included multiple subspecies and/or morphological variants. Species such as *E. longifolia* and *E. polyclada* are very widespread in distribution, and while sampling was limited (three and two specimens respectively), the strongly supported sister relationships of their samples (monophyly) supports our current understanding of their morphology based species taxonomy. Similarly, the strongly supported monophyly of disjunct populations/subspecies of *E. arachnoides*, *E. crassifolia* and *E. obovata*, also indicates that the limits of these species as currently circumscribed are likely to be accurate.

3.4.3.2 Non-monophyletic species based on cpDNA

The remaining 45 species sampled with multiple specimens in the cpDNA analysis were resolved as non-monophyletic. These included: *Eremophila alternifolia* (3), *E. fallax* (2), *E. deserti* (3), *Myoporum sandwicense* (6), *E. gibbifolia* (2), *M. laetum* (2), *M. petiolatum* (2), *E. interstans* (2), *E. simulans* (3), *E. denticulata* (2), *E. hillii* (3), *E. lehmanniana* (2), *E. glabra* (12), *E. decipiens* (3), *E. serrulata* (2), *E. subfloccosa* (2), *E. battii* (2), *E. shonae* (2), *E. hughesii* (2), *E. clarkei* (2), *E. gilesii* (4), *E. petrophila* (2), *E. bowmanii* (2), *E. rhegos* (2), *E. forrestii* (2), *E. latrobei* (2), *E. jucunda* (2), *E. compacta* (2), *E. anomala* (2), *E. cuneifolia* (2), *E. freelingii* (3), *E. platycalyx* (2), *E. tietkensii* (3), *E. flaccida* (2), *E. fraseri* (2), *E. phyllopoda* (2), *E. oldfieldii* (2), *E. maculata* (4), *E. christophorii* (2), *E. mackinlayi* (2), *E. rugosa* (2), *E. youngii* (2), *E. weldii* (2), *E. parvifolia* (2) and *E. scoparia* (5).

The most extensively sampled species found to be non-monophyletic in this study was *E. glabra*. *Eremophila glabra* is currently composed of c. 22 subspecies (Chinnock, 2007; Brown and Buirchell, 2011) that range in distribution from the extremely widespread (*E. glabra* subsp. *glabra*) to narrow endemics (i.e. *E. glabra* subsp. *chlorella*, *E. glabra* subsp. *verrucosa*). Twelve samples of *E. glabra* representing 10 subspecies were included in this cpDNA analysis. While all 12 samples were placed in a single clade (six) in the cpDNA phylogeny, *E. glabra* showed a distinct lack of monophyly, with all samples nested amongst other species from sections *Stenochilus*, *Australophilae* and *Virides* (see Figure 3.5). Even the two samples of a single subspecies, *E. glabra* subsp. *glabra* (MJB2437, RMF251), were highly supported (pp≥0.95) in their placement in separate lineages as sister to other subspecies of *E. glabra* and other species of *Eremophila*.

Morphologically *E. glabra* is known to be a highly variable, and its limits were considered 'tentative' by Chinnock (2007), who suggested more detailed field study would be required to determine the boundaries of the different subspecies that appear to grade into one another. Chinnock also hypothesised a close relationship between *E. glabra* and a number of other species within sect. *Stenochilus* (*E. subfloccosa*, *E. decipiens*, *E. hillii*, *E. veneta*, *E. subteretifolia* and *E. splendens*), stating that they would be best considered a part of a broader *E. glabra* species complex. Morphologically these species all share features such as small, green, scale like sepals, and bird pollinated flowers with narrow corolla tubes composed of a four lobed upper lip and a slender

recurved lower lip (Chinnock, 2007). The relationships of the current cpDNA analysis support Chinnock's hypothesis of close relationships between *E. glabra* and all members of sect. *Stenochilus*. The distinct lack of monophyly, not only in *E. glabra*, but all species in sect. *Stenochilus* sampled with multiple specimens (*E. denticulata*, *E. hillii*, *E. decipiens*, *E. subfloccosa*), may be an indication that the limits of many species may require further taxonomic assessment. However, the lack of monophyly may also be attributed to a number of other evolutionary processes impacting the phylogenetic signal from chloroplast genomes (discussed in detail below).

While *E. glabra* and allied species in the *E. glabra* complex were known to be problematic from a taxonomic perspective, a number of other species not previously identified as in need of further taxonomic work were also resolved as non-monophyletic. Each of *E. mackinlayi*, *E. phyllopoda*, *E. simulans*, *E. petrophila*, *E. hughesii*, *E. shonae*, *E. platycalyx*, *E. youngii*, *E. bowmanii*, *E. forrestii*, *E. latrobei*, *E. jucunda*, *E. maculata*, *E. fraseri*, *E. flaccida*, *E. oldfieldii*, *E. interstans* and *E. parvifolia* were sampled to include two or more of their morphologically distinct subspecies. Though not always the case, these subspecies are often also separated geographically. While the lack of monophyly in each of these species may represent true taxonomic differentiation, it is also possible that evolutionary processes, such as introgressive hybridization and/or incomplete lineage sorting, may be impacting the signal of the chloroplast genome and affecting the observed relationships.

3.4.4 *Evolutionary processes that can affect phylogenetic signal from the chloroplast genome*

Introgressive hybridization resulting in chloroplast capture

The process of introgressive hybridization (introgression) has long been documented as a confounding factor in the use of plastid DNA for phylogenetic reconstruction (Rieseberg and Soltis, 1991; Doyle, 1992; Soltis and Kuzoff, 1995; Tsitrone et al., 2003), and is recognised as a significant contributor to incongruence between chloroplast and morphology/nuclear DNA-based datasets (Acosta and Premoli, 2010; Bayly et al., 2013; Folk et al., 2017; Barrett et al., 2018; Kawabe et al., 2018; Schuster et al., 2018).

Hybridization is relatively common in plants (Rieseberg and Soltis, 1991; Whitney et al., 2010), and results in the movement of genetic material across species boundaries.

While hybridization is often identifiable through morphological analysis of first generation offspring, it can be difficult to detect after multiple generations of backcrossing. Introgression resulting in chloroplast capture can occur following the hybridization of two species, then subsequent back-crossing of the hybrid offspring to one of the parental lineages over many generations (Baum and Smith, 2013). Due to the uniparental (clonal) nature of plastid (and mitochondrial) genome inheritance, these genomes can be transferred directly and unrecombined horizontally between species (Doyle, 1992). In contrast, the biparentally inherited nuclear genome is diluted during subsequent backcrossing, resulting in an organism that may exhibit a nuclear signal (and morphological characters) of one species, but a chloroplast genome of another, and ultimately genealogical discordance (Baum and Smith, 2013).

Incomplete Lineage Sorting

Also known as deep coalescence, the process of incomplete lineage sorting (ILS) can also result in genealogical discordance, where a gene tree may not accurately represent a population or species tree. Incomplete lineage sorting can occur when ancestral genetic polymorphisms are retained variably through time and across speciation events, resulting in the retention of polymorphisms in extant taxa (Doyle, 1992; Mallo & Posada, 2016). This process can be particularly evident in species with large effective population sizes relative to the time between species divergence (i.e. in younger species, Maddison and Knowles, 2006; Gurushidze et al., 2010), and can result in a similar phylogenetic signal to the process of introgression (Zhou et al., 2017).

In the case of Myoporeae, and particularly the 80% of species displaying a marked lack of monophyly relative to relationships expected via morphological species taxonomy, it is difficult to discern between the effects of evolutionary processes such as introgression and incomplete lineage sorting, and uncertainty in taxonomic boundaries (due to taxonomic delimitation based on plesiomorphic characters). In the example of *E. glabra* and closely related species, it seems likely that inaccurate taxonomy may be a major driver of the patterns of polyphyly observed in the cpDNA phylogeny. In contrast, a lack of monophyly was unexpected in many of the other species sampled, and while inappropriate taxonomy cannot be ruled out, the effects of introgression and/or ILS seem plausible in a number of these species. For example, *E. mackinlayi* consists of two

morphologically distinct subspecies, which are also geographically disjunct. In the cpDNA phylogeny, *E. mackinlayi* subsp. *spathulata* (RMF09) is placed sister to *E. hygrophana*, while *E. mackinlayi* subsp. *mackinlayi* (RMF159) is well supported as sister to other species in sect. *Hygrophanae* (*E. fasciata*, *E. ovata* and *E. strongylophylla*). While the two subspecies of *E. mackinlayi* may in fact be distinct, independent species and more closely related to other species of *Eremophila* than to one another, introgression may also be playing a role in the chloroplast signal observed. Interestingly, *E. mackinlayi* subsp. *spathulata* overlaps in distribution with *E. hygrophana*, making contemporary or historic hybridization and chloroplast capture between the two species plausible. Without deeper intraspecific/population level sampling it is not possible to test the validity of this hypothesis, however comparison to alternative genomic datasets (i.e. nuclear DNA) may help to clarify the most probable scenario.

3.4.5 Conclusions

The chloroplast genome phylogeny assessed in the current chapter has revealed some interesting trends of incongruence between the morphology based classification of Myoporeae at generic, sectional and species rank. The nested placement of all allied genera amongst *Eremophila* containing lineages supports the previous findings of Kelchner (2003) and strongly suggests that generic boundaries in Myoporeae require revision. The lack of monophyly at both sectional and species rank is likely to be due to complicated factors; at this stage it is not possible to determine the effects of inaccurate taxonomy, or of evolutionary processes such as introgressive hybridization and incomplete lineage sorting on the chloroplast genome phylogeny. While some understanding of the evolutionary history of Myoporeae can be gained through the interpretation of this cpDNA phylogeny, caution must always be exercised in estimating species trees from a gene tree (Doyle, 1992). In order to tease apart issues of taxonomy, introgression and incomplete lineage sorting, comparison of the current cpDNA analysis to alternative genomic datasets (i.e. analyses based on nuclear/mitochondrial DNA) is critical (Rieseberg & Soltis, 1991), and will be explored in the following chapters (Four and Five).

Chapter Four: Phylogeny of Myoporeae based on nuclear ribosomal DNA

4.1 Introduction

A multi-locus, multi-genome approach has long been recommended for robust phylogenetic estimation (Doyle, 1992; Maddison and Knowles, 2006; Mallo and Posada, 2016). To complement the use of chloroplast markers, regions from the nuclear genome are widely used in plant phylogenetic estimation. Non-coding regions of the nuclear ribosomal cistron such as the internal transcribed spacers (ITS) and external transcribed spacer (ETS) have been the markers of choice for over 25 years for the species-level phylogenetic study of plants (Porter and Collins, 1991; Alvarez and Wendel, 2003; Feliner and Rossello, 2007; Poczai and Hyvonen, 2010). Features of the ITS and ETS regions that make them particularly useful for phylogenetic reconstruction include:

1. High copy number; the nuclear ribosomal cistron occurs in hundreds to thousands of tandem copies and may be present on multiple chromosomal loci (Alvarez and Wendel, 2003; Feliner and Rossello, 2007). This high copy number has allowed ease of isolation and amplification, even in aged/degraded leaf tissue such as that from herbarium specimens. In the era of high throughput sequencing and genome skimming, a high copy number means the nuclear ribosomal cistron is sequenced to significantly greater depth than the majority of the remainder of the nuclear genome, allowing ease of sequence assembly.
2. Variable spacer regions flanked by highly conserved gene regions; the nuclear ribosomal cistron is composed of three RNA genes (18S, 5.8S and 26S), internal transcribed spacer regions (ITS1 and ITS2), the external transcribed spacer (ETS) and the non-transcribed spacer (NTS) – see Figure 4.1. Levels of sequence variation within ITS vary across taxa, but at species level, variation is considered to be relatively high, at 10 – 40% (Baldwin, 1992). The location of ITS1 and ITS2 between the highly conserved genes (18S-5.8S and 5.8S-26S respectively) has allowed for development of universal primers that have made the amplification of the region relatively simple across a diverse range of plants (Baldwin, 1992;

Baldwin et al., 1995; Álvarez and Wendel, 2003; Poczai and Hyvönen, 2010), animals (Hillis and Dixon, 1991; Ji et al., 2003; Wörheide et al., 2004) and fungi (Schoch et al., 2012). This is less important from a high throughput genome skimming perspective as regions of sequence are not targeted for amplification or sequencing; however, close proximity to conserved gene regions does aid in the accurate *de-novo* assembly of sequence data and initiation of sequence assembly by mapping to a conserved reference.

3. Biparental inheritance; unlike plastid or mitochondrial genomes, nuclear ribosomal DNA has maternal and paternal alleles in diploid organisms and is inherited biparentally (Álvarez and Wendel, 2003; Poczai and Hyvönen, 2010). While this can present some challenges when determining gene homology/paralogy, this feature allows for the effects of reticulation/introgressive hybridization to be observed. Particularly with the use of a high throughput sequencing approach, potential variation in copies of ITS/ETS are easily observed across different reads covering the same DNA region, and this variation can be analysed independently (a method previously unavailable using Sanger sequencing data, without laborious and expensive bacterial cloning of large number of amplicons).
4. Concerted evolution; despite the presence of potentially thousands of copies of the nuclear ribosomal cistron repeat, the process of concerted evolution acts as a homogenization mechanism, whereby genes and spacers within the array evolve as a single unit, likely due to unequal recombination, selection, deletions and/or duplications (Hillis and Dixon, 1991; Álvarez and Wendel, 2003; Ganley and Kobayashi, 2007; Xu et al., 2017). This process of homogenization means independent mutations on separate gene copies are jointly shared, and can allow for ease of sequencing and analysis. However this process can be imperfect, and increasingly highly divergent pseudogenes/paralogues within an individual are being reported (Ganley and Kobayashi, 2007; Lunerová et al., 2017; Xu et al., 2017) which result in uncertainties when assembling gene regions and create conflict in phylogenetic reconstruction (Baldwin et al., 2016).

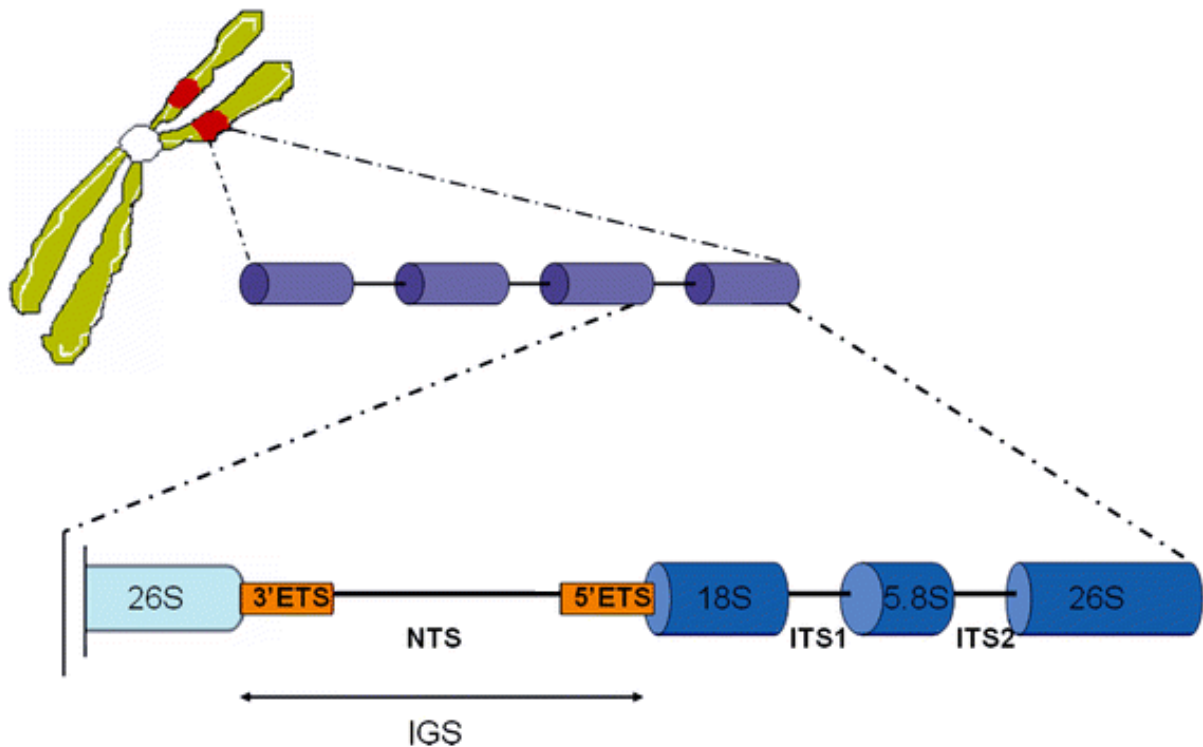


Figure 4.1 Gene map of the nuclear ribosomal cistron, from Poczai and Hyvönen (2010). The non-transcribed spacer (NTS) and external transcribed spacer (ETS) separate the gene blocks, which include internal transcribed spacers 1 and 2 (ITS1, ITS2) and genes 18S, 5.8S and 28S.

There have been no phylogenetic studies of tribe Myoporeae based on nuclear ribosomal DNA to date. Masters student Liu Chang (2008) sequenced the single copy nuclear gene *waxy* for 22 species of *Myoporum* and a small number of related genera from tribe Myoporeae, however, the presence of multiple copies of that gene did not allow for sound interpretation or resolution of species relationships. A comprehensive chloroplast genome-based phylogeny of Myoporeae was undertaken in Chapter Three, which presented a highly supported phylogeny congruent with previous chloroplast analyses of Myoporeae (Kelchner, 2003; Chang, 2008). Despite high topological support, the generic, sectional and species level relationships established in the cpDNA phylogeny were not always congruent with the current morphological classification of the tribe. In particular, a significant lack of monophyly in c. 80% of species represented by multiple accessions indicated that evolutionary processes, such as incomplete lineage sorting and introgression, may be impacting the phylogenetic signal and generating discrepancies between the chloroplast phylogeny and species relationships.

Therefore it was decided to generate a dataset from the nuclear genome in order to compare phylogenetic patterns from reconstructions based on alternative genomes.

4.1.1 Aims

The core aim of this component of research was to generate a comprehensive phylogeny of tribe Myoporeae using sequences from the nuclear ribosomal cistron (nrDNA), which could then be compared to relationships identified using the chloroplast phylogeny.

Specific research questions for this chapter include:

- 1) What are the relationships between the seven genera within Myoporeae based on nrDNA, and are these relationships congruent with those established using complete chloroplast genomes (cpDNA)?
- 2) Are the morphology-based sections of *Eremophila*, proposed by Chinnock (2007), supported by nrDNA, and how do they compare to sectional relationships resolved in the cpDNA analysis?
- 3) Are species monophyletic, and when represented by multiple samples are patterns of monophyly consistent with those established using cpDNA?

4.2 Methods

The methods specific to sampling, DNA assembly and sequence analysis of nrDNA are detailed below. More general details of specimen collection, DNA extraction, library preparation, contig assembly and sequence alignment are outlined in Chapter Two.

4.2.1 Taxon sampling

A total of 355 individuals were included in this part of the study (see Appendix Five), representing 212 species of *Eremophila* (including 7 putatively undescribed species), 20 species of *Myoporum*, all four species of *Diocirea* and representatives of the allied monotypic genera *Calamphoreus*, *Glycocystis*, *Bontia* and *Pentacoelium*. These samples represent all 25 of Chinnock's (2007) described sections of *Eremophila*, and all four sections of *Myoporum*. Members of the sister tribe to Myoporeae, Leucophylleae (*Leucophyllum frutescens* and *L. zygothallum*), were sourced and sequenced as outgroups for this study. In addition, the only two currently available partial ITS/ETS

sequences available on GenBank representing Myoporeae, *Eremophila rotundifolia* (DQ444240) and *Eremophila macdonnellii* (DQ444239), were included for comparison.

4.2.2 Assembly of nuclear ribosomal cistron repeat unit

Once *de-novo* contigs of total genomic DNA had been generated using CLC Genomics Workbench (<https://www.qiagenbioinformatics.com/>) or MegaHit (Li et al., 2015, 2016), as described in Chapter Two, they were imported into Geneious version 9.1.8 (<http://www.geneious.com>, Kears e et al., 2012) along with raw paired read data. Two species of *Eremophila* were available in GenBank for a portion of the nuclear ribosomal cistron, ITS1–5.8S–ITS2 (<https://www.ncbi.nlm.nih.gov/>, NCBI, 2017), however, the aim was to assemble as much of the cistron array as possible. In the absence of a close reference for the entire array, a template array in Geneious was constructed by combining existing sequence data for *Eremophila* ITS1–5.8S–ITS2 with a more completely assembled *Eucalyptus* cistron sourced from GenBank. Due to the highly conserved nature of the 18S, 5.8S and 28S genes, sequence data from even distantly related genera are comparable. To this ‘hybrid’ draft array I was able to map contigs for a number of *Eremophila* species and select the sample with the most complete coverage of the array as an accurate and closely related reference sequence for all other members of Myoporeae. Contigs from all samples were mapped to this *Eremophila* array consensus sequence using custom sensitivity settings in Geneious (gaps allowed set to a maximum of 30% per read, maximum gap size of 3000) with 3–5 iterations of mapping reads back to the consensus of the previous iteration. These settings allow for gaps to be inserted into the mapped contigs or reference sequence during alignment, which is critical when constructing sequences that are often highly variable between individuals due to the prevalence of indels. Once accurately mapped to the reference array, a consensus sequence was generated for each sample, and raw reads were mapped back over the consensus using default settings to confirm accuracy of the sequence. A final consensus sequence for each sample, based on a 75% strict consensus threshold, was generated and extracted with reference sequence annotations. Intra-individual sequence polymorphisms were often detected, which may be attributed to the presence of variable sequences among copies in a single nrDNA array or among arrays on different chromosomes. Such variation could result from a lack of concerted evolution within or between arrays, through hybridisation or through polyploidy. Due to the short

read lengths (125bp) used to assemble sequences, it was not possible to determine links between each of these polymorphic sites or separate cistron copies. For this reason, nrDNA sequences with polymorphisms were assigned DNA ambiguity codes that allow for their inclusion in subsequent Bayesian analyses.

4.2.3 Sequence alignment

All nuclear ribosomal cistron repeat unit sequences were aligned in Geneious using the MAFFT pairwise alignment plug-in (MAFFT version 7.222 (Kato and Standley, 2013)) with the default settings. Once aligned, annotations for each sequence were compared to published annotations of *Eremophila* samples in GenBank (*E. rotundifolia*, DQ444240 and *E. macdonnellii*, DQ444239). Alignments were checked by eye and adjustments made manually. Sections of alignment unable to be resolved (often due to long stretches of repetitive single base repeats or repetitive indels) were recorded and excluded from further analyses. The boundaries of 18S, 5.8S and 28S genes were highly conserved, and the ITS1, ITS2 and ETS regions were constructed in full for all samples. However, the non-transcribed spacer (NTS) region was extremely variable in sequence content within individuals, and the presence of varying copies of this region was evident in all samples. Due to this sequence uncertainty, the lengths of NTS regions included for each sample varied, and a complete sequence of the entire operon was not able to be constructed.

4.2.4 Bayesian inference analysis

Model testing of the nrDNA alignment was conducted in MrModeltest version 2.3 (Nylander 2004). The alignment was partitioned into two character sets representing coding ribosomal DNA sequence (18S, 5.8S and 28S genes) and intergenic spacers (NTS, ETS, ITS1 and ITS2). These partitions were defined as it was assumed that rates of evolution would more likely be similar within genes and spacers and differ between these partitions. Models selected for each partition of BI analysis were based on the Akaike information criterion (AIC). The suggested model of evolution for both the coding DNA sequence and intergenic spacer region partitions was GTR+I+G; and was applied in the following analysis.

A Bayesian Inference analysis for the nrDNA alignment was run in MrBayes version 3.2 (Ronquist et al., 2012) using the default settings. The MCMC analysis was run for 15 million generations with a tree sampling frequency of 1000, and partitions unlinked.

The average standard deviation of split frequencies reached a value less than 0.01 during this analysis, with convergence of MCMC chains checked in Tracer version 1.6 (Rambaut et al., 2014). Twenty five percent of the trees were discarded as burn-in, a consensus tree was generated and Bayesian posterior probabilities estimated for nodes from the consensus tree.

4.3 Results

The BI analysis resulted in a moderately supported phylogeny of tribe Myoporeae. Although not completely resolved, this phylogeny allows for the identification of many major lineages within Myoporeae and comparison with the chloroplast genome phylogeny presented in Chapter Three. Fourteen major clades (labelled clades one to fourteen) are identified with strong support ($pp > 0.95$; for a summary diagram of the major clades in the BI phylogenetic reconstruction see Figure 4.2). Myoporeae is monophyletic and distinct from outgroup species of *Leucophyllum*. A polytomous node joins clade one, clades two+three, clade four and a large clade including the remainder of taxa (clades five to fourteen) (see Figure 4.2). Clade one contains *E. debilis* from the monotypic section *Chamaepogonia*, clade two contains two of the four species from sect. *Duttonia* (*E. gibbifolia*, *E. barbata*), and clade three contains the monotypic genus *Glycocystis*, a single species of *Myoporum* (*M. platycarpum*) and a single representative of *E. deserti* (sect. *Pholidiopsis*) as separate in the tree from all other accessions of the same species (located in clade four). Internal support for all nodes within each of these clades is high (see Figure 4.3).

Clade four ($n=44$, see Figure 4.3) is highly supported as a distinct lineage ($pp=1$) and contains four of the seven genera within the tribe: *Eremophila*, all but one sample of *Myoporum* (the exception being a sample of *M. platycarpum* placed in clade three), *Pentacoelium* and *Bontia*. Monotypic genera *Bontia* and *Pentacoelium* are both nested within this clade; the Caribbean genus *Bontia* is resolved in a clade with Australian *Eremophila* species *E. pentaptera* and *E. polyclada*, while the Asian genus *Pentacoelium* is nested among Australian species of *Myoporum*. Sections of *Eremophila* found in this clade include all representatives of *Platychilus*, *Subscissocarpae*, *Carnosae* and *Platychilus*, and the majority of species from sect. *Pholidiopsis* and *Duttonia*. All species

in this clade that were sampled with multiple accessions (*E. crassifolia*, *E. polyclada*, *E. fallax*, *E. deserti*, *M. petiolatum*, *M. laetum* and *M. sandwicense*) are resolved as monophyletic.

Clade five (n=6, see Figure 4.4) is composed predominantly of samples from sect. *Scariosepalae*, with all individuals of the widely distributed species *E. alternifolia* grouping with *E. purpurescens*. The relationships between these individuals are not well resolved. Sister to sect. *Scariosepalae* in clade five are the Western Australian species, *E. rostrata* (sect. *Amphichilus*) and *E. saligna* (sect. *Platycarpus*). While not labelled as numbered clades, subtending and sister to clades six to 14, are two lineages each containing a single species. The placement of both of those species, *Eremophila oldfieldii* (sect. *Pulchrisepalae*) and *E. adenotricha* (sect. *Australophilae*), in the nrDNA analysis is not well supported (pp=0.82 and 0.8, respectively, see Figure 4.4).

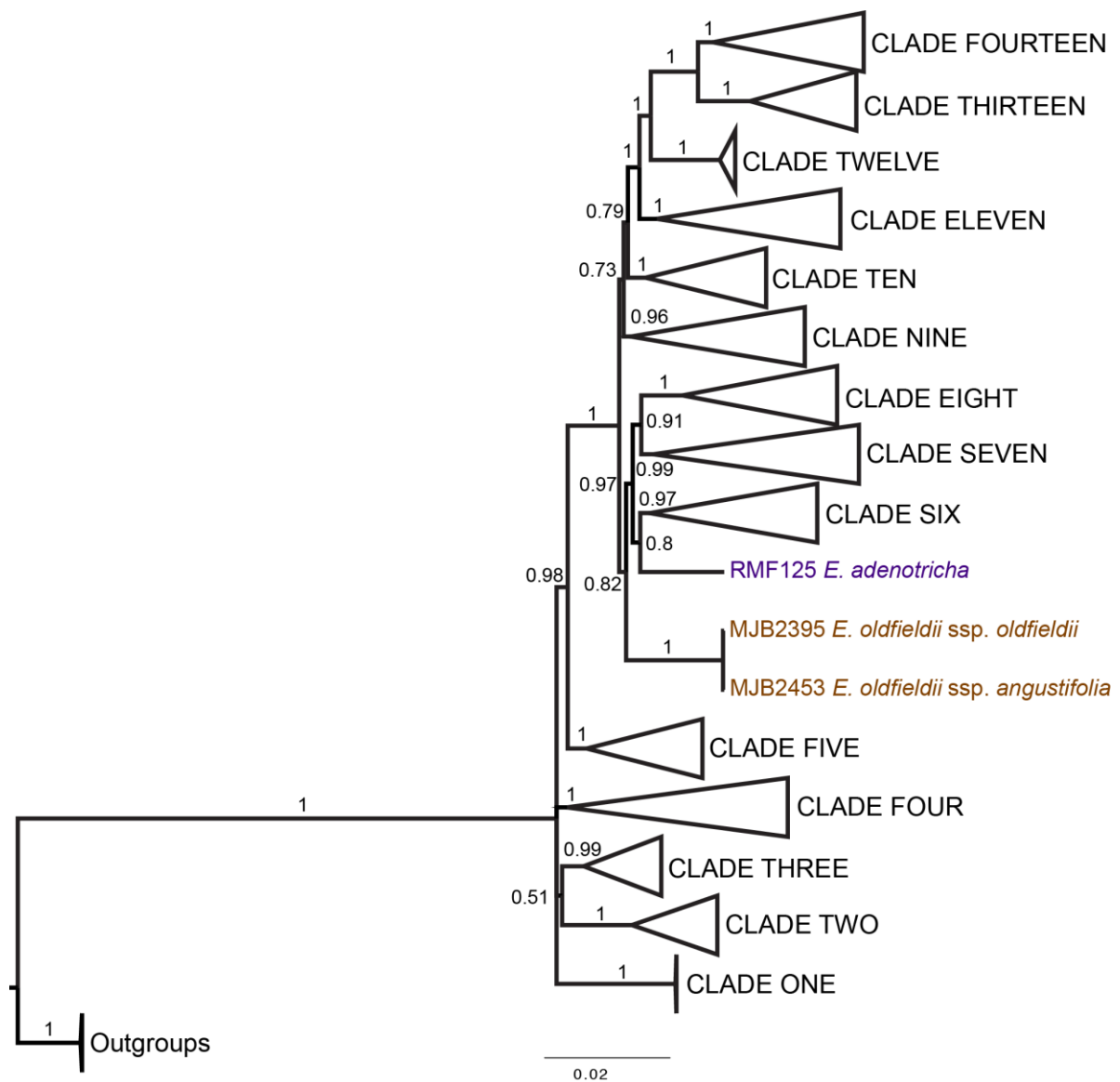


Figure 4.2 Phylogenetic tree based on the Bayesian Inference (BI) analysis of nrDNA, with clades collapsed to summarise. Posterior probability (pp) values are labelled for each branch.

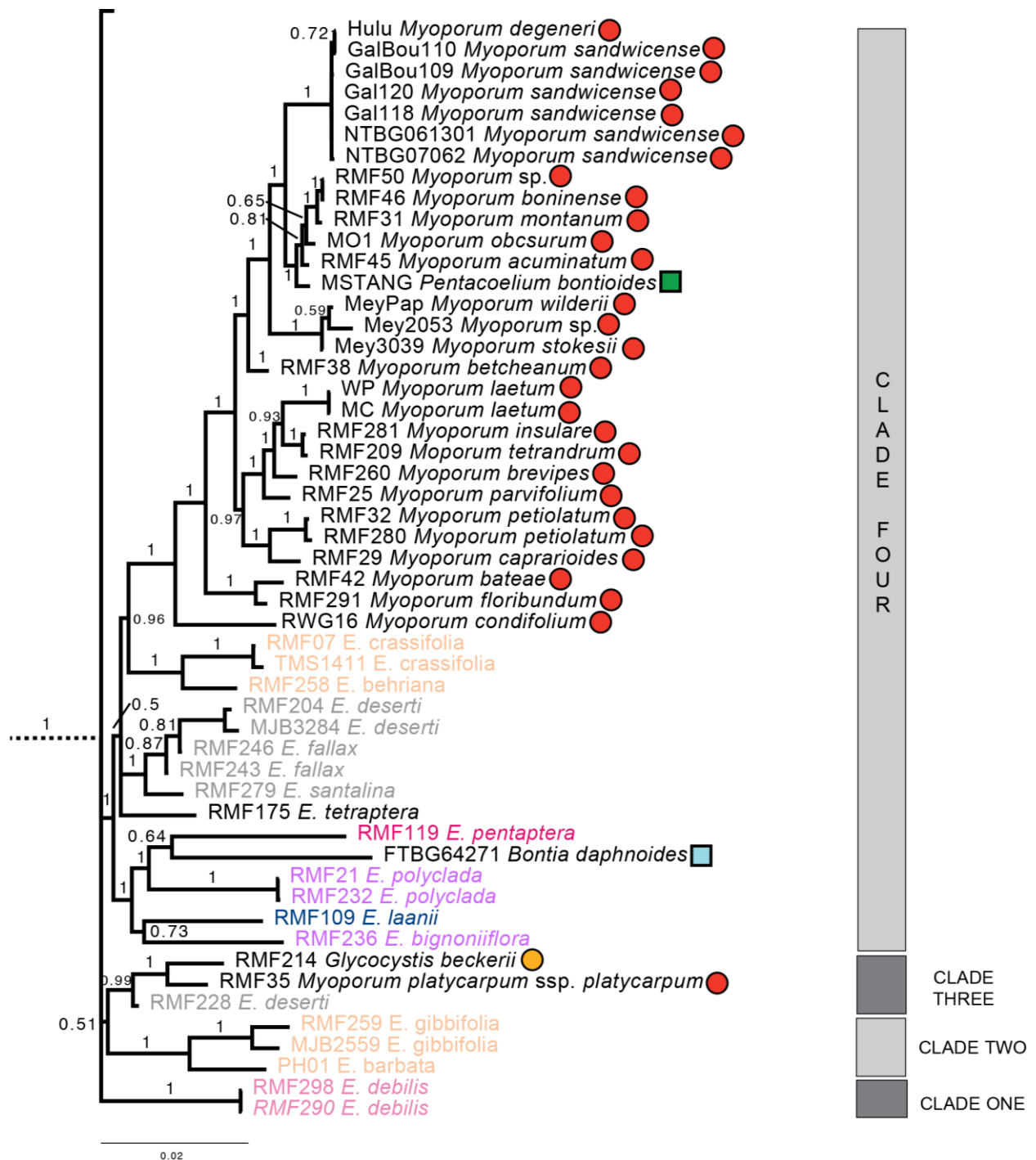


Figure 4.3 Clades one to four of the nrDNA Bayesian Inference (BI) majority-rule consensus tree. Posterior probabilities are included for each branch, *Eremophila* species are coloured according to their sectional classification (*Chamaepogonia*, *Duttonia*, *Pholidiopsis*, *Platyichilus*, *Amphichilus*, *Carnosae*) and other genera are distinguished with coloured symbols.

Clade six (n=29, see Figure 4.4) contains *Eremophila* species from five different sections, as well as the monotypic Western Australian genus *Calamphoreus*. Most internal nodes within this lineage are well supported, allowing for good resolution of relationships between terminal taxa. The largest proportion of this clade is composed of species from sect. *Crustaceae*, which are unique to this lineage. Nested within *Crustaceae* are all members of sect. *Arenaria* (except *E. spuria*), along with *E. lucida*, *E. viscida* and *E. neglecta* (from sect. *Pulchrisepalae*), *E. oppositifolia* and *E. arbuscula* (from sect. *Eremophila*) and one half of sect. *Contortae*, i.e., *E. macgillvrayi*. The Western Australian genus *Calamphoreus* is firmly placed with members of sect. *Crustaceae* and sect. *Arenariae*, though there is insufficient support to determine sister relationships.

Clade seven (n=48, see Figure 4.5) is a well-supported lineage containing members of seven sections of *Eremophila* and all four species of the small Western Australian genus *Diocirea*. While a number of internal branches in the clade lack support, a higher level of resolution is evident among clade terminals. Clade seven is dominated by members of sections *Australophilae* and *Pholidia*. While sect. *Pholidia* is exclusive to this clade, *Australophilae* is a large section scattered throughout four other clades across the phylogeny. Species from sections *Sentis*, *Decussatae* and *Platycalyx* are found only in clade seven, while *E. attenuata* (sect. *Eremaeae*) and *E. bowmanii* subsp. *latifolia* (sect. *Eriocalyx*) represent single, anomalous occurrences of sections that are otherwise monophyletic in other parts of the tree. The genus *Diocirea* contains four species restricted to south-west Western Australia, all four of which are placed in clade seven amongst species of sect. *Australophilae*. *Diocirea violacea* is non-monophyletic, with one sample placed with the other three species of *Diocirea*, and a second sample placed in a separate lineage amongst species of sect. *Australophilae* (sister to *E. weldii*). All species represented from sect. *Decussatae* are non-monophyletic and nested within sect. *Pholidia*. Non-monophyletic species within the clade include *E. youngii* (sect. *Pholidia*) and *E. parvifolia* (sect. *Australophilae*). Notably, *E. scoparia*, which is widely distributed throughout southern Australia and represented with five accessions from right across its range, is resolved as monophyletic.

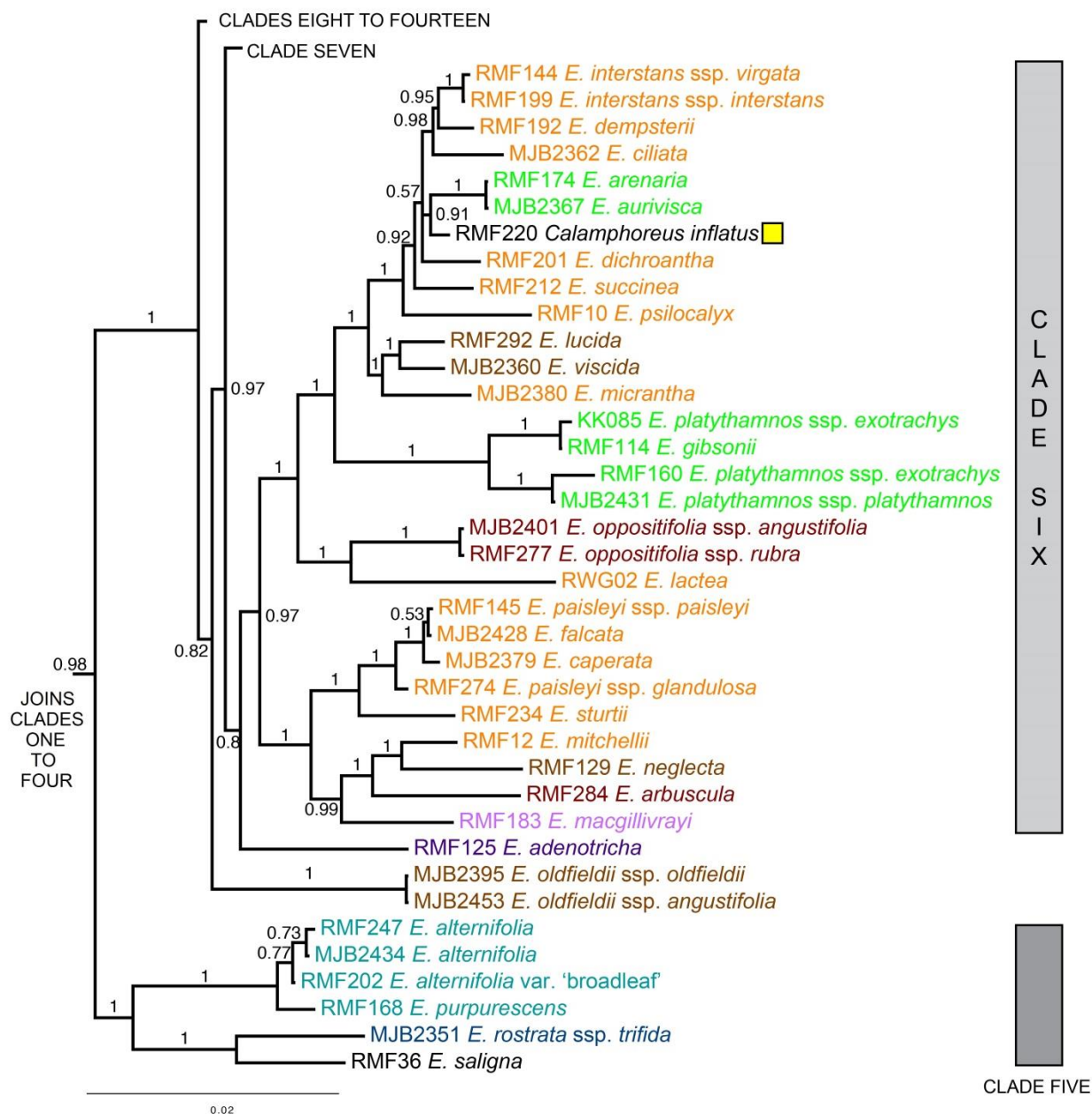


Figure 4.4 Clades five to six of the nrDNA Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification, other genera distinguished with coloured symbol (*Amphichilus*, *Scariosepalae*, *Pulchrisepalae*, *Australophilae*, *Contortae*, *Eremophila*, *Crustaceae*, *Arenariae*).

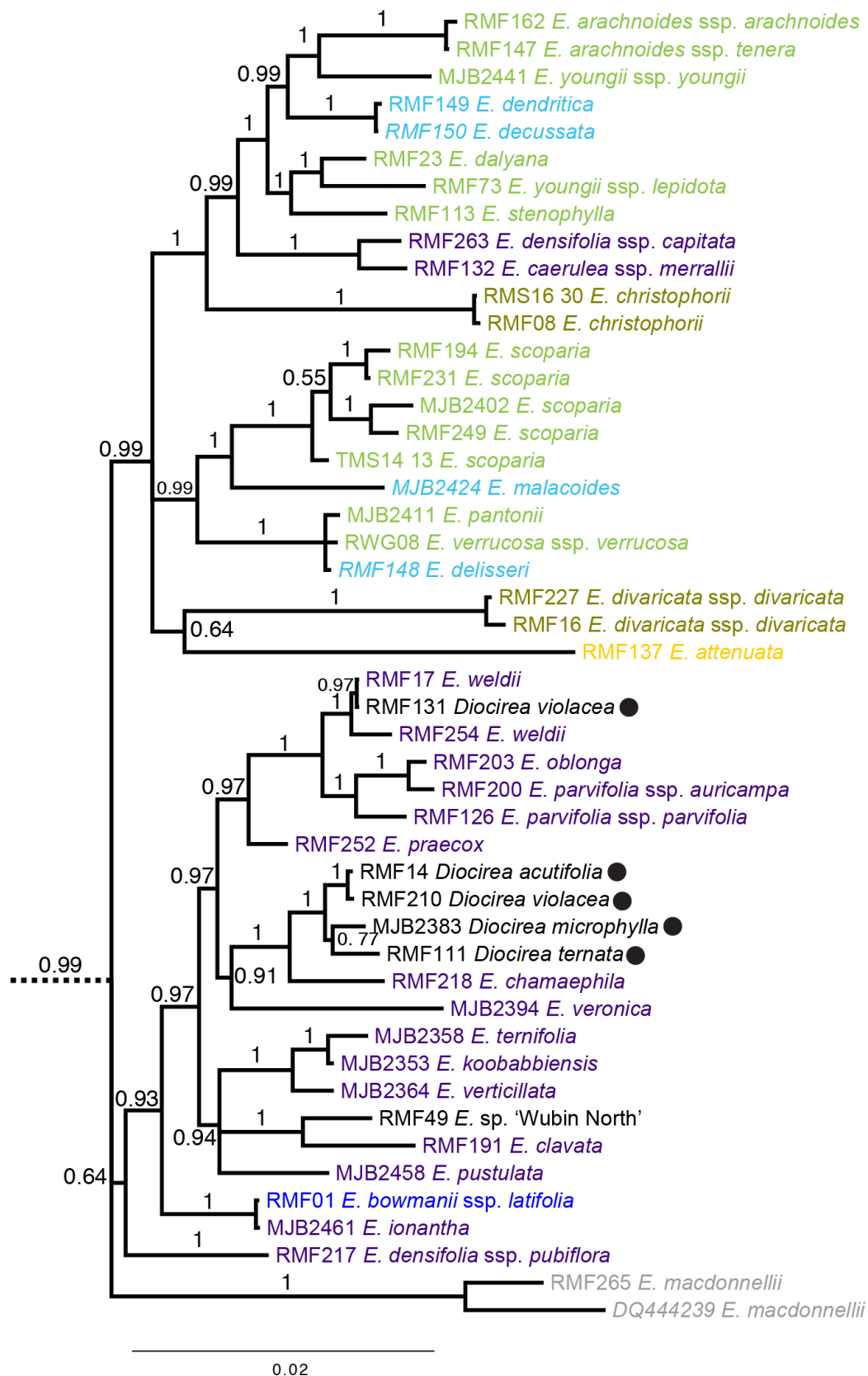


Figure 4.5 Clade seven of Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification, other genera distinguished with coloured symbol (*Platycalyx*, *Australophilae*, *Eriocalyx*, *Eremaeae*, *Sentis*, *Pholidia*, *Decussatae*).

Clade eight (n=49, see Figure 4.6) has strong support as a distinct lineage, although it does lack statistical support at 15 of its 41 internal nodes. Within the clade, three sections of *Eremophila* are represented *Stenochilus*, *Australophilae* and *Virides*. As a section *Virides* is monophyletic and unique to this clade. Eleven individuals from sect. *Australophilae* are placed in clade eight, which represents the largest grouping of sect. *Australophilae* outside of clade seven. All but two species from sect. *Stenochilus* are placed within clade eight, many of which are represented by multiple accessions due to the presence of significant morphological variation evident within species, and wide species distributions. *Eremophila glabra* is the largest species complex within tribe Myoporeae, containing 11 formally described subspecies and a large number of putative subspecies. Thirteen accessions of *E. glabra* were included in this analysis, representing eight named subspecies and three putative subspecies. Those 13 individuals are scattered amongst other species of sect. *Stenochilus* in the tree. A lack of resolution between many samples prevents sound conclusions from being made, but at least some well supported nodes in the clade indicate that *E. glabra* is not monophyletic. Other species from sect. *Stenochilus* with multiple accessions represented within this clade include *E. subfloccosa*, *E. hillii*, *E. decipiens*, *E. denticulata*, and *E. calorhabdos*. Of these, *E. subfloccosa*, *E. decipiens* and *E. denticulata* also show evidence for a lack of monophyly.

Clade nine (n=14, see Figure 4.7) contains three sections of *Eremophila*: *Hygrophanae*, *Stenochilus* and *Platycarpus*. Section *Hygrophanae* is unique to this clade, and forms a well-supported monophyletic lineage (pp=1). *Eremophila mackinlayi* is the only species within the section to have multiple accessions included in the analysis, and is found to be non-monophyletic. *Eremophila compressa* comprises half of sect. *Platycarpus* and is well supported as sister to the members of sect. *Stenochilus* not found in clade six (*E. maculata* and *E. racemosa*). Multiple accessions of *E. maculata* were included in the study due to its wide geographic distribution and division into multiple subspecies; *E. maculata* is supported as monophyletic and sister to *E. racemosa*.

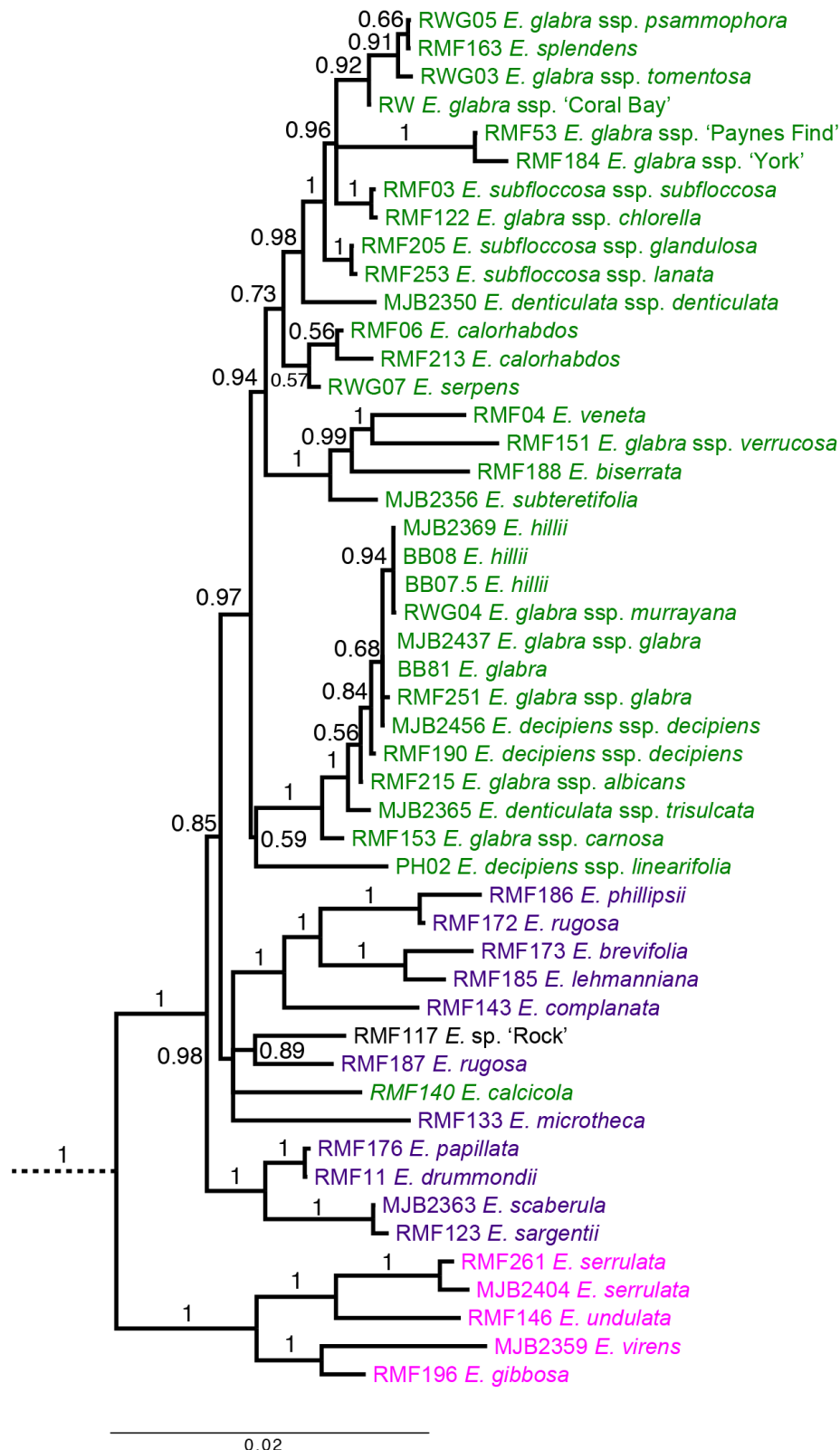


Figure 4.6 Clade eight of Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Virides*, *Australophilae*, *Stenochilus*).

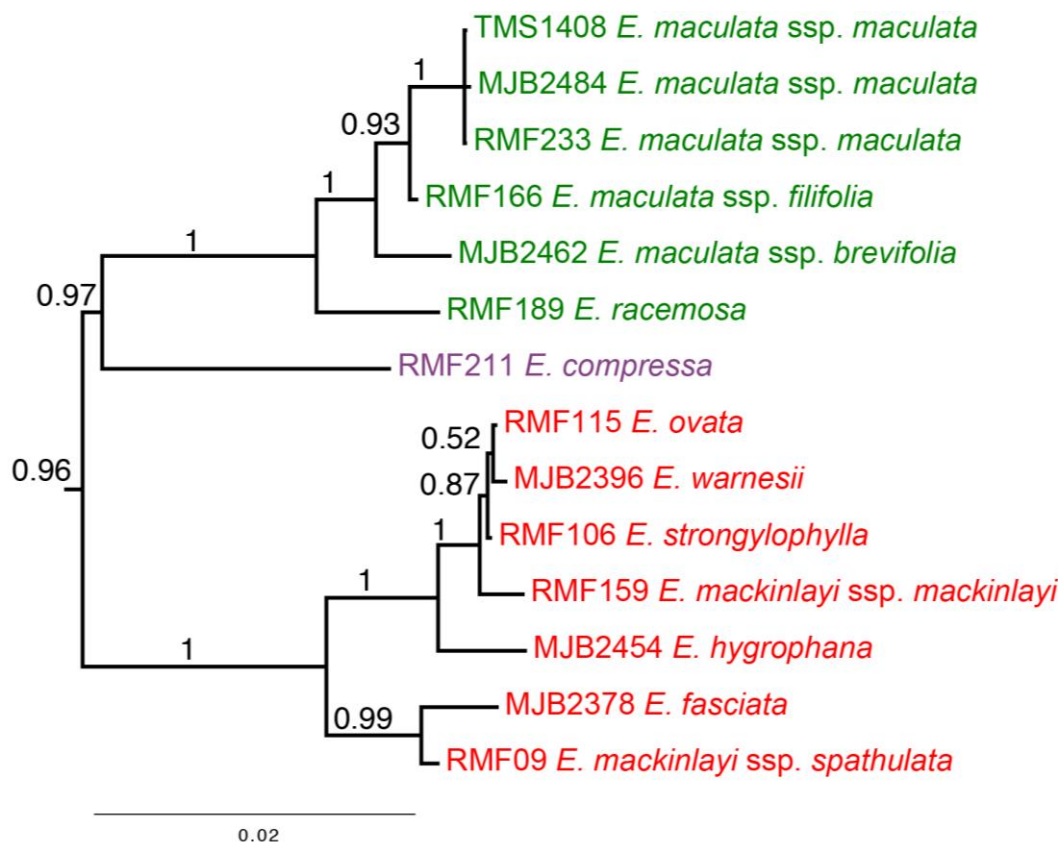


Figure 4.7 Clade nine of Bayesian inference (BI) analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Hygrophanae*, *Platycarpus*, *Stenochilus*).

Clade ten (n=6, see Figure 4.8) includes four samples of *E. longifolia* (sect. *Amphichilus*), and single specimens of *E. perglandulosa* (sect. *Australophilae*) and a putative undescribed species, *E. sp. 'Tropicana'*. *Eremophila longifolia* is one of the most widely distributed species within Myoporeae, spread across the majority of Australia. For this reason, multiple accessions were included from across its range, all of which form a monophyletic group.

Clade eleven is a large lineage (n=54, see Figure 4.8) composed predominantly of species from sect. *Eriocalyx*. Nested within sect. *Eriocalyx* are both members of sect. *Campanulatae*, three species from sect. *Australophilae* (*E. occidentis*, *E. homoplastica*, *E. eversa*) and a single anomalous species from sect. *Eremaeae* (*E. rigens*). Internal node support is low across much of this lineage, so fine scale resolution of relationships within the clade is limited. A large polytomy containing 33 taxa from sect. *Eriocalyx*, both members of sect. *Campanulatae* (*E. humilis*, *E. campanulata*) and *E. rigens* (sect. *Eremaeae*), does not allow for inferences to be made regarding relationships between each of these taxa. Due to poor internal node support there is an almost complete lack of

resolution between species from sect. *Eriocalyx*, however, some species that are almost certainly non-monophyletic include (but are not limited to) *E. rhegos*, *E. forrestii*, *E. jucunda* and *E. latrobei*. *E. miniata* (sect. *Pulchrisepalae*) and *E. pterocarpa* (sect. *Contortae*) are placed as successive sisters to the clade including all species of sect. *Eriocalyx*.

Clade twelve is small (n=4, see Figure 4.9) and well resolved, containing two species of from sect. *Pulchrisepalae* (*E. duttonii* and *E. linearis*); *E. duttonii* is monophyletic and sister to *E. linearis*.

Clade thirteen (n=43, see Figure 4.10) is well supported (pp=1), though more than 50% of internal nodes (19/35) are not well supported (pp<0.95). This clade is made up almost exclusively of samples from sect. *Eremaeae*, the only exceptions being *E. spuria* (sect. *Arenaria*) and an undescribed species, *Eremophila* sp. *Murgoo*, nested within the clade. The lack of internal node support restricts the inferences that can be made about relationships between species within sect. *Eremaeae*, however, species that are almost certainly non-monophyletic include *E. shonae*, *E. pungens*, *E. hughesii* and *E. battii*. Section *Eremaeae* is also non-monophyletic, with species spread predominately across this clade and clade fourteen.

Clade fourteen (n=46, see Figure 4.11) contains species from four sections of *Eremophila*: *Eremaeae*, *Eremophila*, *Pulchrisepalae* and *Eriocalyx*. The majority of individuals from sect. *Eremaeae* not placed in clade thirteen are placed in this lineage, along with the majority of members from sect. *Eremophila*, apart from *E. oppositifolia* and *E. arbuscula*, which fall into clade six. All remaining members of sect. *Pulchrisepalae* are scattered through the lineage. Poor internal node support has resulted in a large polytomy representing the bulk of samples in this clade, meaning many species level relationships are not determined.

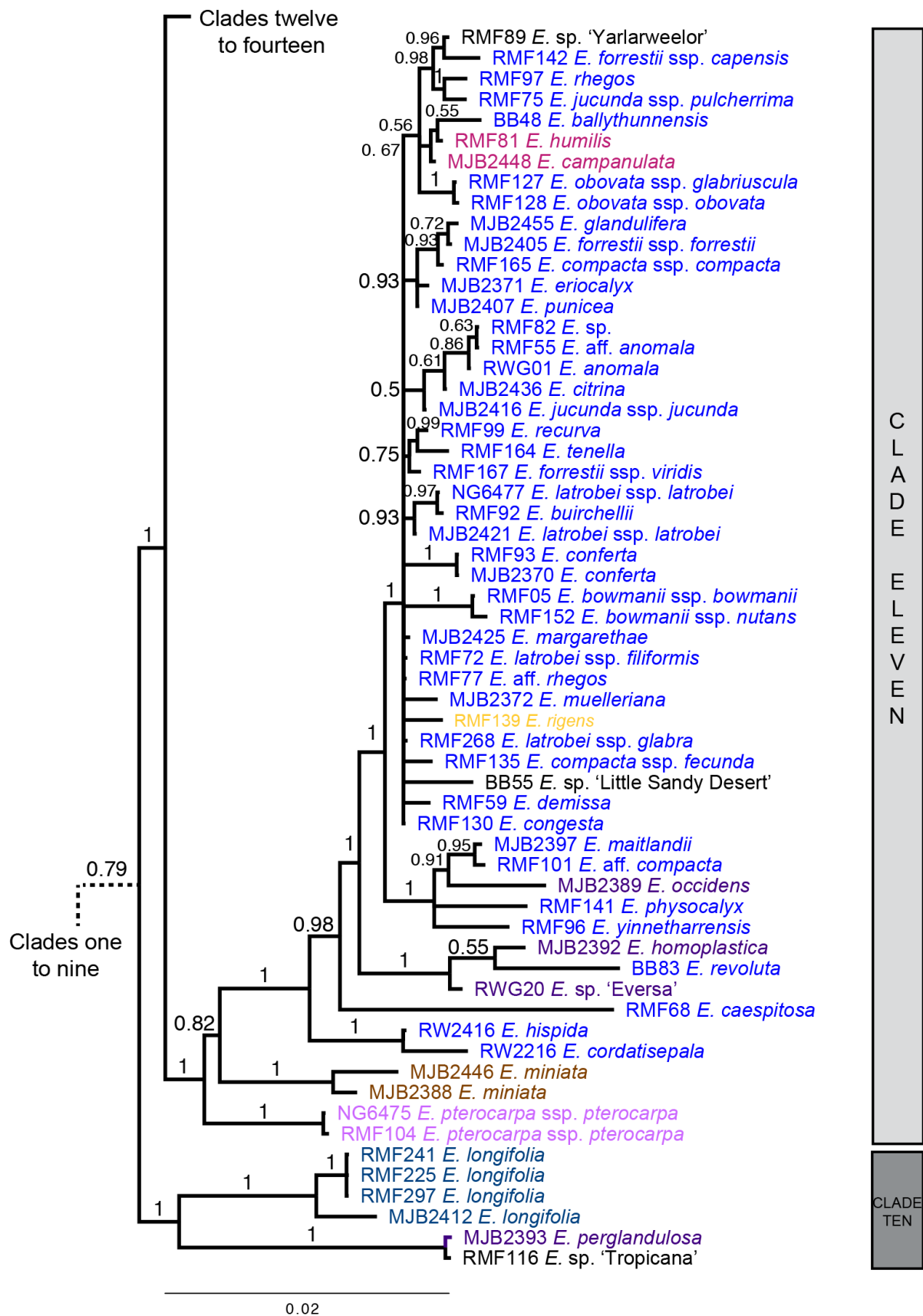


Figure 4.8 Clades ten and eleven of nrDNA Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Australophilae*, *Amphichilus*, *Contortae*, *Pulchrisepalae*, *Eriocalyx*, *Eremaeae*, *Campanulatae*).

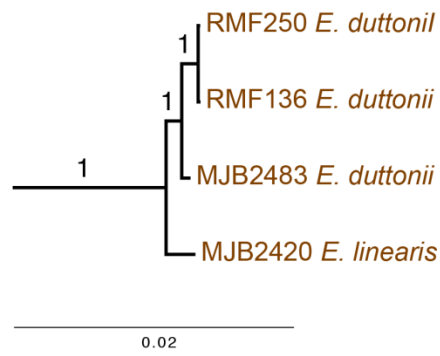


Figure 4.9 Clade twelve of nrDNA Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Pulchrisepalae*).

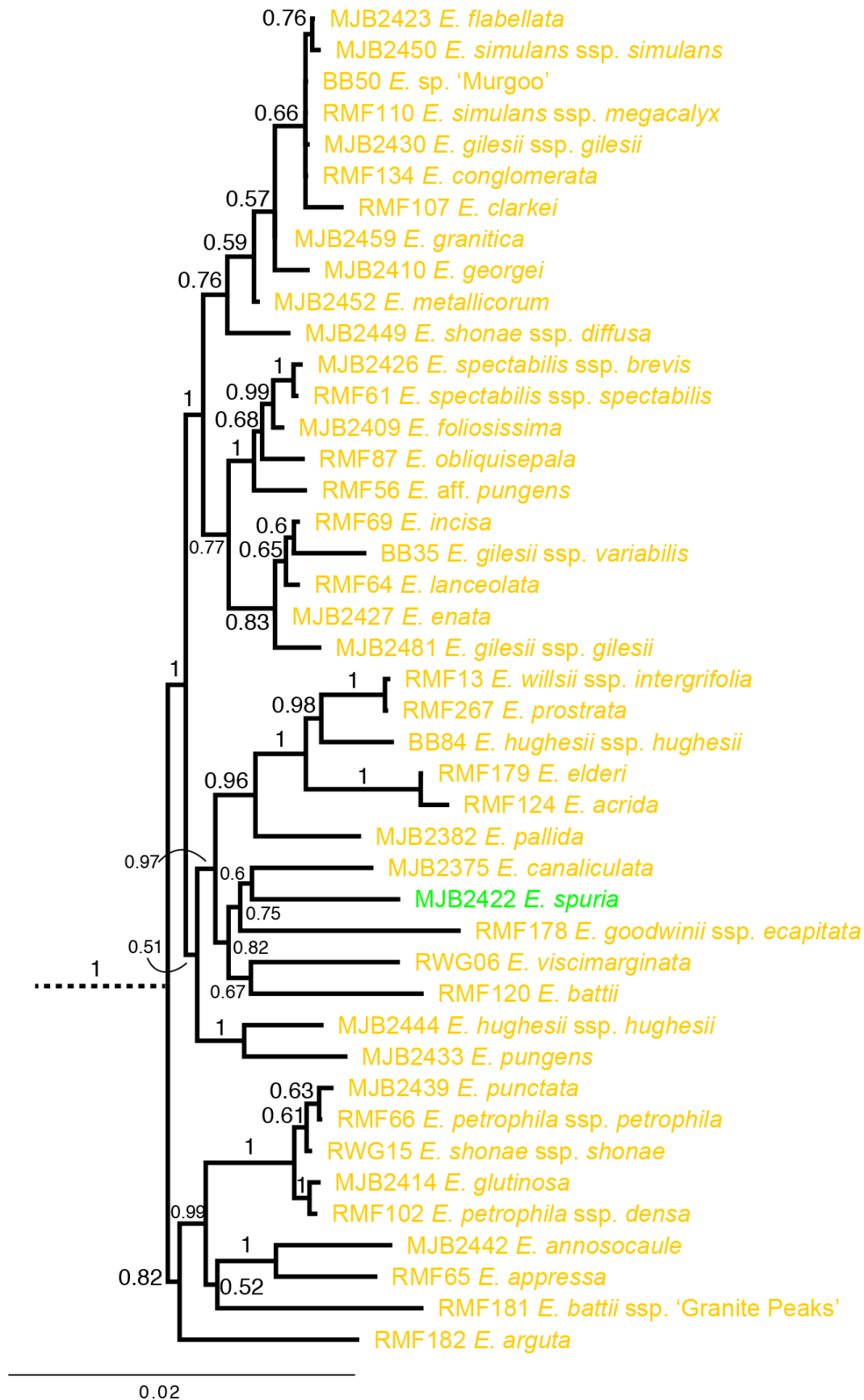


Figure 4.10 Clade thirteen of Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Eremaeae*, *Arenariae*).

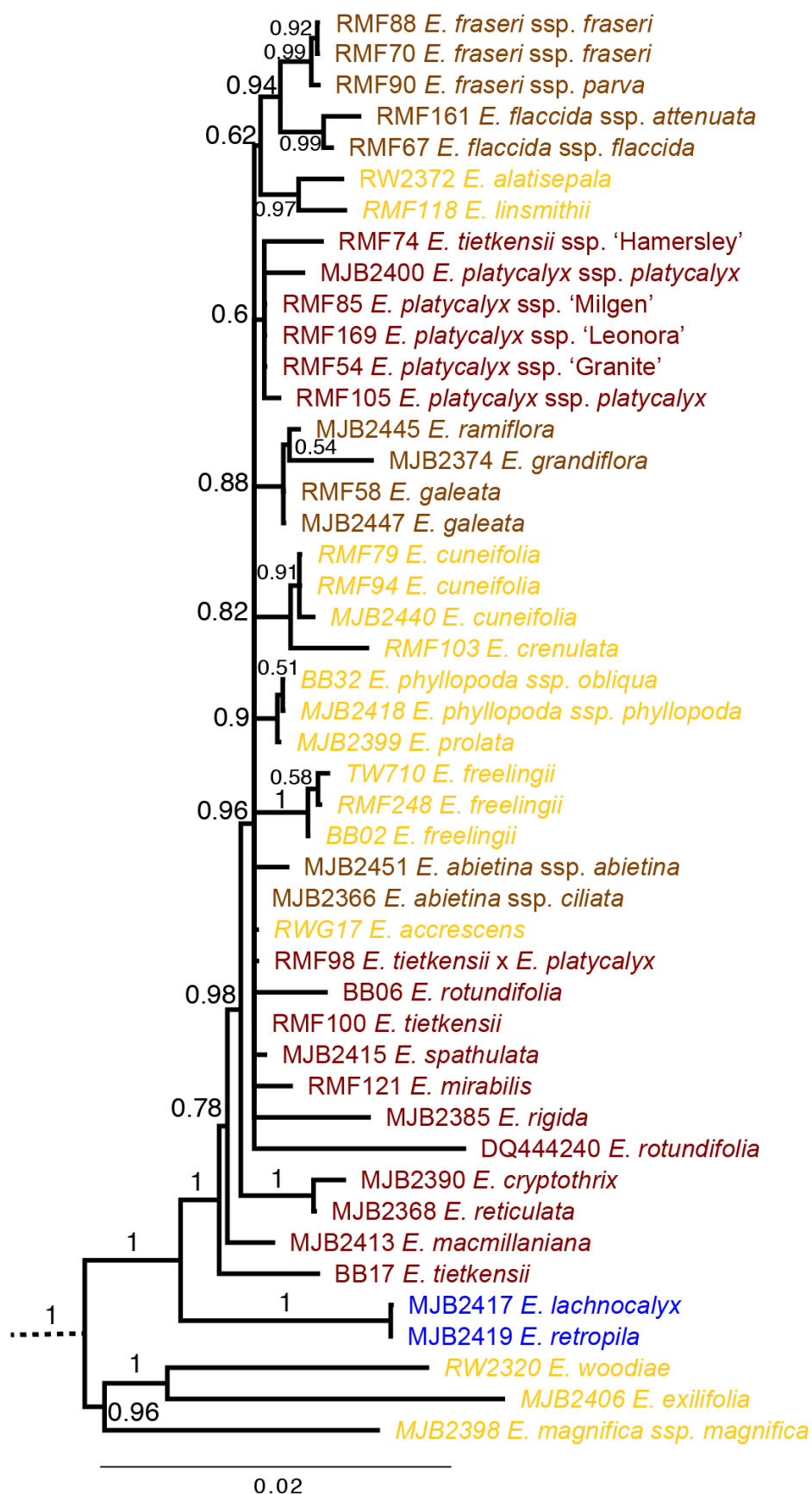


Figure 4.11 Clade fourteen of Bayesian inference analysis. Posterior probabilities included for each branch, *Eremophila* species coloured according to sectional classification (*Eremaeae*, *Eriocalyx*, *Eremophila*, *Pulchrisepalae*).

4.4 Discussion

4.4.1 Comparison of cpDNA and nrDNA datasets

Nuclear ribosomal (nrDNA) and chloroplast (cpDNA) datasets contained largely the same samples and taxa, with 305 specimens (86%) shared between both analyses. More samples were included in the nuclear dataset (355 specimens compared to 317 specimens in the cpDNA dataset), as coverage of reads for the relatively shorter stretch of nrDNA (c. 8000 bp) was more easily obtained than for the assembly of entire chloroplast genomes (c. 150,000 bp), particularly for samples with a lower depth of sequence coverage. Percentage sequence variation in the nrDNA dataset was more than double that of the cpDNA dataset (19% parsimony informative characters compared with 8% in cpDNA dataset). Although percentage sequence variation was higher in the nrDNA dataset, the total number of parsimony informative characters was higher in the cpDNA analysis (11,558 bp vs 1716 bp) due to its greater total length. Overall, 67% (191/283) of nodes in the nrDNA phylogeny displayed high support ($pp \geq 0.95$), compared to 94% of nodes with high support in the cpDNA dataset.

4.4.2 A comparison of generic relationships within Myoporeae: nrDNA versus cpDNA

Species of *Eremophila* were placed in all fourteen major clades in the nrDNA phylogeny of tribe Myoporeae, with all six allied genera nested within these clades. Of the 21 species of *Myoporum* included, 20 species were resolved with high support as a distinct lineage within clade four ($pp=1$), the exception being *M. platycarpum* which is placed sister to the monotypic genus *Glycocystis*, and a single representative of *E. desertii* in clade three ($pp=1$). The general placement of *Myoporum* in this phylogeny is very similar to that of the cpDNA tree. In both cases, the clade containing the majority of *Myoporum* species is sister to species of *Eremophila* from sections *Duttonia* and *Pholidia*, and resolved at a basal node of the phylogeny. The placement of *M. platycarpum* with monotypic genus *Glycocystis* in a separate lineage is also highly supported by both datasets, indicating a relationship between these taxa previously unrecognised by morphological characters. While the general placement of *Myoporum* is similar between chloroplast and nuclear datasets, the relationships within *Myoporum* differ quite significantly. For example, in the cpDNA analysis the two samples of *M. laetum* are placed in separate lineages, sample MC highly supported as sister to *M. insulare* and

sample WP highly supported as sister to *M. cordifolium* (see Figure 3.4). In contrast, in the nrDNA analysis both samples of *M. laetum* are highly supported as sister and most closely related to *M. tetrandrum*, *M. insulare* and *M. brevipes* (see Figure 4.3). Other notable examples of strongly supported incongruence between cpDNA and nrDNA analyses for placement of *Myoporum* species include *M. montanum*, *M. brevipes*, *M. cordifolium*, *M. petiolatum* and *M. parvifolium*. The reasons for this inconsistency in the placement of *Myoporum* species is difficult to confirm based on these data alone, though is likely due to differences in evolutionary processes affecting chloroplast and nuclear datasets. As it stands, the nrDNA analysis most closely reflects the morphological classification of *Myoporum*, though additional sequence data from regions of the nuclear genome could help to confirm which of these gene trees most accurately represents the evolutionary species tree of Myoporeae and what evolutionary processes may be influencing the genomic signal.

Another example of generic incongruence reflected between datasets involves the placement of Asian genus *Pentacoelium* relative to *Myoporum*. *Pentacoelium* is nested deep within the *Myoporum* lineage in the nrDNA phylogeny, sister to mainland Australian species, *M. acuminatum*, *M. boninense* subsp. *australe*, *M. montanum*, and the Norfolk Island endemic *M. obscurum*. The general placement of *Pentacoelium* within the *Myoporum* lineage is consistent with the cpDNA phylogeny also; however, *Pentacoelium*'s placement relative to species of *Myoporum* differs significantly between the datasets. *Pentacoelium* is highly supported as sister to Australian members of *Myoporum* in the nrDNA phylogeny, but based on cpDNA data it is highly supported as sister to all Hawaiian species of *Myoporum*, and *M. obscurum* from Norfolk Island. Once again this strongly supported difference between phylogenies is difficult to interpret and may indicate evidence of different evolutionary processes affecting the phylogenetic signal between chloroplast and nuclear genomes. Despite this lack of certainty in the sister species relationship, *Pentacoelium* is well supported within the core *Myoporum* lineage and will require reconsideration of generic classification (discussed further in Chapter Six).

The monotypic Caribbean genus *Bontia*, is placed in clade four of the nrDNA phylogeny, sister to eastern Australian species, *E. polyclada*, and central South Australian species, *E. pentaptera* (pp=1). In both nrDNA and cpDNA analyses, *Bontia* is placed towards the

base of the phylogeny, however, the sister relationships differ between datasets; in the cpDNA phylogeny *Bontia* is placed with high support (pp=1) as sister to the eastern Australian species *E. debilis*. Relatively long branch lengths for *Bontia* in both analyses suggest significant sequence divergence from related *Eremophila* taxa, although without molecular dating of the phylogeny it is difficult to say whether this is due to a faster rate of sequence variation, longer time since divergence or a combination of both. It is difficult to confirm the most accurate placement of *Bontia* relative to species of *Eremophila*, because both analyses strongly support a close relationship between *Bontia* and different *Eremophila* species from the east of Australia, which is interesting given the significant disjunction between these species distributions. Further discussion surrounding the biogeography of Myoporeae is presented in Chapter Six.

In the nrDNA phylogeny, the small western Australian genus, *Diocirea*, is found to be non-monophyletic and nested within clade seven amongst *Eremophila* species from sect. *Australophilae*. Four of the five representatives of *Diocirea* are placed together in the phylogeny with high support (pp=1), however, the fifth sample (an additional sample of *D. violacea*) is placed separately and with high support (pp=0.97) as sister to *E. weldii* (also sect. *Australophilae*). This placement of *Diocirea* differs to that of the cpDNA phylogeny, where *Diocirea* is non-monophyletic and scattered throughout the large and cosmopolitan clade nine (see Figure 3.8). Until now, placement of *Diocirea* relative to other genera was untested, though Chinnock (2007) predicted a relationship between *Diocirea* and *Eremophila* sect. *Australophilae* based on similarities in growth form and leaf arrangement. *Diocirea violacea* sample RMF131 is placed separately from other species of *Diocirea* and close to *E. weldii* in both cpDNA and nrDNA analyses, which could be attributed to the effects of either introgressive hybridization or incomplete lineage sorting (ILS). Contemporary hybridization has been documented between *D. violacea* and *E. clavata* (also sect. *Australophilae*) (Chinnock, 2007), and hybridization between *D. violacea* and *E. weldii* is equally plausible based on the close proximity of their current distributions (see Figure 4.12) and their relatively close placement in a phylogenetic context. Introgression could explain the morphological features of RMF131 best reflecting *D. violacea*, yet the nuclear ribosomal citron repeat region best matching that of *E. weldii*. To test this hypothesis or that of ILS, more extensive sampling of *D. violacea* across its distribution, in conjunction with additional sampling of closely related species of sect. *Australophilae*, is necessary and could

determine degrees of molecular variation within species and whether there is ancestral polymorphism or more recent gene flow between these taxa.

The monotypic Western Australian genus *Calamphoreus* was nested among species of *Eremophila* from sect. *Crustaceae* and *Arenariae* in clade six of the nrDNA phylogeny. Many of the relationships within this lineage are not well supported, hence the species of *Eremophila* most closely related to *Calamphoreus* cannot be determined. Despite this, the nodes subtending this lineage are highly supported, confirming the placement of *Calamphoreus* with *E. interstans*, *E. dempsterii*, *E. ciliata*, *E. arenaria*, *E. aurivisca*, *E. dichroantha*, *E. succinea* and *E. psilocalyx*. In comparison, the cpDNA phylogeny places *Calamphoreus* sister to *E. succinea* in a lineage also predominately composed of species from sect. *Crustaceae* and *Arenariae*, with all branches highly supported (pp=1). These topologies, though not identical, are consistent, and suggest a close relationship between *Calamphoreus* and *Eremophila* sections *Crustaceae* and *Arenariae*. Taxonomic implications of this placement of *Calamphoreus* relative to *Eremophila* will be discussed further in Chapter Six.

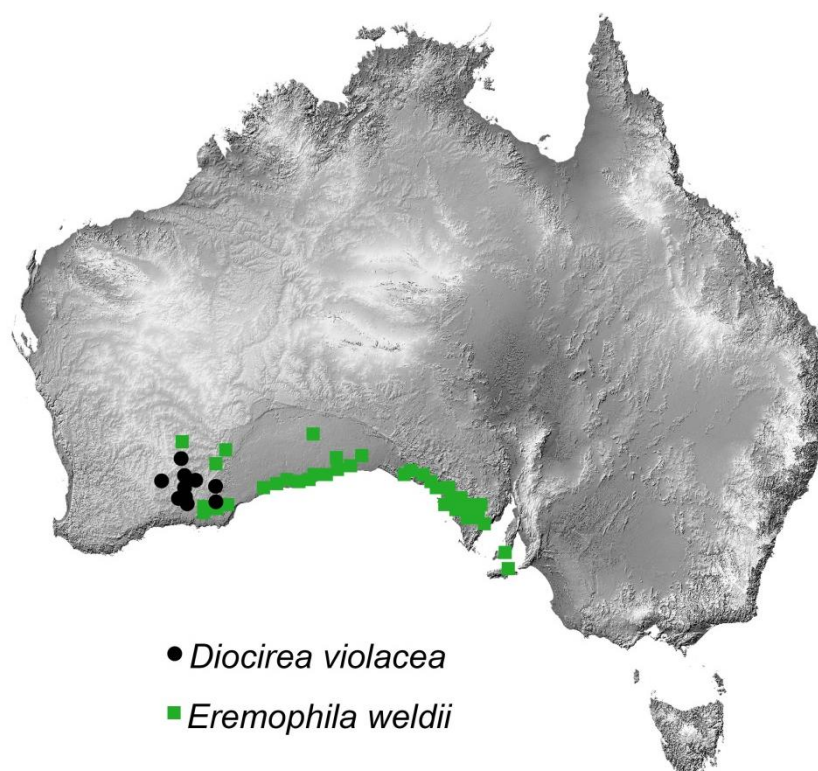


Figure 4.12 Distribution map of *D. violacea* and *E. weldii*; overlapping distributions could allow for contemporary or historical gene flow.

4.4.3 Sectional classification of *Eremophila* based on nrDNA

In the previous chapter it was evident that Chinnock's (2007) morphology-based sectional classification of *Eremophila* was only partly supported by the cpDNA phylogenetic reconstruction. The lack of support for many sections was potentially due to introgressive hybridization, incomplete lineage sorting, or inaccurate taxonomy. In the instance of introgressive hybridization (resulting in chloroplast capture), uniparental inheritance of the chloroplast genome can confound the phylogenetic signal of the most representative species tree (Rieseberg and Soltis, 1991; Doyle, 1992). By complementing the chloroplast analysis with a phylogeny based on the biparentally inherited nuclear ribosomal cistron, it is possible to gain further perspective that may allow for inferences of reticulation and introgressive hybridization (Rieseberg and Soltis, 1991; Degnan and Rosenberg, 2009; Yu et al., 2013; Holmes et al., 2014). If inappropriate taxonomic boundaries are contributing to a lack of sectional support in *Eremophila*, similar patterns of relationships would be expected in the nrDNA phylogeny as found in the cpDNA analysis. As discussed in the previous chapter, Chinnock's (2007) sectional framework groups species of *Eremophila* solely on morphological similarity, but without explicit consideration of the phylogenetic implications of said characters (in a cladistic sense apomorphy/plesiomorphy), hence, this similarity of morphological features does not necessarily reflect underlying evolutionary relationships between species. For example, if the characters on which Chinnock based a particular section reflect an ancestral state (plesiomorphy) rather than a derived character state (apomorphy), a lack of monophyly may be observed. Additionally, incomplete lineage sorting, the variable retention of genetic polymorphisms over speciation events and time (Doyle, 1992), may also account for inconsistencies between the morphology-based classification and the chloroplast genome phylogeny; however, if evident this process will also affect the nuclear-based reconstruction too. Through comparing results from the cpDNA and nrDNA reconstructions we hope to clarify the impact of each of these issues on the respective phylogenies and provide a clearer picture of species relationships. In the following discussion I will sequentially work through the implications of the phylogenetic results on Chinnocks (2007) sectional classification of *Eremophila*.

4.4.3.1 Sections of *Eremophila* supported by the nrDNA phylogeny

Overall, the nrDNA phylogeny more closely reflects the morphological taxonomy of *Eremophila* than the chloroplast genome phylogeny of Chapter Three. In the nrDNA dataset, five sections are resolved as strictly monophyletic (*Chamaepogonia*, *Platycalyx*, *Hygrophanae*, *Virides* and *Scariosepalae*). Section *Scariosepalae* is defined as having an enlarged calyx with complex venation, and a corolla with a four lobed upper lip with flabellate arrangement (Chinnock, 2007); all four accessions of this section, represented by both *E. alternifolia* and *E. purpurescens*, were placed in a monophyletic lineage in clade five with high support (pp=1). Likewise, section *Hygrophanae* is defined as having a dense tomentum on branches and leaves, alternate leaf arrangement and an unusual red-brown pigment of the corolla upon drying (Chinnock, 2007). All seven accessions, representing the six species in *Hygrophanae*, were well supported and monophyletic in clade nine. Section *Virides* is defined by its narrow corolla tube, long pedicels, green flowers and large sepals (Chinnock, 2007). All five accessions representing the four species of *Virides* are highly supported as monophyletic in clade eight. The monophyly of each of these sections indicates that the morphological characters used to unite species in each section are also evolutionarily meaningful and likely to be synapomorphic.

A number of other sections, while not strictly monophyletic, show distinct species clustering in clades of the nrDNA phylogeny, and while these sections may require some taxonomic revision they are still likely to represent evolutionarily meaningful lineages. For example, 11 of the 13 species from sect. *Stenochilus* (including the type species, *E. glabra*) are placed in a well-supported monophyletic lineage within clade eight, while the remaining two species (*E. maculata* and *E. racemosa*) are placed in their own well-supported monophyletic lineage of clade nine. A similar pattern was observed in the cpDNA phylogeny, and morphologically, Chinnock (2007) identified the distinctiveness of *E. maculata* and *E. racemosa* based on the presence of spotting patterns on their corolla, relative to other species in the section. In this instance the section (*Stenochilus*) is still relevant, but will need revision to exclude *E. maculata* and *E. racemosa*. A number of other large sections that are not strictly monophyletic, but still at least partly represent evolutionary meaningful sections for the purposes of classification (albeit, with some revision) include, sections *Eriocalyx* and *Eremaeae*.

With the addition of seven newly described species (Buirchell and Brown, 2016), section *Eriocalyx* now contains 36 species of *Eremophila*. In the nrDNA phylogeny, 44 accessions represent 31 of these species, and all but two species (*E. lachnocalyx* and *E. retropila*), including the type species, *E. eriocalyx*, are positioned in clade eleven (see Figure 4.8). Interspersed with the bulk of species from sect. *Eriocalyx* in clade eleven, are a number of species assigned to other sections; three from sect. *Australophilae* (*E. occidentis*, *E. homoplastica* and *E. sp. 'eversa'*), both species from sect. *Campanulatae* (*E. humilis* and *E. campanulata*) and a single species from sect. *Eremaeae* (*E. rigens*). The link between these species and the core *Eriocalyx* lineage is not immediately clear, but morphologically some characteristics can be identified that unite them. Chinnock (2007) separated sect. *Campanulatae* from sect. *Eriocalyx* based on differences in corolla shape (campanulate vs bilabiate respectively), however, vegetatively both sections are very similar (small compact shrubs, vegetative parts clothed in stellate to dendritic hairs). Chinnock placed *Eremophila occidentis*, *E. homoplastica* and *E. sp. 'eversa'* in sect. *Australophilae* but recognised that they had features uncharacteristic of the section more broadly (further discussion surrounding the utility of sect. *Australophilae* is addressed below). A morphological assessment of these species is required to determine synapomorphic features that may unite them with species in sect. *Eriocalyx*. Overall, both cpDNA and nrDNA phylogenies largely support the group of species in sect. *Eriocalyx*, with the inclusion of the above species and the exclusion of those discussed below.

Eremophila lachnocalyx and *E. retropila* (sect. *Eriocalyx*) are positioned quite distantly to clade eleven, and are highly supported as sister to the bulk of species in clade fourteen (belonging to sections *Eremaeae*, *Eremophila* and *Pulchrisepalae*). Section *Eriocalyx* is defined by the presence of a sparse to dense covering of hairs on vegetative and floral parts, relatively uniform flower structure and large, glabrous fruit (Chinnock, 2007). However, *E. lachnocalyx* is a notable exception in the section, displaying lanate fruit, and a subwhorled spiral arrangement of leaves (Chinnock, 2007). Chinnock suggested *E. lachnocalyx* did not appear to be closely related to any other species in sect. *Eriocalyx*, and this is supported by its placement in both cpDNA and nrDNA phylogenies. *Eremophila retropila* is also unique in its presence of eglandular hairs on the ovary and fruit, in a section otherwise characterised by glabrous fruit. Based on the placement of both of these species separate to other members of sect. *Eriocalyx* in both cpDNA and

nrDNA analyses, and the presence of morphological features uncharacteristic of sect. *Eriocalyx*, both species should be removed from sect. *Eriocalyx*.

Containing 54 species, section *Eremaeae* is the largest section in *Eremophila*. In the nrDNA phylogeny, 59 accessions representing 45 species were included for analysis, and the majority of these species split into two large sister clades; 32 species are largely monophyletic in clade thirteen, and 11 species somewhat scattered in clade fourteen (see Figures 4.10 and 4.11). Only two species (*E. rigens* and *E. attenuata*) are highly supported elsewhere in the phylogeny (discussed further below). Overall, this large section has 70% of species placed in a single clade, and although it will require some revision to achieve strict monophyly, it is likely to be predominantly based on synapomorphic morphological characters.

Clade thirteen is made up, almost exclusively, of species from sect. *Eremaeae*, the exceptions being a putative undescribed species (*Eremophila* sp. Murgoo) and *E. spuria*. *Eremophila spuria* is placed in section *Arenariae*, based on its resinous habit, inflated flowers and enlarged sepals and fruit (Chinnock, 2007). However, as its name alludes to, *E. spuria* resembles a number of species from other sections. For example, its swollen sepal apex is a feature shared with both of *E. setacea* (sect. *Arenariae*) and *E. hughesii* (sect. *Eremaeae*). Based on the high support with which *E. spuria* is placed with other species from sect. *Eremaeae* in both cpDNA and nrDNA reconstructions, and the ambiguity surrounding morphological character synapomorphy, it seems likely that *E. spuria* has been misplaced in sect. *Arenariae* based on plesiomorphic characters. Based on this molecular evidence, *E. spuria* would be best placed in sect. *Eremaeae*.

Clade fourteen contains *Eremophila alatisepala*, *E. linsmithii*, *E. cuneifolia*, *E. crenulata*, *E. phyllopoda*, *E. prolata*, *E. accrescens*, *E. woodiae*, *E. exilifolia*, *E. magnifica* and sect. *Eremaeae* type, *E. freelingii*. The highly supported placement of these species, as distinct from all other species in sect. *Eremaeae*, indicates further morphology-based study is required in order to determine possible synapomorphic characters that unite these particular species from sect. *Eremaeae* and other members of clade fourteen. Resolution of relationships within clade fourteen is low, with short branch lengths displayed in much of the lineage (see Figure 4.11) indicating minimal molecular variation between species in the nuclear ribosomal cistron. To resolve the relationships of the above species (along with other species from sect. *Pulchrisepalae*, *Eremophila* and *Eriocalyx*

also present in clade fourteen), additional sequence data from low copy nuclear regions and further morphological revision will likely be needed.

Eremophila attenuata and *E. rigens* may be considered anomalous in their placement in the nrDNA phylogeny, being the only two specimens from sect. *Eremaeae* not placed in either clade thirteen or fourteen. *Eremophila attenuata* is known only from a single location, and was noted by Chinnock (2007) to be of uncertain relationship to other species, and was questioned to be as of hybrid origin by Brown and Buirchell (2011). Placement of *E. attenuata* (RMF137) in clade seven, separate from all other members of *Eremaeae* and sister to sections *Pholidia*, *Decussatae* and *Sentis*, warrants further morphological study. *Eremophila rigens* is placed in clade eleven with species from sect. *Eriocalyx*. Likewise, morphological characters uniting these species are unclear and further morphological study is also recommended to assess this relationship.

4.4.3.2 Sections of *Eremophila* not supported by the nrDNA phylogeny

Unlike the sections outlined above (resolved as being strictly monophyletic, or monophyletic after minor revision), the five sections of *Eremophila* described below show a distinct lack of monophyly. Species in these sections are scattered throughout multiple lineages of the nrDNA phylogeny, and are unlikely to have been classified using synapomorphic characters, rendering these sections less informative from an evolutionary perspective. These sections include *Australophilae*, *Pulchrisepalae*, *Amphichilus*, *Contortae* and *Eremophila*.

Section *Australophilae* contains 32 species of *Eremophila*, and is the second largest assemblage of species defined by Chinnock (2007). Twenty eight of these species were included in the nrDNA phylogeny, and were found to be scattered throughout clades six, seven, eight, ten and eleven. A similar lack of monophyly in this section was found in the cpDNA analysis. In this instance, it seems likely that the morphological characters used to unite species in sect. *Australophilae* do not represent synapomorphies, but are rather plesiomorphic or homoplastic characters. Species in sect. *Australophilae* are characterised by Chinnock (2007) as having 'relatively small, densely clustered, spiral, opposite or whorled leaves, usually simple hairs and corollas often with unspecialised bases, while the rounded lobes, although irregularly arranged, are similarly dissected into the tube.' Many of these characters are quite general, and Chinnock suggests that,

overall, the vegetative features observed in sect. *Australophilae* are similar to those seen in other sections. Vegetatively, sect. *Duttonia* shares features with *Australophilae*, while characteristics of the flower and fruit are similar in sections *Carnosea*, *Eremaeae*, *Arenariae* and *Australophilae*.

Section *Pulchrisepalae* contains 13 species, all of which are represented (22 accessions) in the nrDNA phylogeny. Similarly to sect. *Australophilae*, species from *Pulchrisepalae* are scattered throughout the phylogeny in clades six, eleven, twelve and fourteen. Species in sect. *Pulchrisepalae* are defined by their enlarged calyx with complex venation and unequal lobes, resin production on leaf and stem surfaces and a deeply dissected corolla (Chinnock, 2007). However, a number of characteristics are similar or shared between *Pulchrisepalae* and other sections. For example, leaf and stem resin production is also a characteristic of the large section *Eremaeae*, similar calyx and fruit structure are observed in sect. *Scariosepalae*, and similar corolla and sepal structure occurs in sect. *Eremophila* (Chinnock, 2007). *Eremophila fraseri*, *E. flaccida*, *E. ramiflora*, *E. galeata*, *E. grandiflora* and *E. abietina* are all found to be nested with species from sections *Eremaeae* and *Eremophila* in clade fourteen, while *E. duttonii* and *E. linearis* form their own well supported lineage (clade twelve), sister to clades thirteen and fourteen. In these instances it would appear that the similarity in characters between *Pulchrisepalae* and *Eremaeae*/*Eremophila* represent evidence of a shared evolutionary history. At this stage the basis for placement of *E. miniata* in clade eleven (with sect. *Eriocalyx*), and *E. lucida*, *E. viscida* and *E. neglecta* in clade six (with sect. *Crustaceae* and *Arenariae*), is not clear from a morphological perspective.

The other three sections of *Eremophila* highly supported as non-monophyletic, include sect. *Amphichilus*, *Contortae* and *Eremophila*. Sections *Amphichilus* and *Contortae* contain three and two species respectively, and in each case the species are in vastly different clades in both cpDNA and nrDNA reconstructions. Section *Eremophila* contains eleven species, all of which were included in the nrDNA analysis, and were found to be scattered throughout clades six and fourteen. Relationships between species from sect. *Eremophila* and other sections placed in clade fourteen show low support values and are often not well resolved.

Overall, each of these sections found to be non-monophyletic will require additional study, particularly from a morphological perspective, to ascertain features/characters

that unite species relationships uncovered in the current study. Chinnock's sectional system of classification is still useful in identifying species in the field, but potentially an alternative system of classification would better reflect the evolutionary relationships within the genus and allow a predictive classification to be generated (discussed further in Chapter Six).

4.4.4 *Species relationships in tribe Myoporeae based on nrDNA*

Overall, 240 species from across tribe Myoporeae were sampled in the nrDNA dataset, and of these, 65 species (27%) were sampled with multiple accessions. The inclusion of multiple accessions for a subset of taxa has allowed for exploration and interpretation of intraspecific variation, species level monophyly and comparison to the cpDNA dataset. Approximately 40% of species with multiple accessions were highly supported as monophyletic, while the monophyly of an additional 23% of species was uncertain due to a lack of support or resolution in the phylogeny. The remaining 37% of species were found to be non-monophyletic; the majority of these non-monophyletic species were characterised in their relatively close placement of accessions (i.e. species paraphyletic due to the placement of a morphologically similar species, most often from the same section, as sister). Only three non-monophyletic species had accessions resolved in vastly different parts of the phylogeny.

As it stands, approximately 176 species have been sampled with a just single accession in the nrDNA dataset, meaning monophyly remains untested for the majority of species. However, it is worth noting that the 65 species within tribe Myoporeae selected for deeper sampling were not selected at random, but to test the monophyly of species with particularly widespread or disjunct distributions and/or significant intraspecific morphological variation (i.e. species with delimited subspecies or varieties). As such, rates of monophyly in this subset of species may not accurately reflect patterns of species-level monophyly more broadly across Myoporeae.

When comparing species-level relationships between analyses, it is evident that the cpDNA dataset contains a much higher proportion of species resolved as non-monophyletic (80%) than the nrDNA dataset (37%). This difference in proportion represents a large degree of incongruence in the signal of the two datasets, as well as stronger support for cpDNA relationships (based on a greater number of parsimony

informative characters) than nrDNA relationships. Of the 46 species supported as non-monophyletic in the cpDNA dataset, 21 (46%) species were also found to be non-monophyletic in the nrDNA dataset, 13 (28%) lacked adequate resolution and/or support to confirm monophyly, while 12 (26%) species were highly supported as monophyletic. Incongruence between datasets based on different genomic regions is not uncommon (Rieseberg and Soltis, 1991; Barrett et al., 2014; Holmes et al., 2014; Folk et al., 2016; Mallo and Posada, 2016) and is likely due to different evolutionary processes acting on the chloroplast and nuclear genomes. These processes will be considered for specific examples from across the nrDNA phylogeny in the discussion below.

4.4.4.1 Species monophyly in the nrDNA dataset

The 26 species found to be strongly supported as monophyletic ($pp \geq 0.95$) in the nrDNA analysis include: *Eremophila debilis* (2), *E. gibbifolia* (2), *E. polyclada* (2), *E. crassifolia* (2), *Myoporum petiolatum* (2), *M. laetum* (2), *E. oldfieldii* (2), *E. oppositifolia* (2), *E. interstans* (2), *E. macdonnellii* (2), *E. divaricata* (2), *E. scoparia* (5), *E. christophorii* (2), *E. arachniodes* (2), *E. serrulata* (2), *E. maculata* (5), *E. longifolia* (4), *E. pterocarpa* (2), *E. miniata* (2), *E. conferta* (2), *E. obovata* (2), *E. duttonii* (3), *E. spectabilis* (2), *E. freelingii* (3), *E. flaccida* (2) and *E. fraseri* (3). These monophyletic species are scattered throughout the tree, with representatives found in almost every clade of the nrDNA phylogeny. All species found to be monophyletic in the cpDNA phylogeny were also found to be monophyletic in this phylogeny. In contrast, many species resolved as monophyletic in this nrDNA phylogeny were highly supported as non-monophyletic in the cpDNA phylogeny. This incongruence in datasets is explored below.

A number of species resolved as monophyletic based on nrDNA were specifically targeted for more comprehensive sampling due to their widespread geographic distributions. Species such as *Eremophila maculata*, *E. longifolia*, *E. scoparia*, *E. duttonii* and *E. freelingii* are extremely widespread across Australia (see Figure 4.13). These species are also considered to exhibit significant intraspecific variation across their ranges (Chinnock, 2007) and were ideal candidates to test for intraspecific molecular variation, the possible presence of cryptic species/lack of monophyly. For each of these species, 3-5 accessions were collected from as widely as possible across their distributions, with the aim to capture possible genetic variation. Although the presence

of unsampled yet significantly divergent populations/individuals cannot be ruled out, the current finding strongly supports the monophyly of each of these particularly widespread, morphologically variable species of *Eremophila*.

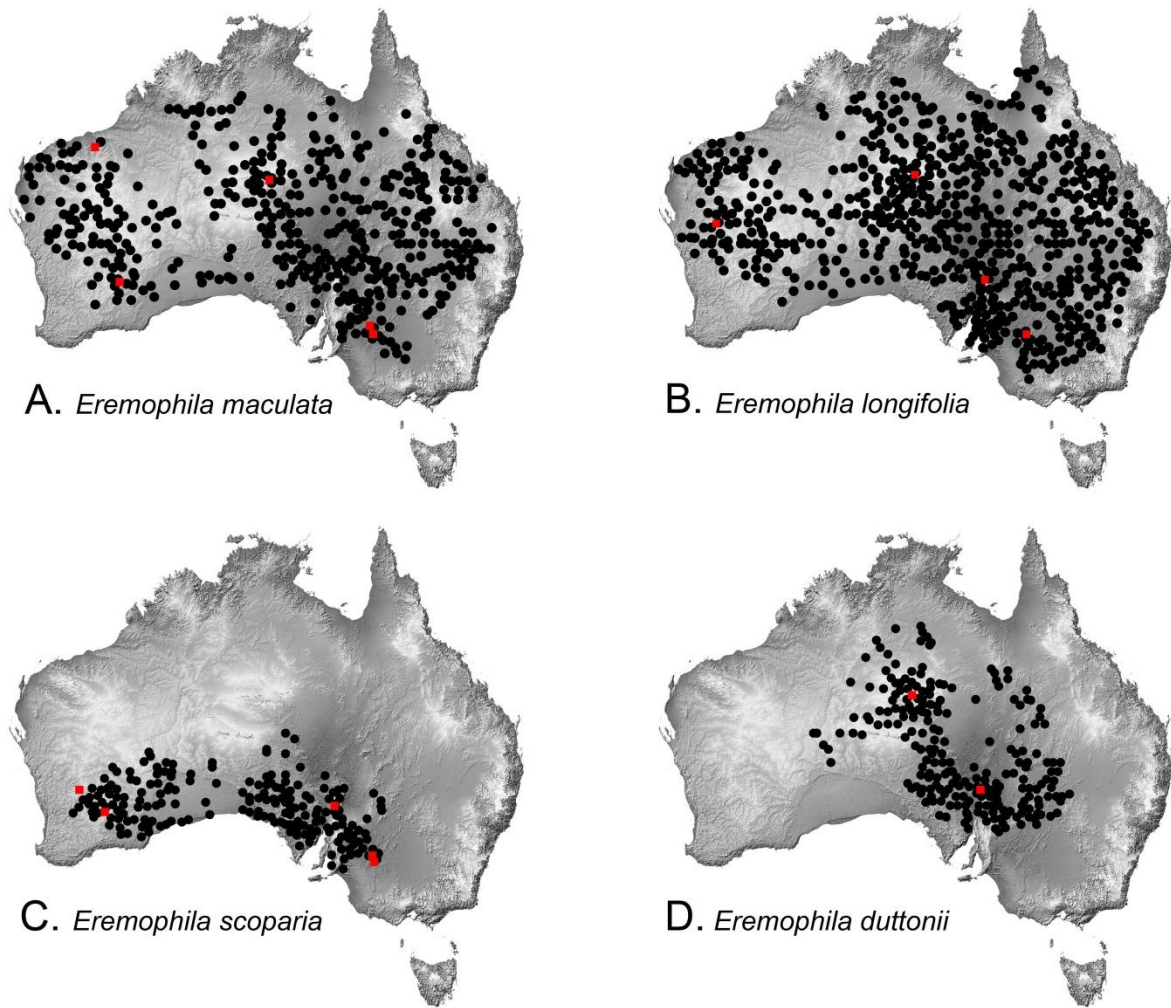


Figure 4.13 Distributions of *Eremophila maculata*, *E. longifolia*, *E. scoparia*, and *E. duttonii* based on data downloaded from the Australasian Virtual Herbarium (as at January 2018). Location of samples included in nrDNA analysis indicated by ■.

A number of *Eremophila* species with particularly disjunct distributions were also the focus of additional sampling, again due to the possibility of significant genetic divergence between geographically isolated populations. *Eremophila gibbifolia*, *E. crassifolia*, *E. oppositifolia*, *E. divaricata* and *E. arachnoides* all exhibit such disjunct distributions (see Figure 4.14), with their populations often separated by hundreds to

thousands of kilometres. These species were sampled from across their ranges, and were found to be monophyletic despite potential geographic barriers to gene flow. Of these species, *E. crassifolia*, *E. divaricata* and *E. arachnoides* were also resolved as monophyletic in the cpDNA analysis (this couldn't be assessed for *E. oppositifolia* as only a single sample was included in the cpDNA analysis). *Eremophila gibbifolia* stands out as an exception, having previously been resolved as non-monophyletic in clade three of the cpDNA phylogeny. A possible reason for this change in monophyletic status between the cpDNA and nrDNA datasets is that of introgressive hybridization. The distribution of *E. gibbifolia* overlaps with a number of species of *Myoporum* (with which it is most closely placed in the cpDNA analysis, see Figure 3.4), allowing for the possibility of hybridization and chloroplast capture. Being uniparentally inherited, the chloroplast genome signal could be reflecting that of the *Myoporum* species of historical hybridization events, while the nuclear signal more closely reflects the species morphology, placing both samples of *E. gibbifolia* as monophyletic and most closely related to other morphologically similar species from the same section (*E. barbata*). To confirm this hypothesis, further population level sampling would be required for *E. gibbifolia* across its range using both chloroplast and nuclear markers.

Eremophila oppositifolia is another example of a species with highly disjunct populations, though based on the nrDNA analysis is confirmed as a well-defined, monophyletic species. *Eremophila oppositifolia* is divided into three subspecies based on variation in leaf shape and arrangement (Chinnock, 2007). A single specimen of *E. oppositifolia* subsp. *angustifolia* was collected from Western Australia, and a cultivated collection of *E. oppositifolia* subsp. *rubra* was sampled from the Australian Arid Lands Botanic Garden. Subspecies *rubra* is restricted in distribution to central QLD and NSW, and is over 2000 km in distance from the WA sample of subspecies *angustifolia*. The monophyletic placement of these two samples supports the morphology-based classification of this species, despite significant geographic separation.

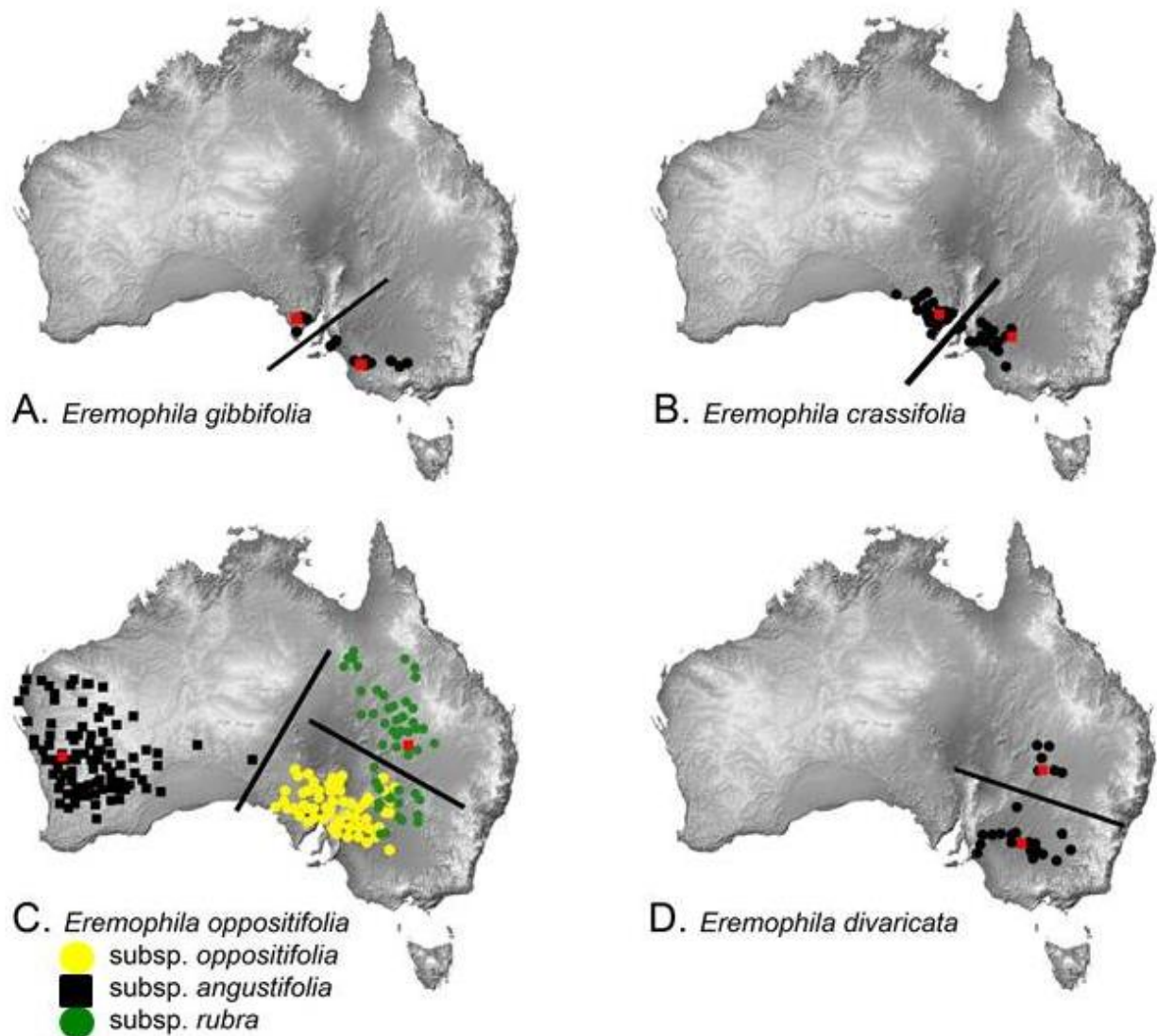


Figure 4.14 Disjunct distributions of A) *Eremophila gibbifolia*, B) *E. crassifolia*, C) *E. oppositifolia*, and D) *E. divaricata*. Location of samples included in nrDNA phylogeny indicated by ■. Disjunct distributions based on data from the Australasian Virtual Herbarium (as at January 2018). Lines indicate population disjunctions.

4.4.4.2 Lack of species monophyly in the nrDNA dataset

The 24 species found to be strongly supported as non-monophyletic ($pp \geq 0.95$) in the nrDNA phylogeny include: *E. deserti* (3), *E. paisleyi* (2), *E. platythamnos* (3), *E. densifolia* (2), *E. bowmanii* (3), *E. parvifolia* (2), *E. youngii* (2), *E. rugosa* (2), *E. glabra* (13), *E. denticulata* (2), *E. decipiens* (3), *E. subfloccosa* (3), *E. mackinlayi* (2), *E. rhegos* (2), *E. forrestii* (2), *E. jucunda* (2), *E. latrobei* (4), *E. battii* (2), *E. shonae* (2), *E. hughesii* (2), *E. petrophila* (2), *E. tietkensii* (4), *E. pungens* (2) and *Diocirea violacea* (2). Although all of

these species are strictly non-monophyletic, it is worth distinguishing between the different degrees of non-monophyly represented. The majority of these non-monophyletic species are characterised by their relatively close placement of accessions (i.e. species paraphyletic due to the sister status of morphologically similar, yet distinct species). Only 3 non-monophyletic species (*E. deserti*, *E. densifolia* and *E. bowmannii*) had accessions resolved in different parts of the phylogeny. Such placement indicates the highly divergent DNA sequences between individuals, and it contradicts our current morphology-based classification of these species, as explored below.

Eremophila deserti is a widespread species, with significant variation in leaf, fruit and flower morphology across its range (Chinnock, 2007). Three specimens of *E. deserti* were included in the nrDNA analysis (see Figure 4.15), two samples from Western Australia (RMF204, MJB2384) and one from Victoria (RMF228). The nrDNA analysis placed the two Western Australian samples of *E. deserti* as sister (and most closely related to other species from the same section) in clade four, but RMF228 was placed in clade three, sister to *Glycocystis beckeri* and *Myoporum platycarpum*. The same three samples were also included in the cpDNA analysis, and though also resolved to be non-monophyletic, Victorian sample RMF228 was placed sister to *E. fallax* (of the same section, *Pholidiopsis*), while Western Australian sample MJB2384 was resolved as sister to species of *Myoporum* (*M. montanum*, *M. boninense*, see Figure 3.4).

Although variable in morphology, *E. deserti* is morphologically distinct from other species of *Eremophila* and *Myoporum*, readily distinguished by its small, *Myoporum*-like corolla and unique anther type (Chinnock, 2007; Brown and Buirchell, 2011). The nrDNA placement of the two Western Australian specimens of *E. deserti* with other species in sect. *Pholidiopsis* is congruent with the current morphological understanding of the section, however, the placement of RMF228 is unexpected. There is an apparent lack of morphological characters to unite *E. deserti* to either *G. beckeri* or *M. platycarpum*. Geographically, RMF228 is highly disjunct from *G. beckeri* (which is limited in distribution to south-west Western Australia), though it does occur within the range of *M. platycarpum* (see Figure 4.15).

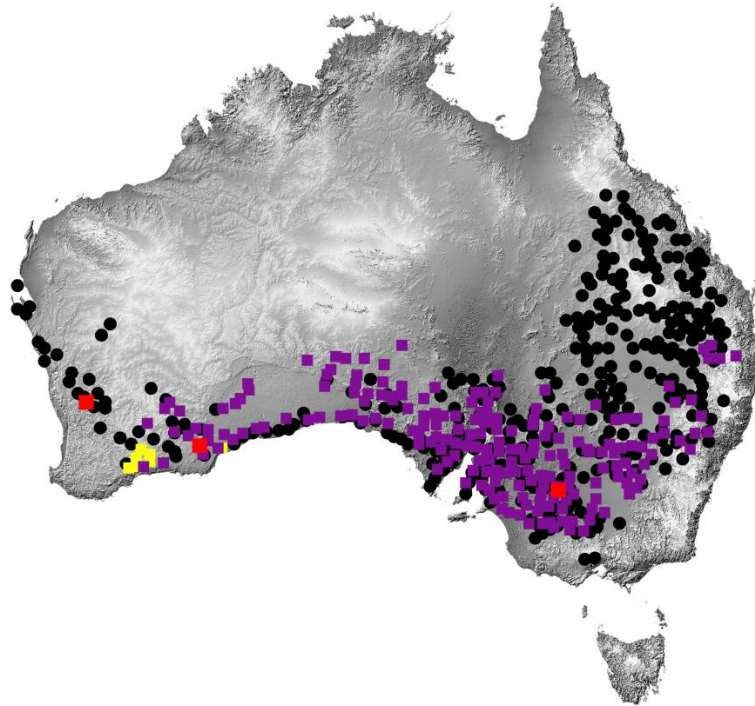


Figure 4.15 Distribution of *Eremophila deserti* (●), *Myoporum platycarpum* (■) and *Glycocystis beckeri* (■). Samples of *E. deserti* included in nrDNA analysis marked with ■.

One explanation for the observed lack of monophyly is possible introgressive hybridization between *E. deserti* and *M. platycarpum* lineages in Victoria. Their overlapping distributions could allow for ancient or more contemporary hybridization, resulting in the transfer of nrDNA across species boundaries. In the instance of hybridization or introgression, a geographic pattern to the genomic signal should be observed, for example the absence of shared DNA in populations of *E. deserti* isolated from *M. platycarpum*. However, under this scenario of introgression, some signal might also be seen in cpDNA relationships, depending on which species were involved as maternal parent(s) – and this is not the case. Based on cpDNA, RMF228 is placed most closely to *E. fallax* and other species from sect. *Pholidiopsis*; while *G. beckerii* and *M. platycarpum* are placed in a very separate lineage, apart from all basal members of the tribe. While this does not rule out introgression, the alternative relationships observed in the cpDNA analysis don't provide evidence for this scenario. An alternative hypothesis for the separate placement of RMF228 from other specimens of *E. deserti* is incomplete lineage sorting (ILS) of nrDNA. In this scenario the variation in each nrDNA lineage of *E. deserti* may predate speciation, and have been variably retained in some

individuals or populations, though the depth of split between *E. deserti* containing lineages and the number of species in between also makes this scenario less probable. To test the validity of either of these scenarios, it will be important to sample more intensively across the distribution of *E. deserti* to determine if further variation is detected in nrDNA and/or if the spatial east-west distinction within *E. deserti* is maintained. The complex, and incongruent signals between both cpDNA and nrDNA datasets indicate that the evolutionary history of *E. deserti* is also likely to be complex, exhibiting combinations of introgression/ILS and possibly a reticulate, non-bifurcating evolutionary history.

Another case of non-monophyly in which samples of the same species are spread across multiple clades in the nrDNA phylogeny is that of *E. densifolia*. *Eremophila densifolia* is restricted in distribution to the south-west of Western Australia (see Figure 4.16). Variation in indumentum supports the classification of four subspecies, two of which were sampled in the nrDNA phylogeny. *Eremophila densifolia* subsp. *pubiflora* was positioned sister to the bulk of species from sect. *Australophilae* in clade seven with low support (pp=0.64), although it is highly supported as a member of the clade (pp=0.99). In contrast, *E. densifolia* subsp. *capitata* is placed sister to *E. caerulea*, also in clade seven, though nested in a well-supported lineage dominated by species of *Eremophila* from sect. *Pholidia*. While comparison cannot be made to the cpDNA analysis (where only a single sample was included), the positioning and branch length of each of these subspecies in the nrDNA indicates significant sequence divergence between subspecies, despite their morphological similarity. A number of hypotheses could explain this variation, including incorrect application of taxonomic boundaries, incomplete lineage sorting, introgressive hybridization, or a combination of these causes. Placement of *E. densifolia* subsp. *capitata* with *E. caerulea*, nested within a clade made up predominantly of species from sect. *Pholidia*, is morphologically unexpected based on Chinnock's sectional classification, however, sect. *Australophilae*, to which they belong, does not seem to be evolutionarily meaningful (based on the non-monophyly of species in sect. *Australophilae* which are spread throughout multiple clades in the phylogeny - discussed previously). Species from sect. *Pholidia*, to which *E. densifolia* subsp. *capitata* is most closely related based on nrDNA (*E. youngii*, *E. arachnoides*, *E. dalyana*, *E. stenophylla*), exhibit quite distinct geographical distributions with no current overlap in ranges with *E. densifolia* or *E. caerulea*, making contemporary hybridization implausible

and historical introgression also unlikely. Perhaps the best explanation for the observed pattern is incomplete lineage sorting, whereby variable copies of nrDNA have been retained within *E. densifolia*, and are exhibited in the non-monophyletic placement of each individual. To test these hypotheses, more extensive sampling of each subspecies right across their distributions would be necessary, as would be the addition of additional nuclear markers to help confirm or refute the most plausible process.

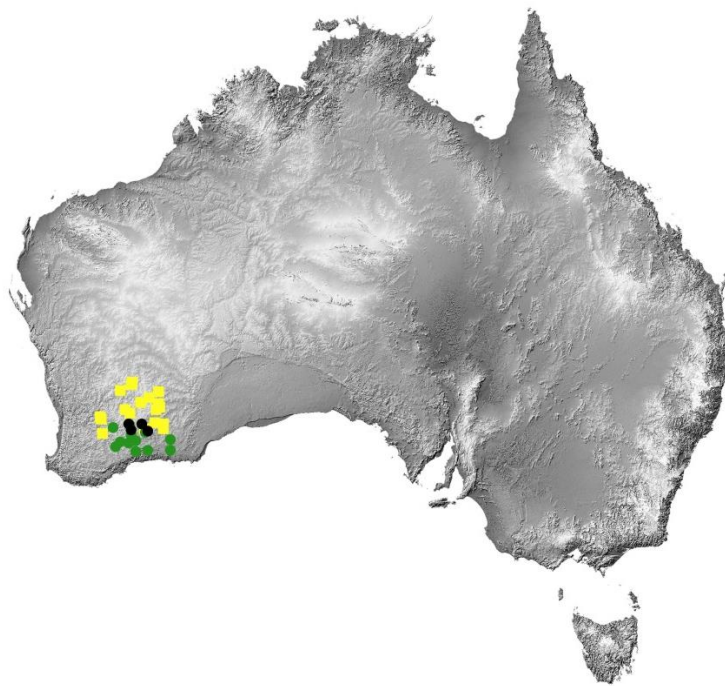


Figure 4.16 Distribution of *Eremophila densifolia* subsp. *pubiflora* (●), *E. densifolia* subsp. *capitata* (●) and *E. caerulea* (■).

4.4.4.3 Species paraphyly in nrDNA clades eleven, thirteen and fourteen

Unlike the previous two examples, the majority of non-monophyletic species recovered in the nrDNA analysis were not highly divergent. For 21 of the 24 non-monophyletic species, accessions were located in the same clade, often separated by a morphologically similar species from the same section. The evolutionary processes affecting these paraphyletic species are different to those hypothesised to generate the examples of highly non-monophyletic species outlined above. Many species exhibiting this pattern of paraphyly are located in particular clades of the nrDNA phylogeny (specifically clades eleven, thirteen and fourteen). Clade eleven is predominately

composed of species from sect. *Eriocalyx*, whereas clade thirteen is almost entirely made up of species from sect. *Eremaeae*, and clade fourteen is a combination of species from sections *Eremophila*, *Pulchrisepelae* and *Eremaeae*. The branches that subtend each of these clades show high support values (pp=1), and their relatively long branch lengths indicate a significant number of molecular characters unite taxa within each lineage. But within each clade, branches are generally short, and node support is low, resulting in a lack of resolution and the presence of large polytomies. Short branches in each of these clades are a symptom of a lack of molecular variation within the nrDNA region. This lack of variation may reflect relatively recent divergence between taxa within these lineages, or slow rates of sequence divergence in this region of nrDNA. Non-monophyletic species in these clades are characterised by the presence of one or more specimens placed in an unresolved polytomy, and a single specimen resolved as sister to a different species with high support. A good example of this is *E. latrobei*; four accessions of *E. latrobei* are included in the nrDNA dataset, all of which fall in clade eleven. Three of these accessions (RMF268, RMF72, MJB2421) are either unresolved or lack support along a large species polytomy (see Figure 4.8). A single sample (NG6477) is also placed along this polytomy, but is highly supported as most closely related to *E. buirchellii* (RMF92) rather than other species in the polytomy. Further population level study of both *E. latrobei* and *E. buirchellii*, potentially using additional nuclear DNA markers, would be required to determine possible reasons for this suggested relationship, which may include contemporary or historical hybridization events, incomplete lineage sorting, homoplasy in the nrDNA sequences, or that *E. latrobei* is paraphyletic with respect to *E. buirchellii*. Other similar examples of non-monophyly from these clades include: *E. forrestii*, *E. rhegos*, *E. jucunda*, *E. compacta*, *E. battii*, *E. hughesii*, *E. shonae*, *E. pungens*, *E. petrophila* and *E. tietkensii*. Similar to the *E. latrobei* scenario outlined above, in order to confirm the boundaries of each of these species further molecular study would be required at population level and across species ranges.

Outside of the clades mentioned above, a number of species are found to be non-monophyletic in the nrDNA analysis. *Eremophila platythamnus* (sect. *Arenariae*) is distributed throughout central Western Australia, South Australia and the Northern Territory (see Figure 4.17), and is separated into three subspecies based on leaf and sepal morphology (Chinnock, 2007). Two of the three subspecies were included in the

cpDNA analysis, *E. platythamnus* subsp. *platythamnus* (MJB2431) and *E. platythamnus* subsp. *exotrachys* (RMF160), and an additional sample of *E. platythamnus* subsp. *exotrachys* (KK085) was included in the nrDNA analysis. Results from the cpDNA analysis placed both subspecies of *E. platythamnus* in a polytomy with *E. gibsonii*, a morphologically similar species, also classified in sect. *Arenariae*, and overlapping in geographic distribution (see Figure 4.17). The nrDNA analysis similarly places *E. gibsonii* in a well-supported clade containing all specimens of *E. platythamnus*. Interestingly, the two accessions of *E. platythamnus* subsp. *exotrachys* are not sister to one another, with KK085 well-supported as most closely related to *E. gibsonii* rather than to either sample of *E. platythamnus*. The paraphyly of *E. platythamnus*, and close relationship to *E. gibsonii* is recovered with high support in both cpDNA and nrDNA datasets, and while it may be attributed to the evolutionary processes outlined for other examples of non-monophyly (ILS, introgression) the boundaries of these two species may need revision. *Eremophila platythamnus* and *E. gibsonii* are morphologically very similar (small, mauve entomophilous flowers, outer surface of corolla lobes glandular pubescent, inner surface glabrous, Chinnock, 2007) and overlap in distribution. Significant morphological variation is observed in *E. platythamnus*, allowing for the delimitation of three subspecies, so perhaps *E. gibsonii* may be best included in *E. platythamnus*, potentially warranting subspecies status, or alternatively, the subspecies of *E. platythamnus* could be elevated to species rank. To test the limits of each of these species and subspecies, more extensive sampling of *E. gibsonii* and all subspecies of *E. platythamnus* across their geographic ranges would be required, ideally using additional molecular markers (i.e. low copy nuclear regions).

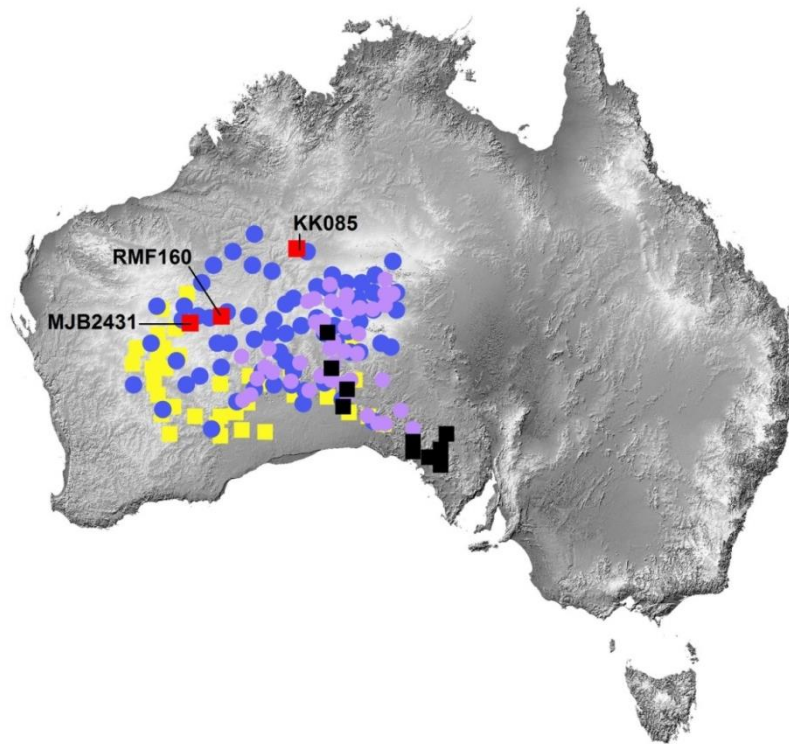


Figure 4.17 Distribution of *Eremophila platythamnus* and *E. gibsonii* (○). *Eremophila platythamnus* divided into subspecies; subsp. *platythamnus* (□), subsp. *extrachys* (●), and subsp. *villosa* (■). Samples of *E. platythamnus* included in nrDNA analysis marked with ■.

4.4.4.4 Species level incongruence between cpDNA and nrDNA datasets

When comparing datasets at the level of species, it is apparent that the cpDNA and nrDNA phylogenies differ significantly in topology and species relationships. This incongruence is particularly evident when contrasting degrees of species monophyly between the datasets. Of the 46 species highly supported as non-monophyletic in the cpDNA analysis, 21 are also non-monophyletic in the nrDNA phylogeny, 13 are unable to be determined as monophyletic/non-monophyletic due to lack of support or resolution, and 13 are monophyletic with high support in the nrDNA analysis. The 13 species that are supported as non-monophyletic in the cpDNA analysis versus supported as monophyletic in the nrDNA analysis are particularly interesting, and can potentially help understand the evolutionary processes that differentially affect chloroplast and nuclear genomes. These species are: *Eremophila alternifolia*, *E. gibbifolia*, *E. serrulata*, *E. oldfieldii*, *E. interstans*, *E. scoparia*, *E. christophorii*, *E. maculata*, *E. fraserii*, *E. flaccida*, *E. freelingii*, *Myoporum laetum* and *M. petiolatum*.

Eremophila alternifolia is a widespread species, distributed in southern Australia (see Figure 4.18). Significant morphological variation in leaf form and size is observed across the species range, and Brown and Buirchell (2011) recognise two variants of the species characterised by either broad or narrow leaf forms. Chinnock (2007) placed *E. alternifolia* with *E. purpurescens* in their own section (*Scariosepalae*), based upon their unique morphology. In the nrDNA phylogeny, *E. alternifolia* is moderately supported as monophyletic (pp=0.77) and strongly supported as sister to *E. purpurescens*. However, these relationships are contradicted by the cpDNA tree, which separates the South Australian sample of *E. alternifolia* (RMF247) from Western Australian samples (MJB2434, RMF202). RMF247 is resolved with high support as sister to *E. pentaptera*, a morphologically distinctive species of *Eremophila* placed in monotypic section *Carnosae*, with a geographic distribution of two to three disjunct populations in central South Australia. The distribution of *E. alternifolia* overlaps that of *E. pentaptera* in South Australia, allowing the possibility of introgressive hybridization, through which the maternally inherited chloroplast genome may have been transferred from *E. pentaptera* to *E. alternifolia*. To test this hypothesis, and rule out the effects of other evolutionary processes such as ILS, more extensive sampling of *E. alternifolia* and *E. pentaptera* would be required from across their species ranges, and particularly from the area of geographic overlap. Introgression would be supported if only specimens of *E. alternifolia* located in close proximity to *E. pentaptera* share closely related chloroplasts. In contrast, for ILS to be supported, there would be equal chance of cpDNA matching that of *E. pentaptera* right across the distribution of *E. alternifolia* (i.e. presence in Western Australia).

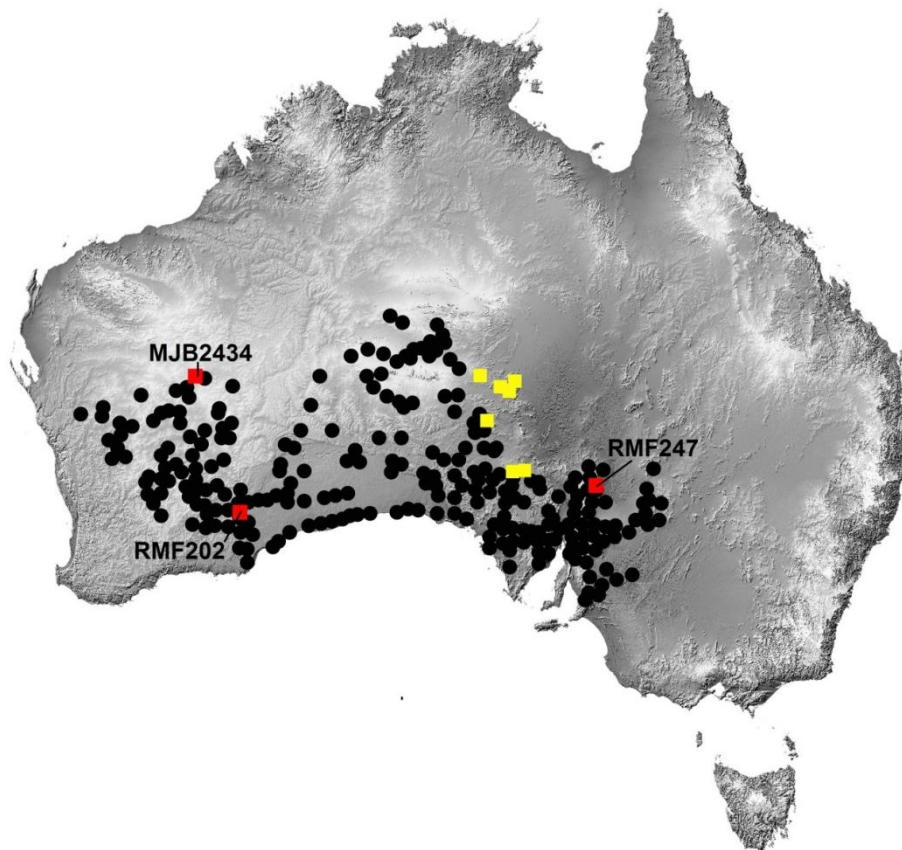


Figure 4.18 Distribution of *Eremophila alternifolia* ● and *E. pentaptera* ■. Samples of *E. alternifolia* included in nrDNA analysis marked with ■.

A second example of incongruence between cpDNA and nrDNA datasets is found in *Myoporum laetum*, a species restricted in distribution to coastal areas of both the North Island and South Island of New Zealand (see Figure 4.19). Two samples of *M. laetum* (MC and WP) were included in both of the cpDNA and nrDNA analyses, but their placement in a phylogenetic context differs between each dataset. The nrDNA phylogeny resolves *M. laetum* as monophyletic (pp=1), and highly supported as sister to Australian (coastal) species of *Myoporum*; *M. insulare*, *M. tetrandrum* and *M. brevipes* (pp=1). This placement supports the previous work of Chinnock (2007), who aligned *M. laetum* with the majority of species of *Myoporum* from Australia, Hawaii, islands of the Pacific and Mauritius, in sect. *Myoporum*. However the results of the cpDNA analysis in part differ in their placement of *M. laetum*; sample MC is resolved with high support (pp=1) as sister to *M. insulare*, while sample WP is strongly supported (pp=1) as sister to *M. condifolium*. *Myoporum condifolium* is a species restricted in distribution to south

west Western Australia (see Figure 4.19), and was recognised as morphologically distinct to all other species of *Myoporum* by Chinnock (2007), who placed it in its own section (sect.). The alignment of sample WP with *M. condifolium* is unlikely to be due to the process of introgression, which would require individuals to be in close enough proximity for hybridization to occur (contemporary populations are separated by thousands of km's of land and ocean). A more plausible explanation for this pattern is the process of ILS, whereby ancestral variation of the chloroplast genome has been retained within the species, resulting in the presence of multiple chloroplast genome types, and thus differing phylogenetic signal, among individuals of *M. laetum*. As is the case with many hypotheses proposed from this research, more in depth population level sampling would be required for *M. laetum*, to determine the degree of chloroplast genome variation and confirm the likelihood of either of these scenarios.

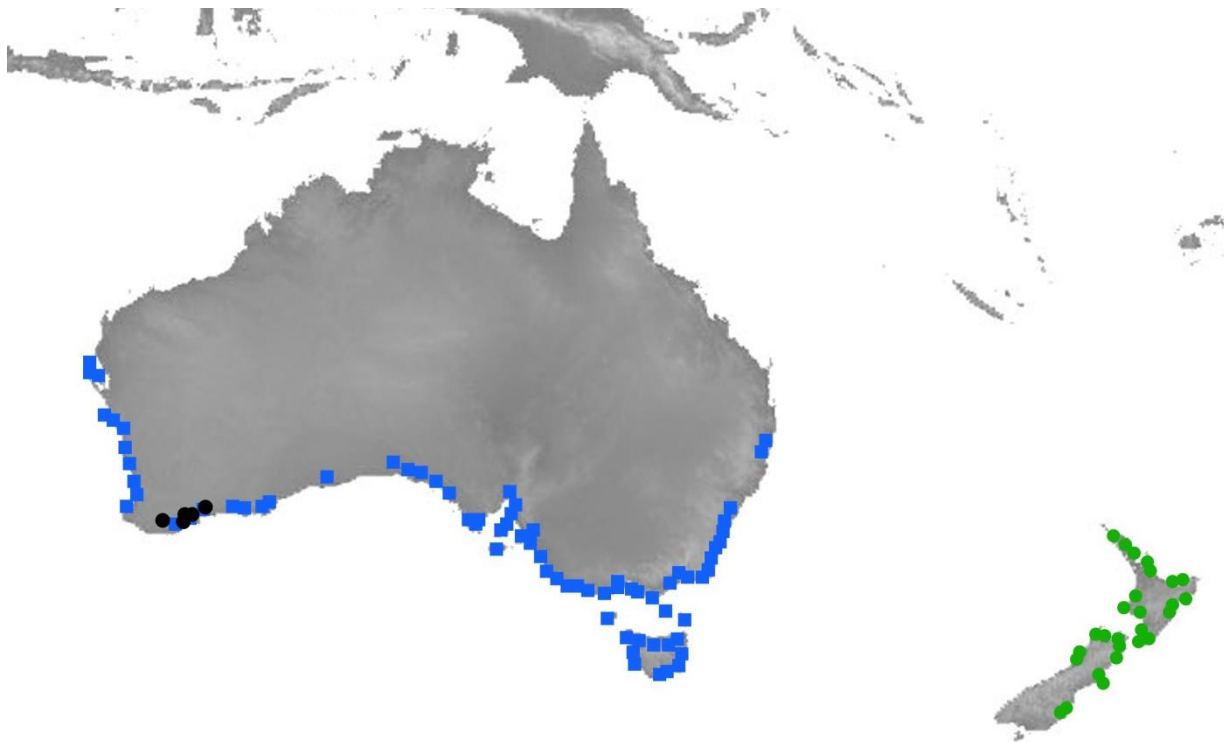


Figure 4.19 Distributions of *Myoporum insulare* (■), *M. cordifolium* (●) and *M. laetum* (●).

4.4.5 Conclusions

Overall, in generating a nuclear-based phylogeny of Myoporeae we have been able to observe and compare an alternative genome pathway of evolution, which in this instance, better reflects the current morphological classification of the tribe than the chloroplast genome phylogeny of the pervious chapter. Some unexpected relationships have been uncovered, at generic, sectional and specific levels. It appears that the effects of introgressive hybridization, ILS and possibly inappropriate taxonomy are evident in different species and lineages of the phylogeny. Additional research, namely increased sampling densities across taxon ranges is required to clarify the effects of these processes, particularly at the level of species. The genetic relationships of each species are potentially shaped by different combinations of processes, and with c. 250 taxa this could mean many different scenarios in tribe Myoporeae. As is often the case, this study has just scratched the surface in determining possible evolutionary scenarios responsible for contemporary species diversity, and has likely raised more questions than it has addressed. Going forward, we now have a better idea of species in need of further population level study.

Chapter Five: Exploration of the mitogenome for use in phylogenetic reconstruction of tribe Myoporeae

5.1 Introduction

To date, the field of molecular plant phylogenetics has focused mostly on the use of chloroplast and/or nuclear ribosomal markers to infer relationships at various taxonomic levels (Álvarez and Wendel, 2003; Shaw et al., 2005, 2014; Feliner and Rosselló, 2007). These regions are usually highly variable, and due to their presence in high copy numbers within the cell and their proximity to highly conserved gene regions, they have proven relatively easy to amplify and sequence (discussed further in Chapters Three and Four) using “universal” primers. In contrast, the plant mitochondrial genome (mitogenome) has been relatively understudied from a phylogenetic perspective (Govindarajulu et al., 2015; Wicke and Schneeweiss, 2015), after early exploration of the plant mitogenome was critical of its use in phylogenetics, because studies demonstrated high levels of gene rearrangement accompanied by limited sequence variation (Palmer, 1992). Now with the development and uptake of high throughput sequencing (HTS) and a genome skimming approach, the plant mitogenome is becoming more accessible for in-depth phylogenetic study (Duminil, 2014; Smith, 2016), and in many plant groups appears to be more complex, and in some cases more informative, than originally anticipated (Knoop, 2012; Wang and Wang, 2014; Fan et al., 2016; Warren et al., 2016; Rydin et al., 2017). Therefore it is now worthwhile investigating the possible utility of the mitogenome in plant phylogenetic studies on a case-by-case basis.

Regions of the mitogenome have long been used for species delimitation, phylogeography and phylogenetics in animal systems (Avice 2000; Galtier et al., 2009; Smith, 2016). In metazoans, the mitogenome is highly conserved in size, composition and structure. The metazoan mitogenome is generally circular, small in size (c. 16 kilo base pairs; kbp), and contains a consistent set of 37 genes (13 protein coding genes, 22 tRNA and two rRNA) (Boore, 1999; Knoop 2012). It does not generally undergo recombination (Birky, 2001), is uniparentally inherited (though some cases of biparental inheritance have been recorded, Ballard and Whitlock, 2004) and evolves

approximately ten times faster than the rate of nuclear DNA (Ballard and Whitlock, 2004). Like the plant chloroplast genome, the mitogenome occurs in high copy numbers in the cell, and due to the presence of highly variable DNA sequence flanked by highly conserved gene regions, is relatively easy to amplify and sequence (Boore, 1999; Smith 2016). For these reasons, the mitogenome is the most popular molecular marker sequenced for animals, and the mitochondrial region cytochrome oxidase subunit one (*cox1*) selected as a universal animal barcode (Galtier et al., 2009). In 2014, c. 82% of sequenced organelle genomes deposited in GenBank were metazoan mitogenomes (Smith and Keeling, 2015), and as of 2015, 92% of all complete mitogenomes in GenBank were from metazoans, with the remaining 8% of mitogenomes spread between plants, fungi and protists (Smith, 2016).

The mitogenome had been relatively understudied in plants for a number of reasons. Compared to chloroplast and nuclear genomes, the nucleotide substitution rate of the plant mitogenome is generally low, with the relative rate of substitutions of mitochondrial, chloroplast, nuclear genes in seed plants approximately 1:3:10 (Druin et al., 2008). Unlike the animal mitochondrion, the plant mitogenome varies considerably in size. The mitogenomes of green algae range from 13–200 kbp, non-vascular land plants from 67–209 kbp and flowering plants from 222–c. 2700 kbp (Burger and Nedelcu, 2012; Knoop, 2012; Liu et al., 2012; Smith and Keeling, 2015). Although the number and organisation of genes within plant mitogenomes are highly variable (Knoop, 2012; Smith and Keeling, 2015), the vast differences in plant mitogenome size are largely attributed to differing amounts of non-coding DNA (Richardson et al., 2013), the result of repeats, introns, insertion of foreign DNA (plasmid or nuclear origin) and horizontal transmission (Knoop, 2012; Smith and Keeling, 2015; Warren et al., 2016). Structurally, the plant mitogenome also varies considerably, exhibiting one to multiple genomes of circular and/or linear structure (Smith and Keeling, 2015).

While the low nucleotide substitution rate of the plant mitogenome affects the resulting phylogenetic signal used to infer relationships, the highly variable size and structure of the genome, presence and organization of genes, and lack of universal primers, has made the sequencing and assembly of the genome problematic (Wicke and Schneeweiss, 2015). Repetitive regions of DNA are difficult to sequence or assemble, and frequent insertions, deletions and structural rearrangements make genome

construction and alignment difficult. Despite these issues, and with the growing application of high throughput sequencing, exploration of the plant mitogenome is gaining momentum (Wang and Wang, 2014; Govindarajulu et al., 2015; Wicke and Schneeweiss, 2015; Rydin et al., 2017; Sawicki et al., 2017). Interestingly, results from recent studies indicate that not only can the plant mitogenome be informative from a phylogenetic perspective, but are also challenging the traditional views of mitogenome (maternal) inheritance – with the phylogenetic signal often incongruent with chloroplast and nuclear datasets (Wang and Wang, 2014; Govindarajulu et al., 2015; Rydin et al., 2017).

Within this context, the main aims of this chapter are to:

- 1) Explore the mitogenomic DNA from tribe Myoporeae, and identify variable regions for use in phylogenetic reconstruction;
- 2) Generate a mitochondrial DNA (mtDNA) phylogeny of tribe Myoporeae for comparison with cpDNA and nrDNA phylogenies.

5.2 Methods

Details of specimen collection, DNA extraction, library preparation, DNA sequencing, contig assembly and sequence alignment are outlined in Chapter Two: General Methods.

5.2.1 *Taxon sampling*

A total of 31 individuals, representing 26 species of *Eremophila*, two species of *Myoporum* and single species of *Diocirea* and *Pentacoelium*, were included in the tribe-wide exploration of Myoporeae using mitochondrial DNA (see Appendix Five). These samples were specifically selected to represent each of the major clades identified in both the nuclear and chloroplast phylogenies generated in Chapters Three and Four, to compare phylogenetic patterns. These samples were also selected to represent a broad range of the 25 sections of Chinnock (2007). A sample of *Leucophyllum frutescens* (sister group to tribe Myoporeae, (Gandara and Sosa 2013)) was sourced and sequenced for this study, and used as the outgroup.

5.2.2 Marker selection and assembly of mitochondrial DNA

Complete mitochondrial genomes are often difficult to assemble in plants due to highly variable genome size and structure, and the presence of significant gene rearrangements, even at low taxonomic levels (Knoop, 2012; Wicke and Schneeweiss, 2015). Rather than attempting to construct complete mitogenomes, the aim of this study was to identify variable regions of the genome that were common between samples of the dataset, and could be reliably assembled for analysis. Initially, to explore the mitogenome, *de-novo* assembled contigs from a random subset of 100 samples, broadly representing tribe Myoporeae, were mapped to the closest available complete mitochondrial genome from GenBank (<https://www.ncbi.nlm.nih.gov/>, NCBI, 2017). At the time of this work (October 2017), the closest reference mitogenome to Myoporeae was from *Castilleja paramensis*, family Orobanchaceae, order Lamiales (Fan et al., 2016). Geneious version 9.1.8 (<http://www.geneious.com>, Kearse et al., 2012) was used to map contigs using custom sensitivity settings (gaps allowed set to a maximum of 30% per read, maximum gap size 3000). These more ‘relaxed’ mapping settings allowed for mitochondrial contigs from Myoporeae to align with the distantly related reference genome. This mapping process resulted in contigs from Myoporeae that spanned the majority of the reference mitochondrial genome (see Figure 5.1). Gene regions were particularly well covered, and though contigs often extended beyond exons into non-coding spacer regions, not all spacer regions were covered. The absence of contigs in these spacer regions is likely due to significant sequence divergence between taxa of Myoporeae and the distantly related reference *Castilleja paramensis*, meaning contigs are unable to be recognised and mapped as homologous against the reference sequence. Additionally, changes to mitogenome gene order or loss of DNA could also account for the absence of contigs in these spacer regions. Regions of highest sequence variability were assessed by eye. Four regions of the mitochondrial genome were selected for analysis based on the high contig coverage for a range of Myoporeae samples to the reference (indicating conservation of the region across Myoporeae), in conjunction with relatively high numbers of nucleotide polymorphisms in each of these regions (indicating sequence variability across Myoporeae). Looking across the contigs mapped to the reference mitogenome, the majority of regions showed a complete lack of variation, or were unable to be properly aligned due to structural rearrangement.

Regions selected include *nad4-trnS_{GAA}*, *sdh3-nad2*, *cob-rp15* and a spacer region (spacer2, located at c. 133000 bp of the *Castilleja paramensis* reference sequence, see Figure 5.2). A fifth region (*nad1-cox1*) was also selected for further analysis based upon the use of *cox1* in other studies (Strauss et al., 1993; Tomaru et al., 1998). For each of these regions, the longest contig (i.e. spanning the most sequence) was selected as a reference to which all other samples of Myoporeae were mapped.

For each of the 31 samples included, *de-novo* assembled contigs from total genomic DNA were mapped to the Myoporeae reference sequences generated in the exploration exercise. All raw (paired) reads for each sample were then mapped to their contig(s) using custom sensitivity settings (gaps allowed at 20% per read, maximum gap size 1000) and 3–5 iterations. This mapping of raw reads to contigs acts as a checking mechanism (see Figure 2.2), and any issues in sequence construction can be easily identified visually in Geneious. Once an accurate alignment of reads was mapped to a draft consensus, a final consensus sequence was generated for each sample, based on a 50% strict consensus threshold.

5.2.3 Sequence alignment

Alignments for each of the five mitochondrial regions were generated in Geneious v9.8.1 using the MAFFT pairwise alignment plug-in (MAFFT version 7.222, Katoh and Standley, 2013) and default settings. Structural rearrangements are common in the mitochondrial genome and these were particularly evident when aligning sequences from across the tribe. Where non-homologous sequence was identified, sequence ends were removed to where sequence was able to be unambiguously aligned. Alignment issues were checked by eye and adjustments made manually. Alignments were concatenated into a single file in Geneious for subsequent analyses.

Constant, parsimony uninformative and parsimony informative characters were calculated for coding and non-coding regions of each of the five mtDNA regions (Table 5.1), as well as for equivalent cpDNA and nrDNA datasets (i.e. alignments of same 32 specimens, see Table 5.2) in PAUP* version 4 (Swofford, 2002).

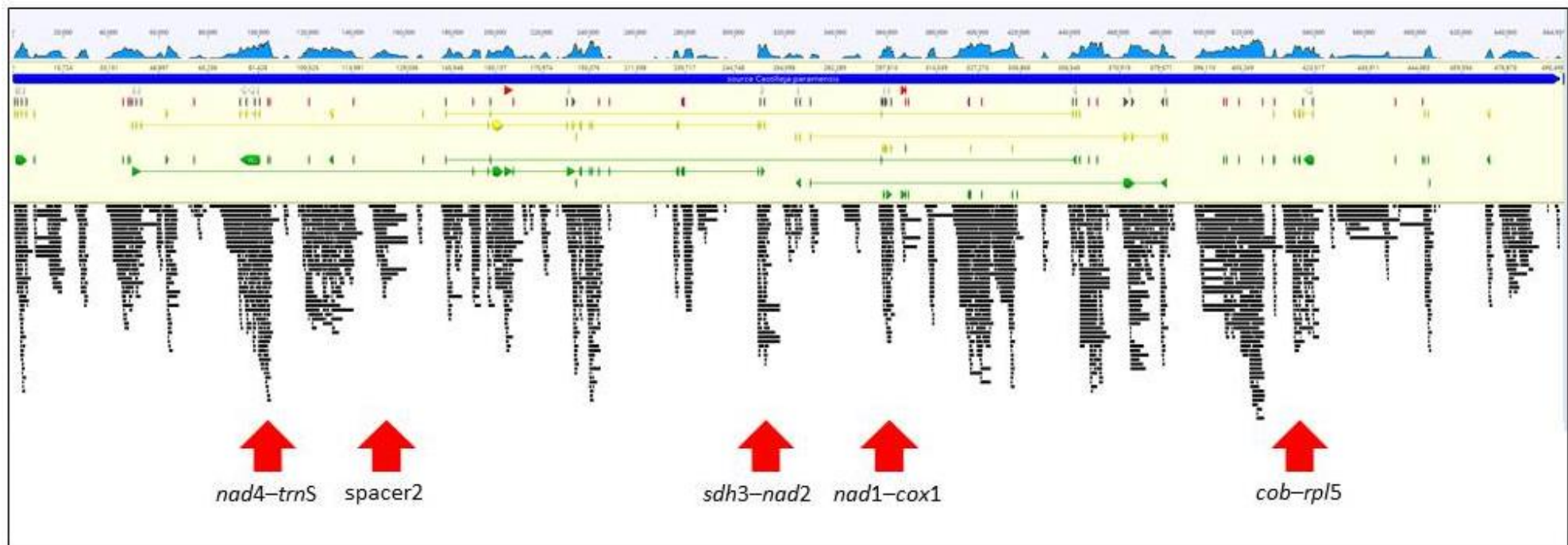


Figure 5.1 Contigs from 100 samples of tribe Myoporeae mapped to a complete mitogenome from reference species *Castilleja paramensis* (NC_031806.1, downloaded from GenBank) as displayed in Geneious. Blue graph at the top of the figure represents coverage of the reference genome; green, yellow and pink bands below the dark blue bar indicate the placement of gene regions in the reference genome; stacks of black bars represent placement of individual contigs from Myoporeae samples; red arrows indicate location of regions selected for mitogenome analysis in Myoporeae.

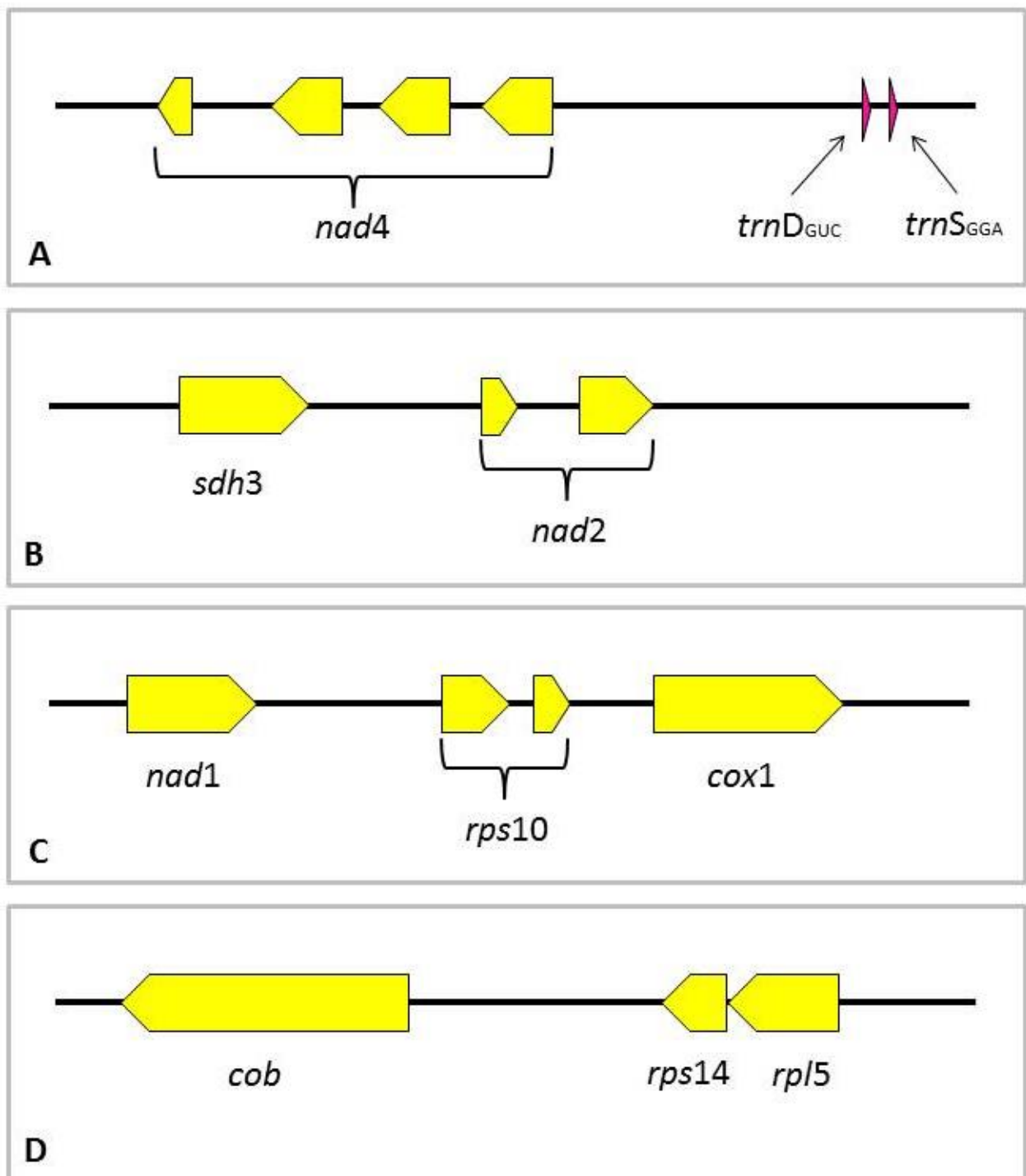


Figure 5.2 Diagram of placement, order and relative size of genes and spacers (including flanking tails) of the mitogenome used for analysis; A) *nad4-trnS_{GGA}*, B) *sdh3-nad2*, C) *nad1-cox1*, D) *cob-rpl5*. Spacer2 is absent as not flanked by any gene regions.

5.2.4 Bayesian Inference analysis

Model testing of the concatenated mtDNA alignment was conducted in MrModeltest version 2.3 (Nylander 2004). The alignment was partitioned into two character sets representing coding DNA sequence (CDS) and spacers/introns (SPCR). The partitions were selected based on the assumption that rates of evolution would more likely be similar within these regions than between them. Models selected for each partition of the BI analysis were based on the Akaike information criterion (AIC). The suggested model of evolution for both the CDS and SPCR partitions was SYM+G, which was applied in the following analysis.

A Bayesian Inference analysis for the mitochondrial DNA alignment was run in MrBayes version 3.2 (Ronquist et al., 2012) using the default settings. The MCMC analysis was run for 3 million generations with a tree sampling frequency of 500, and partitions unlinked. The average standard deviation of split frequencies reached less than 0.01 over the course of this analysis, with convergence of MCMC chains checked in Tracer version 1.6 (Rambaut et al., 2014). Twenty five percent of the trees were discarded as burn-in, a consensus tree was generated and Bayesian posterior probabilities estimated for nodes of the consensus tree.

5.3 Results

5.3.1 Mitochondrial marker selection and analysis

The closest reference genome selected from Genbank (*Castilleja paramensis*, family Orobanchaceae) was 495,499 base pairs in length, and contained 39 genes, 29 tRNA regions and 3 rRNA regions. All genes, tRNA and rRNA regions appeared to be conserved within Myoporeae, based on the presence of contigs spanning each of these regions.

The five mtDNA regions selected for analysis ranged in length, proportion of coding to non-coding DNA sequence and proportion of parsimony informative characters (see Table 5.1). Marker length ranged from 2723–12829 base pairs, and proportions of parsimony informative characters ranged from 0.12% (*nad1-cox1*) to 3.19% (spacer2). Non-coding regions tended to be more variable, displaying more parsimony informative

sites, than coding/gene regions (see Table 5.1). High numbers of variable, yet non-parsimony informative characters were also present in the non-coding DNA regions of each marker, indicating that with the inclusion of additional taxa further parsimony informative characters would likely be present. When combined, the proportion of parsimony informative characters from all five mtDNA regions analysed (number of PICs/total number of base pairs) is low (0.4%), relative to proportions observed in both chloroplast (1.5%) and nuclear (7.4%) datasets analysed with the same 32 samples (see Table 5.2).

Table 5.1 Summary of length, gene/spacer composition and sequence variation of mitochondrial markers selected for analysis.

Marker	Total length of region	Length of CDS (bp)	CDS parsimony informative characters	CDS variable (parsimony uninformative) characters	Length of spacer (bp)	Spacer parsimony informative characters	Spacer variable (parsimony uninformative) characters
<i>nad4</i> – <i>trnS_{GGA}</i>	12829	1479	1 (0.07%)	5	11350	24 (0.21%)	223
<i>sdh3</i> – <i>nad2</i>	12574	1125	2 (0.18%)	29	11449	14 (0.12%)	173
<i>nad1</i> – <i>cox1</i>	5102	2210	2 (0.09%)	9	2892	4 (0.14%)	26
<i>cob</i> – <i>rpl5</i>	3177	2082	5 (0.16%)	14	1095	12 (1.09%)	51
<i>spacer2</i>	2723	0	-	-	2723	87 (3.19%)	104
Total	36405	6896	10	57	29509	141	577

Table 5.2 Summary statistics for mtDNA analysis relative to cpDNA and nrDNA analyses (including total and reduced sampling to reflect specimens included in mtDNA dataset).

	mtDNA total 32 samples	cpDNA (reduced sampling n=32)	cpDNA (total 317 samples)	nrDNA (reduced sampling n=32)	nrDNA (total 355 samples)
Total characters	36405	143324	143324	8832	8832
Constant characters	35620 (97.9%)	137829 (96.2%)	124555 (87%)	7647 (86.6%)	6703 (75.9%)
Parsimony uninformative characters	634 (1.7%)	3268 (2.3%)	7211 (5%)	535 (6%)	413 (4.7%)
Parsimony informative characters	151 (0.4%)	2227 (1.5%)	11558 (8%)	650 (7.4%)	1716 (19.4%)

5.3.2 Phylogenetic analyses

Overall, the Bayesian Inference (BI) analysis of the mtDNA dataset produced a moderately supported phylogeny (see Figure 5.3), with 17 of the 28 branches (61%) supported with pp values ≥ 0.95 . A lack of support for 11 branches does restrict the interpretation of relationships for a number of species, but overall this mtDNA tree still shows enough resolution to be compared with patterns of chloroplast and nuclear phylogenies.

For ease of interpretation, the mtDNA phylogeny can be divided into seven well supported clades (pp ≥ 0.95). Two species of *Eremophila* (*E. debilis* and *E. duttonii*) are placed with high support (pp=0.98) at the base of the mtDNA phylogeny, sister to the remainder of the tribe. Clade one is highly supported (pp=1), and contains both species of *Myoporum* included, monotypic genus *Pentacoelium*, and all species of *Eremophila* from sections *Pholidiopsis* (*E. deserti* and *E. fallax*) and *Duttonia* (*E. gibbifolia*). All nodes within clade one are highly supported (pp=1).

Clade two is well supported (pp=0.97), and contains two species of *Eremophila*, *E. maculata* (sect. *Stenochilus*) and *E. caperata* (sect. *Crustaceae*). Clade three (pp=1) contains both species representing sect. *Hygrophanae* (*E. hygrophana* and *E. mackinlayi*), while clade four (pp=0.99) is an assemblage of five species: both species represented from sect. *Pholidia* (*E. scoparia* and *E. arachnoides*), one of the two species included from sect. *Australophilae* (*E. parvifolia*), one of the two species included from sect. *Crustaceae* (*E. interstans*), and the single representative included from the Western Australian genus *Diocirea* (*D. violacea*). The relationships between clades three and four, and samples of *E. flaccida* and *E. platythamnos* are uncertain due to low node support.

Clade five is well supported (pp=1) and contains both species from sect. *Eriocalyx* (*E. latrobei* and *E. congesta*) and *E. viscida* from sect. *Pulchrispalae*. Clade six is also well supported (pp=0.98), however relationships within the lineage are uncertain. The clade contains three of the five taxa included from sect. *Stenochilus* (*E. subfloccosa* and two samples of *E. glabra*) and a single species, *E. complanata*, from sect. *Australophilae*. Clade seven (pp=1) contains all species from sect. *Eremaeae* included in the phylogeny (*E. conglomerata*, *E. gilesii*, *E. cuneifolia*). The relationships between clades five, six,

seven, and samples of *E. macdonnellii* and *E. racemosa* are uncertain due to low node support connecting each of these lineages.

5.4 Discussion

5.4.1 Utility of the mitogenome for use in phylogenetic reconstruction

The plant mitogenome is characterised by its highly variable size, composition and structure, yet slower rate of evolution relative to other genomes or animal mitochondrial DNA (Druin et al., 2008; Knoop, 2012; Richardson et al., 2013). These features have typically discouraged use of the mitogenome for plant phylogenetics, though this is changing in the era of HTS and genome skimming. Being of high copy number in the cell (much like the chloroplast genome), the mitogenome is easily recovered when genome skimming is implemented even to very low depths of coverage (Malé et al., 2014; Wicke and Schneeweiss, 2015; Zhang et al., 2015; Zhu et al., 2016). The current study implemented this technique, and was able to recover large stretches of the mitogenome from right across tribe Myoporeae. While an entire mitogenome was not assembled, this could feasibly be achieved if samples were sequenced to increasing depths of coverage. Comment cannot be made on genome size or structure, but the presence of contigs spanning all gene regions of the reference sequence (*Castilleja paramensis*) indicates that gene composition is likely to be consistent in the order Lamiales. Interestingly, these gene regions were highly conserved, both between species in tribe Myoporeae, and between Myoporeae and the reference genome, supporting the general hypothesis of slow rates of mitogenomic sequence evolution, and providing anchor points from which the less conserved non-coding (spacer) regions of DNA could be constructed and aligned. Evidence of significant structural rearrangement became obvious in these spacer regions, which were invariably unable to be aligned as non-homologous sequence occurred the further away from the coding regions, even between closely related species in the tribe. Such rearrangement/structural plasticity is well document (Kubo and Newton, 2008; Knoop, 2012; Wicke and Schneeweiss, 2015) and would likely be a hurdle to the process of entire mitogenome assembly in tribe Myoporeae going forward.

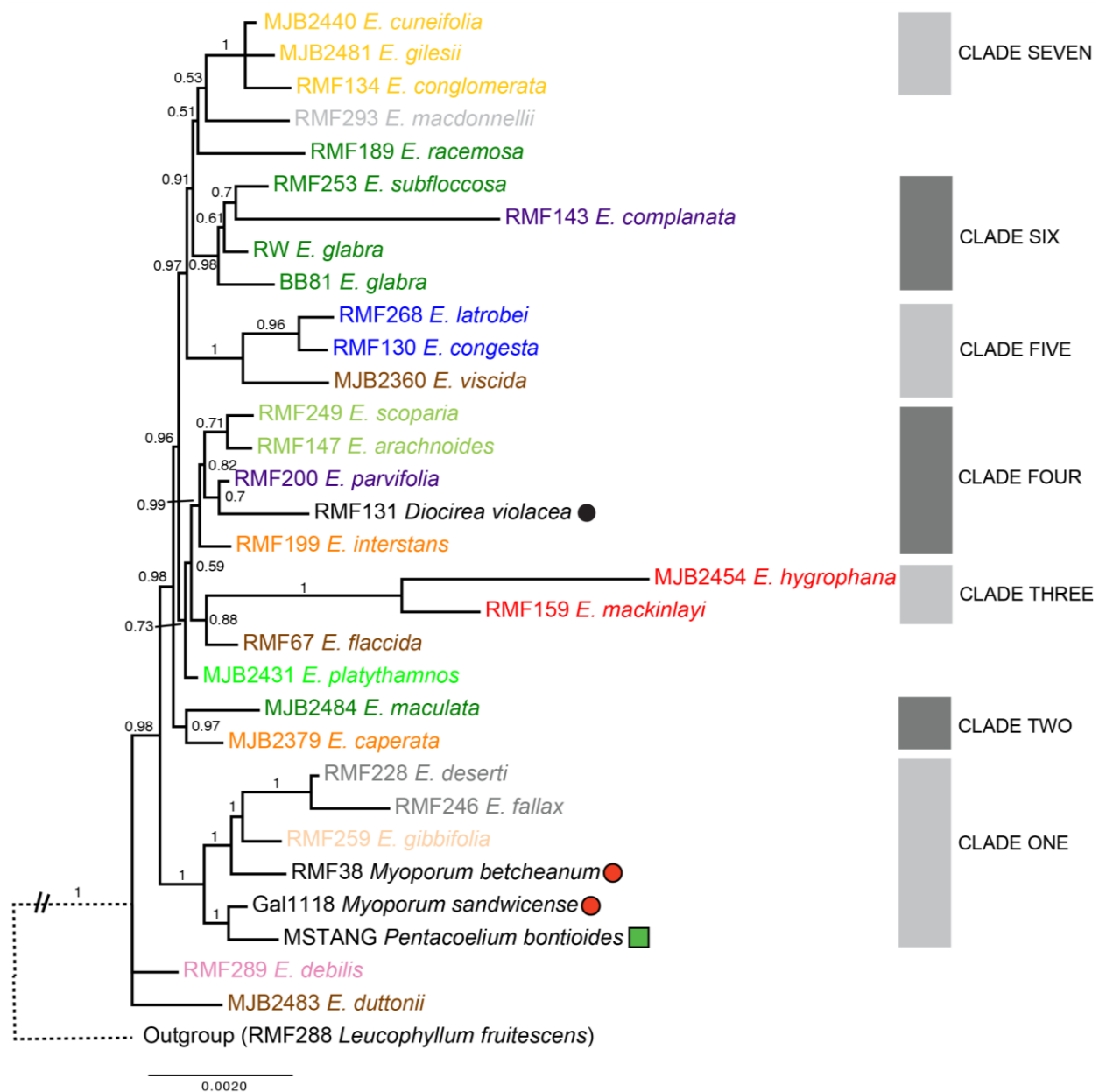


Figure 5.3 Bayesian inference phylogeny of all five regions of mtDNA analysed. Posterior probability (pp) values included beside each branch/node. *Eremophila* species are coloured according to Chinnock's sectional classification (*Pulchrisepalae*, *Chamaepogonia*, *Duttonia*, *Pholidiopsis*, *Crustaceae*, *Stenochilus*, *Arenariae*, *Hygrophanae*, *Australophilae*, *Pholidia*, *Eriocalyx*, *Platycalyx*, *Eremaeae*) and other genera identifies with coloured symbols. Clades are labelled according to grey bars on right side of figure.

Research to date indicates that the nucleotide substitution rate of the plant mitogenome is unusually low compared to chloroplast, nuclear and metazoan mitochondrial genomes (Wolfe et al., 1987; Druin et al., 2008; Richardson et al., 2013). Estimates suggest that rates of plant mitogenome evolution are on average 2–3× lower than that

of the chloroplast genome and 10–20× lower than the nuclear genome (Parkinson et al., 2005; Duminil, 2014; Malé et al., 2014), and exceptionally high or low rates are hypothesised to exist in 10–20% of plant species (Mower et al., 2007; Sloan et al., 2012; Duminil, 2014). In general, tribe Myoporeae appears consistent with most plant groups in displaying low levels of mitogenomic sequence variation, with an average proportion of 0.4% parsimony informative characters (PICs) across all coding and non-coding regions of the mitogenomic alignment analysed. This compares with average proportions of 1.5% and 7.4% PICs exhibited respectively, in chloroplast and nuclear ribosomal datasets analysed with the same 32 specimens as the mtDNA analysis. The relatively high levels of variable yet parsimony uninformative characters (i.e. characters that varied in only a single individual) observed across the majority of non-coding mtDNA regions analysed indicates that with the inclusion of additional samples from across Myoporeae, the number of PICs, and thus phylogenetic signal, may also increase (as seen between reduced and complete cpDNA and nrDNA datasets of Table 5.2). That said, the five mtDNA regions were specifically selected for this analysis as having displayed the highest levels of variation from across the mitogenome. With this in mind, additional samples and/or additional mtDNA regions may still not result in a well-resolved and informative phylogeny.

Aside from the average rate of sequence divergence observed in Myoporeae, it is interesting to note the variation in rate between different parts of the mitogenome. Not surprisingly, on average gene (coding) regions of the mitogenome displayed lower rates of divergence (0.1%) than non-coding DNA regions (0.95%), though significant heterogeneity was also observed within these regions (see Table 5.1). For example, the highest proportions of PICs among the regions sampled here occurred in the non-coding spacer regions of *spacer2* (3.19%) and *cob-rpl5* (1.09%) which are comparable to the levels of variation seen for the same samples across the chloroplast genome (1.5%). In other plant groups, rate heterogeneity within the mitogenome is hypothesised to occur for a number of different reasons, including horizontal gene transfer, insertions from other (chloroplast, nuclear) genomes, or mutations that affect the nuclear gene control of replication and repair in the mitochondrial genome (Parkinson et al., 2005; Warren et al., 2016).

The propensity of the mitochondrial genome to gain significant amounts of ‘foreign’ DNA has long been recognised (Palmer, 1992), with current estimates suggesting 1–12% of angiosperm mitochondria is of plastid origin (Mower et al., 2012; Zhu et al., 2016) and 0.1–13% is of nuclear origin (Kubo and Newton, 2008; Rodriguez-Moreno et al., 2011). While transfers of DNA regions of chloroplast origin are relatively easy to discern within the mitogenome, DNA from the highly variable, large, are relatively uncatalogued nuclear genome is considerably more difficult to detect (Knoop, 2012). In addition, the vast majority of non-coding DNA in the mitogenome is of unknown origin, presumed to be the result of horizontal transfer from bacteria, viruses, and genomes from other plant species (Kubo and Newton, 2008; Knoop, 2012; Park et al., 2014; Warren et al., 2016). Linear plasmids thought to have been originally derived via horizontal transmission from fungi are thought to be relatively common elements in the plant genome, either stand-alone or integrated into the mitogenome, and are hypothesised as one of the major contributors to the variable rates of sequence evolution in the mitogenome (Warren et al., 2016). In Myoporeae, both regions of highest mtDNA sequence diversity (spacer2 and *cob-rpl5*) are non-coding, and in the instance of spacer2, placed quite distantly from gene regions. A ‘blast’ search of the spacer2 region ruled out chloroplast origin, but nuclear transfer or linear plasmid origin, are possible explanations for elevation in the rate of sequence diversity observed. As more of the mitochondrial genome is sequenced across plants more generally, it seems likely that light will be shed on the origin of much of the unknown content of the plant mitogenome, which will be critical for better understanding the processes that shape its structure and composition.

5.4.2 *Phylogeny – incongruence between mitochondrial and chloroplast/nuclear phylogenies*

Based on their shared, uniparental, usually maternal, mode of inheritance and lack of recombination, organellar genomes of the chloroplast and mitochondrion are assumed to reflect the same evolutionary signal (Rieseberg and Soltis, 1991; Govindarajulu et al., 2015). But until recently, this theory was largely untested, with the majority of plant phylogenetic studies focusing on chloroplast and/or nuclear DNA only. As comparative studies of the chloroplast and mitochondrial genomes are gradually completed, it is becoming apparent that these two organelles do not always produce congruent

phylogenetic signals (Wang and Wang, 2014; Govindarajulu et al., 2015; Zhu et al., 2016; Rydin et al., 2017), and such incongruence between chloroplast, nuclear and mitochondrial DNA phylogenetic reconstructions in the tribe Myoporeae is similarly apparent.

While some relationships established in the mtDNA phylogeny of Myoporeae are well supported in both the cpDNA and nrDNA phylogenies (Chapters Three and Four), the placements of a number of taxa in the mtDNA phylogeny are unique, differing substantially, and with high support, from both the cpDNA and nrDNA reconstructions. Looking specifically at strongly supported nodes in the mtDNA analysis, 13/16 are incongruent when compared with relationships of the cpDNA analysis and 9/16 are incongruent when compared with the nrDNA analysis. For example, in clade five of the mtDNA analysis (Figure 5.2) the relationship between *E. latrobei* and *E. congesta* (both from sect. *Eriocalyx*) is evident in both cpDNA and nrDNA reconstructions, but the sister placement of *E. viscida* (sect. *Pulchrisepalae*) has not been recovered in either cpDNA nor nrDNA analyses. In the cpDNA analysis *E. viscida* is well supported in clade nine, more closely related to *E. platythamnus* than to *E. latrobei* or *E. congesta* which are placed in cpDNA clade seven. Similarly, in the nrDNA analysis *E. latrobei* and *E. platythamnus* are well supported as sister in clade eleven, while *E. viscida* is placed in clade six, more closely related to *E. interstans* and *E. platythamnus*. Similar incongruence can also be observed for the relationship between *E. maculata* and *E. caperata* in clade two of the mtDNA analysis. Though well supported as sister in the mtDNA analysis (pp=0.97), in the cpDNA analysis both *E. caperata* and *E. maculata* are placed in clade nine, but most closely related to other species of *Eremophila* (*E. parvifolia*/*E. scoparia*/*E. arachnoides* and *E. racemosa* respectively), and in the nrDNA analysis *E. caperata* is placed in clade six, while *E. maculata* placed in clade nine.

Also evident throughout the mtDNA phylogeny are several instances in which the phylogenetic reconstruction differs from the cpDNA analysis, yet supports the nrDNA analysis. Clade six of the mtDNA phylogeny encompasses three of the five samples representing section *Stenochilus* (*E. subfloccosa* and two subspecies of *E. glabra*), as well as *E. complanata* (sect. *Australophilae*). The cpDNA analysis supports the close relationship between *E. subfloccosa* and *E. glabra*, however, *E. complanata* differs significantly in its position between mtDNA and cpDNA analyses. Based on the cpDNA

analysis, *E. complanata* should be placed in clade four with species from sections *Pholidia*, *Crustaceae*, *Australophilae* and genus *Diocirea*. Interestingly, the placement of *E. complanata* actually best reflects the relationships established in the nrDNA phylogeny, whereby section *Stenochilus* is resolved as sister to a subset of species from sect. *Australophilae*, including *E. complanata* (see Figure 4.6). A similar instance of congruence between the nrDNA and mtDNA analyses is observed in the relationships of clade four. The composition of clade four largely represents clade seven of the cpDNA analysis, and clade five of the nrDNA analysis, but the presence of *E. interstans* is only congruent with the nrDNA reconstruction. In the cpDNA analysis, *E. interstans* (RMF199) is placed in an anomalous position, distant from all species in sect. *Crustaceae* (see Figure 3.5), but the nrDNA reconstruction places all species from sect. *Crustaceae* sister to sections *Pholidia*, *Australophilae* and genus *Diocirea*.

Congruence between the nrDNA and mtDNA phylogenies rather than between cpDNA and mtDNA phylogenies is unexpected based the presumed differing modes of inheritance of each genome. The nuclear genome is biparentally inherited (Mendelian), while the chloroplast and mitochondrial genomes are generally expected to follow uniparental (clonal) inheritance (Birky, 2001). Mitochondria were traditionally thought to be exclusively maternally inherited, but increasingly instances of paternal inheritance, heteroplasmy and paternal leakage have been recorded (for example conifers, Neale et al., 1989; bananas, Fauré et al., 1994; cucumber, Havey, 1997; green algal genus *Chlamydomonas*, Aoyama et al., 2006; Plantago, Levensen et al., 2016), indicating alternative modes of inheritance may be more common than previously thought (Barr et al., 2005; McCauley, 2013).

A number of hypotheses may explain the observed patterns of congruence between the nrDNA and mtDNA analyses. Although the mtDNA phylogeny was generated based upon five different markers from across the genome, the bulk of parsimony informative characters were located in the 'spacer2' region, which is placed in an unknown location of the mitochondrial genome (see Figure 5.1). Movement of DNA between genomes is well documented, including from the nuclear and/or chloroplast genome to the mitochondrion and from mitochondria to the chloroplast and/or nucleus (Palmer, 1992; Palmer et al., 2000; Duminil, 2014). It seems plausible that a transfer of DNA from the nuclear genome to the mitogenome could explain a signal most reflective of the nrDNA

analysis. A second possibility is that of paternal leakage, resulting in biparental inheritance of the mitogenome, or alternatively, a paternal mode of inheritance. Instances of biparental and paternal mitogenomic inheritance are well documented in plants (Weihe et al., 2009; Zhu et al., 2016) and could explain the congruence between nrDNA and mtDNA phylogenies, and differences between the cpDNA and mtDNA phylogenies. At this stage, mode of inheritance is assumed in the Myoporeae, further work to clarify the origin of patterns of phylogeny in the tribe would be best focused on some foundational work on inheritance.

5.4.3 *Conclusion*

Overall, the genome skimming approach implemented for the exploration of tribe Myoporeae was successful in generating not only chloroplast and nuclear DNA, but also large stretches of the mitogenome. Within Myoporeae, the mitogenome appears to be fairly conventional in its low rate of sequence variation and presence of significant structural rearrangements. A moderately supported mtDNA phylogeny was generated, and many of the relationships established mirror those of the cpDNA and nrDNA analyses. A number of the relationships uncovered were unique to the mitogenome (not seen in previous analyses), or were matched the nrDNA phylogeny but not the cpDNA phylogeny. Such incongruence between the cpDNA and mtDNA was unexpected, but it adds to the growing body of research that indicates the evolution of the mitogenome is more complex than previously thought (Sloan et al., 2012; Gualberto et al., 2014; Park et al., 2014; Folk et al., 2017).

Initial assessment of the mitogenome by Palmer (1992) dissuaded its use in plant phylogenetics, largely due to proposed low levels of sequence variation and high degrees of structural rearrangement. These features do appear to be consistent across the many plant groups now analysed, though with access to HTS technology and the ability to sequence the entire genome, the mitogenome can provide an additional line of evidence from a different organelle from which evolutionary relationships (and processes such as ILS and introgression) can be explored. Going forward, the plant mitogenome is a good candidate for further research, particularly using a relatively cost-effective genome skimming approach to sequence all high copy regions of the genome. While coding regions of DNA were somewhat uninformative for phylogenetic

reconstruction at species level in Myoporeae, this does not always hold true across other plant groups. Likewise, the mitogenome is likely to be more informative at higher levels of classification (i.e. family, order). Non-coding regions of the mitogenome were more variable than gene regions, but due to the highly variable size and composition of the genome, the identification and alignment of homologous DNA sequence between individuals can be difficult and time consuming. In the case of Myoporeae, time spent sequencing species at greater depth in order to construct complete mitochondrial genomes for the purposes of phylogenetic analyses may be better spent focusing on other more informative genomic DNA, such as low copy regions of the nuclear genome.

Chapter Six: General discussion, biogeography and final conclusions

6.1 Phylogeny of tribe Myoporeae

This research aimed to construct a phylogeny of the largely Australian tribe Myoporeae, including comprehensive coverage of the large (c. 233 species) and mostly arid-distributed Australian endemic genus, *Eremophila*, and all six allied genera from Australia, islands of the Pacific, southern China and the Caribbean. High throughput sequencing (HTS) was used, taking a genome (shotgun) skimming approach to minimise the sequencing costs while obtaining data from all three plant genomes (plastome, nuclear, mitochondria) targeted in this study. The library preparation method developed specifically for this study was successful in generating the quality and quantity of DNA required to construct entire chloroplast genomes and high copy regions (ribosomal cistron) of the nuclear genome for 317 and 355 specimens of *Eremophila* and Myoporeae, respectively. In addition, large regions of DNA from the mitochondrial genome (mitogenome) were also assembled, and were analysed for a subset of 31 samples.

The chloroplast genome (cpDNA) dataset (c. 143000 bp) produced a highly supported and well resolved phylogeny. All six allied genera (*Myoporum*, *Diocirea*, *Calamphoreus*, *Glycocystis*, *Bontia* and *Pentacoelium*) are nested within clades of a polyphyletic *Eremophila*. A small number of Chinnock's (2007) 25 sections are supported as monophyletic in the cpDNA analysis, though the majority of species do not cluster into lineages based upon the morphology-based sections. Similarly, while some species represented by multiple accessions are resolved as monophyletic, the vast majority were found to be non-monophyletic, despite evidence of clear morphological features uniting them. The processes of introgressive hybridization and incomplete lineage sorting, and some instances of inaccurate taxonomy, are all likely to be contributing to issues of non-monophyly in the cpDNA dataset.

The nuclear ribosomal (nrDNA) dataset (c. 8000 bp) displayed fewer well supported nodes than the cpDNA analysis, and was less well resolved overall, but was informative

in reconstructing species, section and generic level relationships in the tribe. As in the cpDNA analysis, all six allied genera were nested within *Eremophila*, however, sections were much more clearly delimited into distinct lineages and fewer instances of non-monophyly were observed at species level. The effects of introgression and incomplete lineage sorting were still evident in the nrDNA phylogeny, but the combined interpretation of both cpDNA and nrDNA results allowed for the most complete (and likely informative) interpretation of phylogeny in Myoporeae.

The mitogenome was explored, and five regions of mtDNA (c. 36,000 bp) were constructed and analysed for a subset of samples, and results were compared with cpDNA and nrDNA phylogenies. Low levels of sequence variation and significant structural rearrangements within the mitogenome made phylogenetic analysis within Myoporeae difficult, however, a moderately supported phylogeny was still reconstructed. Interestingly, the mtDNA analysis was most congruent with the previous nrDNA analysis, rather than the cpDNA analysis. Based on these relatively low levels of sequence variation and difficulties with full mitogenome assembly, for the purposes of phylogeny reconstruction in Myoporeae, mtDNA is interesting to explore but probably less useful at present than other more variable regions of DNA, such as low copy regions of the nuclear genome.

6.2 Implications for generic classification of tribe Myoporeae

In the most recent and comprehensive revision of tribe Myoporeae, Chinnock (2007) recognised Myoporeae to contain seven genera: *Eremophila*, *Myoporum*, *Diocirea*, *Calamphoreus*, *Glycocystis*, *Bontia* and *Pentacoelium*. *Bontia* was the first of these genera to be formally described (Linnaeus, 1753), followed by *Myoporum* (Forster, 1786). *Eremophila* was not formally described until 1810 by Robert Brown, though the earliest species, *E. debilis*, was first collected in the 1790s and described under the illegitimate genus name *Pogonia* (Andrews, 1802). In his monograph, Chinnock (2007) identified and formally described *Diocirea*, *Calamphoreus* and *Glycocystis* for the first time. The monophyly of tribe Myoporeae was initially inferred from vegetative morphology and anatomy (Chinnock, 2007), and was supported phylogenetically by the preliminary work of Kelchner (2003). Although the tribal status of Myoporeae and sister

relationship to Central American tribe Leucophylleae is now well established (Olmstead et al., 2001; Oxelman et al., 2005), generic limits and relationships between the seven genera in Myoporeae were hypothesised but untested by formal analysis until now. The current thesis is the first comprehensive molecular analysis of the tribe and reveals a number of well supported generic relationships not recognised previously.

Representatives from all seven genera were included in this study for both chloroplast and nuclear ribosomal analyses, and though the topology of each phylogeny was partially incongruent (see Chapters Three and Four), in both analyses *Eremophila* was paraphyletic, with all allied genera nested amongst species of *Eremophila* with strong support (see Figure 6.1). The cpDNA phylogeny is well resolved, highly supported, and clearly places *E. tetraptera* as sister to the remainder of the tribe. The nested placement of *Bontia*, *Calamphoreus*, *Diocirea*, *Glycocystis*, *Myoporum* and *Pentacoelium* within lineages of *Eremophila* in the cpDNA phylogeny indicates the need for significant taxonomic revision of Myoporeae. In contrast, the nrDNA phylogeny is less well resolved, particularly at the base of the phylogeny. Clades one to four in the nrDNA phylogeny form a polytomy, and are equally likely as sister to the remainder of the tribe. The positions of *Calamphoreus* and *Diocirea* in the nrDNA phylogeny are consistent with the cpDNA analysis, suggesting that they should be congeneric with the core groups of species currently included in *Eremophila*. The taxonomy of *Bontia*, *Glycocystis*, *Myoporum* and *Pentacoelium* also clearly requires revision, but decisions about that are more complex and changes should not be undertaken at the current time. This is because uncertainty remains about their relationships, in particular that they all fall at a basal polytomy in the nrDNA analysis, making any taxonomic decisions premature and not necessarily stable. Without additional nuclear datum, it is not possible to confirm which of *Bontia*, *Eremophila*, *Glycocystis*, *Myoporum* or *Pentacoelium* is sister to the remainder of the tribe, which has taxonomic implications for the entire lineage.

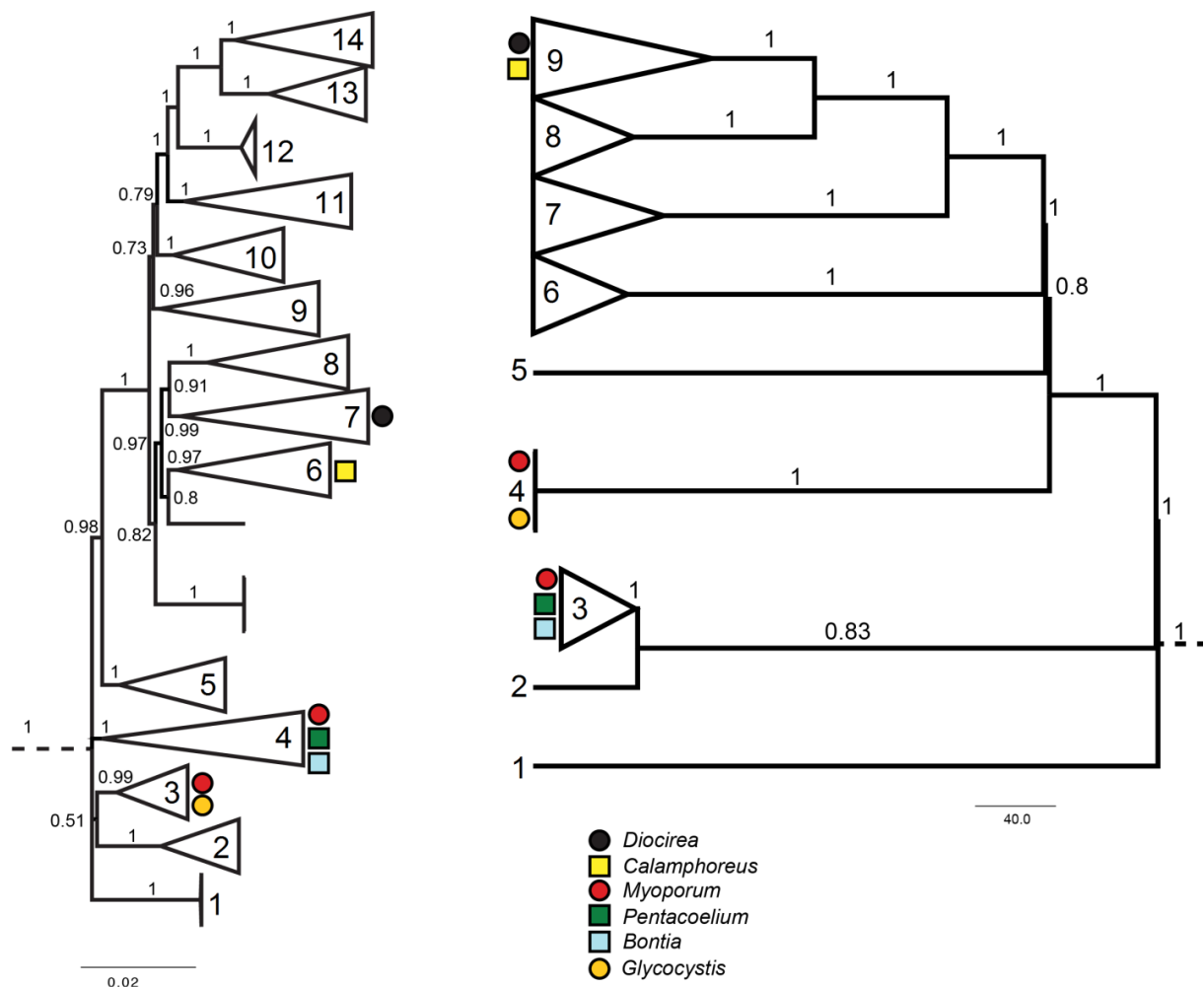


Figure 6.1 Comparison of the summary trees based on the Bayesian phylogenetic analyses of nuclear ribosomal (left) and chloroplast genome (right) datasets. Posterior probabilities are assigned to each branch/node. Species of *Eremophila* are present in every lineage except clade four of the chloroplast genome phylogeny; coloured symbols represent the presence of allied genera, as indicated. Clade numbers on trees match those of Figure 4.2 (nuclear ribosomal DNA tree) and Figure 3.3 (chloroplast DNA tree).

6.2.1 Rules and conventions surrounding plant nomenclature and implications for tribe *Myoporeae*

The International Code of Nomenclature for Algae, Fungi and Plants (ICN) outlines a standard set of rules and recommendations to be followed when naming plant taxa (McNeill et al., 2012; most recent Shenzhen Code (2017) not yet published). At its core, the ICN ensures that all taxa, at a given rank and circumscription, have a unique name, supported for future reference with a designated type specimen (ideally accessible in a public collection) and that the name is effectively and validly published. Application of

the ICN is not always straightforward, with a number of articles in the code competing in the outcome. For example, if a group is described more than once, the earliest legitimate name of the same rank is given priority (Melbourne Code, Chapter II, Section 3, Article 11.3), but to maintain stability of nomenclature, the concept of nomenclatural conservation can be implemented, for example, a later published name can be conserved over the original (Melbourne Code, Chapter II, Section 4, Articles 13–15), or a name re-typified to reflect a preferred use of name. Such exceptional cases are well documented (i.e. conservation of the name *Acacia* for Australian taxa over the South African type; Orchard and Maslin, 2003; Luckow et al., 2005; Dyer, 2014), and seem likely to increase in number as molecular phylogenetic studies continue to uncover previously unrecognised relationships.

While the ICN provides a code of nomenclatural rules to regulate taxonomic naming, the delimitation of taxonomic boundaries to which names are applied is somewhat subjective, and is largely dependent on the taxonomic criteria being followed, about which there is much debate at all levels of the taxonomic hierarchy, especially with respect to species (de Queiroz, 2007), genera, families and orders (APG, 2016; Stevens, 2017). Since the development of the field of phylogenetic systematics the concept of monophyly has given taxonomists an objective basis with which taxa can be grouped (Baum and Smith, 2013). The identification of evolutionary relationships and organisation of taxa following the concept of monophyly (i.e. groups that include all descendants of a common ancestor, Wiley and Lieberman, 2011), is central to modern systematics, and though can be problematic at species rank and below (Rieseberg and Brouillet, 1994; de Queiroz, 2007; Knowles and Carstens, 2007) at higher taxonomic levels monophyly is commonly agreed as the best method to guide classification based on natural groups (Entwistle and Weston, 2005; Thiele et al., 2015). For this reason, monophyly will be used to direct the limits of genera in the current study.

All three datasets explored in the current thesis (cpDNA, nrDNA, mtDNA) indicate a lack of monophyly in all non-monotypic genera, and therefore the need for significant taxonomic revision at generic rank, in tribe Myoporeae. Unfortunately, due to incongruence between cpDNA and nrDNA datasets, including the uncertain relationships of key taxa at the base of the nrDNA analysis, at this stage it is not possible to confirm the most appropriate revision/framework for the tribe. To ensure an

evolutionarily accurate, robust and long-lasting classification, additional phylogenetic support is recommended, ideally, in the form of multiple independent nuclear loci. Despite the limitations of the current nrDNA phylogeny (i.e. ambiguous placement of *Myoporum*, *Pentacoelium*, *Bontia* and *Glycocystis* relative to *Eremophila* due to a lack of resolution at the base of the nrDNA phylogeny), it is still possible to explore some of the most likely taxonomic revision scenarios for Myoporeae below.

6.2.2 Option One – All seven genera of tribe Myoporeae reduced to a single genus

The well supported placement of *E. tetraptera* in clade one of the cpDNA analysis, and the inclusion of *Eremophila* species in each of the four clades in a basal polytomy of the nrDNA phylogeny (see Figure 6.1), highlights the paraphyletic nature, and likely sister status of some *Eremophila* species relative to all other genera in Myoporeae. The most straightforward solution to achieve monophyly in this instance would be to reduce all genera in Myoporeae to a single genus (see Figure 6.2). If that genus was called *Eremophila*, it would serve to minimise taxonomic change (resulting in c. 38 name changes for all the species in the six allied genera, see Table 6.1), but would violate the ICN rule of priority, with both *Bontia* and *Myoporum* described prior to *Eremophila*. If the rule of priority was upheld and either *Bontia* or *Myoporum* was used as the name for the one large genus of Myoporeae, name changes, as new combinations, would have to be published for c. 360 taxa. The iconic status and the sheer number of *Eremophila* species in Australia, compared with the relatively obscure and monotypic genus *Bontia*, could justify a proposal to conserve the name *Eremophila* over the legitimate names of the same rank, *Bontia* and/or *Myoporum* under Article 14 of the ICN (Melbourne Code).

6.2.3 Option Two – Maintaining *Eremophila* and *Bontia*/*Myoporum* at generic rank

An alternative option is to divide tribe Myoporeae into two (or more) genera, based on the topology of the nrDNA phylogeny. The base of the phylogeny (clades one to four, Figure 6.2) would represent one or more genera, while the bulk of ‘core’ *Eremophila* (clades five to fourteen) would be maintained as *Eremophila*. As is stands, clades one to four in the nrDNA analysis form a polytomy, making it unclear as to whether they constitute a single clade or a series of clades that form a paraphyletic group. Ideally, further nuclear datum would be obtained to confirm the order of these basal clades, then depending on what scenario is revealed, the clade(s) named to best represent

species relationships. While clades one and two consist entirely of *Eremophila* species, clades three and four contain species from *Myoporum*, *Bontia*, *Pentacoelium* and *Glycocystis*. *Bontia* and *Myoporum* and represent the two earliest names in the tribe, so depending on their placement are likely candidates for generic names of this clade(s). An additional consideration is that of minimising taxonomic change; if resolved as a single lineage the later named *Myoporum* would result in fewer taxonomic changes (20) across the lineage than if the genus name *Bontia* was used (49 changes)(see Table 6.1). Regardless of the scenario implemented for clades one to four, naming of clades five to fourteen would be straightforward, with *Eremophila* (based on the type, *E. oppositifolia*, placed in clade six) maintained and the small Australian genera *Calamphoreus* and *Diocirea* sunk into *Eremophila* based on their nested positions. Overall, under this scenario the monophyly of each genus could be maintained and taxonomic change would be minimised across the large Australian genus *Eremophila*.

Both of the options outlined above require significant change to the classification of the tribe, and it should be emphasised again that until the nuclear phylogeny is more fully resolved, an informed decision cannot be made as to whether either of these options, or a different scenario entirely, is most favourable. Based on the congruence between the cpDNA and nrDNA phylogenies, certainty does surround the reduction in rank of *Calamphoreus*, *Diocirea* and *Pentacoelium*, while the maintenance of *Eremophila* in some form is favourable to minimise taxonomic change based on the sheer number of species currently described. The fate of *Bontia* and *Myoporum* depends entirely upon the outcomes of further research.

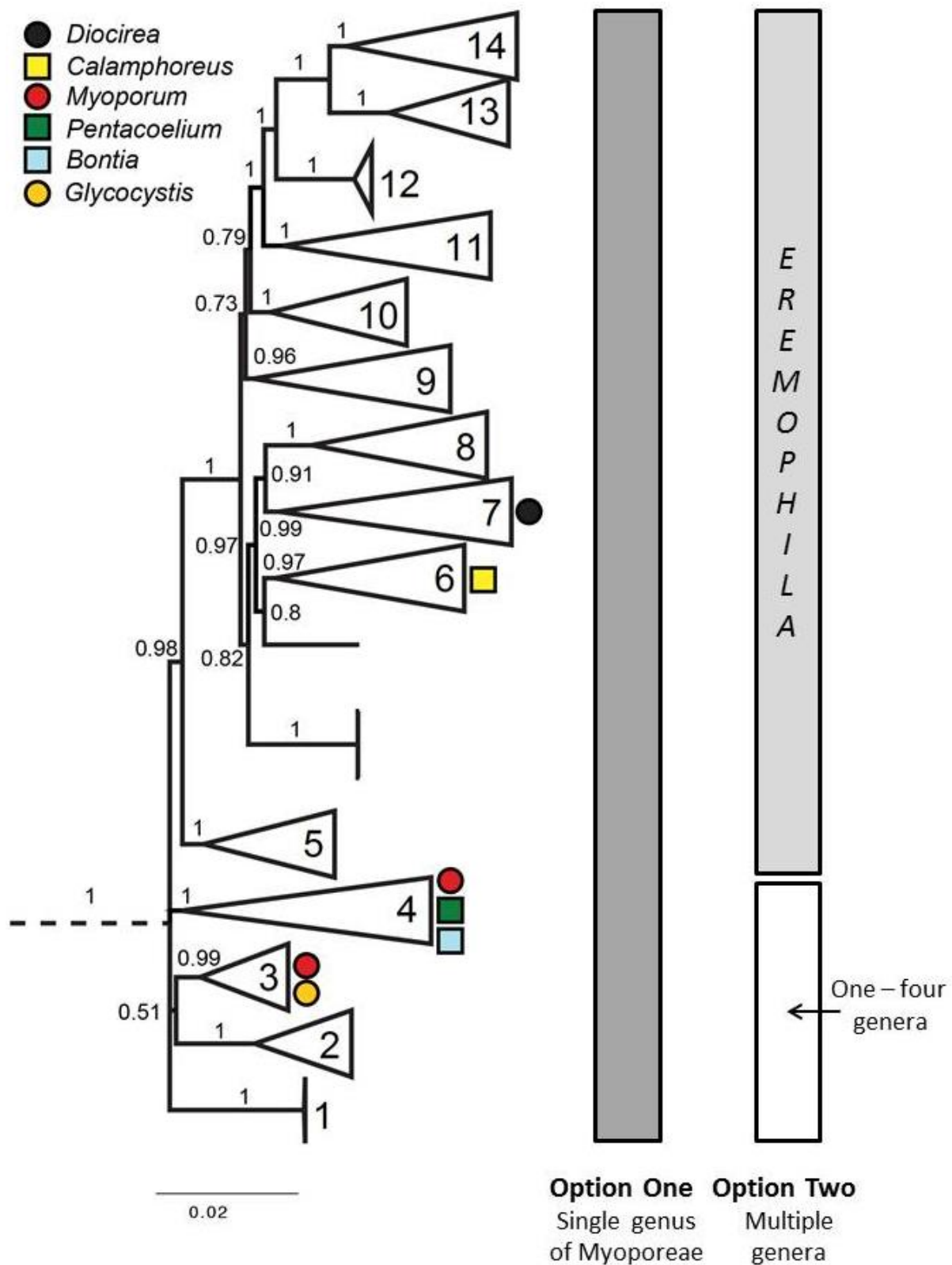


Figure 6.2 Summary of two possible scenarios for taxonomic revision of tribe Myoporeae at generic rank, based on nuclear ribosomal (nrDNA) Bayesian Inference majority-rule consensus tree. Posterior probability values are associated with branches and clade numbers given in clade summary triangles. All clades except clade three contain species of *Eremophila*, coloured symbols next to summarised clades represent the presence of allied genera (refer to key in top left corner).

Table 6.1 Number of changes in species names associated with potential scenarios for taxonomic revision of Myoporeae. Option one refers to the reduction of all genera to a single genus (preferably *Eremophila*). Option two refers to the division of Myoporeae into two or more genera; (nrDNA) clades five to fourteen being *Eremophila*, and clades one to four to one or more of *Bontia*, *Myoporum*, *Pentacoelium*, *Glycocystis*. If clades one to four are supported through further analyses as a single lineage, option two(a) *Myoporum* or two(b) *Bontia* would be favoured being the earliest described names of the tribe.

Option One		Option Two (a) - <i>Myoporum</i>		Option Two (b) - <i>Bontia</i>	
<i>Calamphoreus</i>	1	<i>Calamphoreus</i>	1	<i>Calamphoreus</i>	1
<i>Diocirea</i>	4	<i>Diocirea</i>	4	<i>Diocirea</i>	4
<i>Bontia</i>	1	<i>Bontia</i>	1	<i>Bontia</i>	0
<i>Glycocystis</i>	1	<i>Glycocystis</i>	1	<i>Glycocystis</i>	1
<i>Pentacoelium</i>	1	<i>Pentacoelium</i>	1	<i>Pentacoelium</i>	1
<i>Myoporum</i>	30	<i>Myoporum</i>	0	<i>Myoporum</i>	30
<i>Eremophila</i>	0	<i>Eremophila</i>	3	<i>Eremophila</i>	13
Total	38	Total	20	Total	49

6.3 Research implications for Chinnock's (2007) sectional classification of *Eremophila*

In his monograph of tribe Myoporeae, Chinnock (2007) divided *Eremophila* into 25 sections based on vegetative, floral and fruit characters. Though not stated clearly, it is assumed this infra-generic classification was established both as a means to organise the large genus, particularly for the purposes of field-based species identification, and to reflect evolutionary groupings of species. Chinnock's sectional classification is undoubtedly successful in organising and categorising species of *Eremophila* for identification purposes, however, the molecular datasets generated in this thesis indicate that these sections do not necessarily reflect evolutionarily groupings.

The chloroplast dataset from Chapter Three only partially supports Chinnock's sectional classification of *Eremophila*, which is likely attributed to the effects of introgressive hybridization and incomplete lineage sorting. In comparison, the nuclear ribosomal dataset of Chapter Four more closely reflects morphological based taxonomy of *Eremophila*, and it is thus more likely to represent an accurate estimate of species relationships within sections. For this reason, the nuclear ribosomal phylogeny has been selected as a framework to view Chinnock's sectional classification.

Unlike the cpDNA analysis, the nrDNA phylogeny presented in Chapter Four, supported many of Chinnock's sections as monophyletic (i.e. the morphological characters used to delimit these sections are likely synapomorphies), and thus representative of phylogenetic relationships. In total, 13 of Chinnock's 25 sections are supported as monophyletic based on nuclear ribosomal DNA (see Table 6.2). For example, all species in sect. *Hygrophanae* are supported as monophyletic in the nrDNA analysis and are united morphologically by Chinnock based on the unique red/brown pigmentation of the corolla upon drying. Similarly, with the exclusion of *E. maculata* and *E. racemosa*, all species of sect. *Stenochilus* are supported as monophyletic and united through their small, scale-like sepals and uniquely marked ornithophilus flowers (*E. maculata* and *E. racemosa* are also supported as monophyletic though in their own distinct lineage). In contrast, 12 sections in the nrDNA phylogeny were found to be non-monophyletic. In these instances, the characters used to delimit the section are likely plesiomorphic/homoplastic and thus not useful for phylogenetic reconstruction.

Ideally classifications should reflect evolutionary relationships, as this aids in species identification, organisation and allows predictions to be made (Entwistle and Weston, 2005). While a sectional classification approach based solely on molecular datum is not ideal, the nrDNA phylogeny presented in Chapter Four provides a preliminary framework for evaluating the utility of Chinnock's sections. This molecular dataset would be best paired with further morphological assessment of the genus to identify possible synapomorphic morphological characters that support the sectional relationships. Such a morphological assessment of *Eremophila* in the context of molecular findings was beyond the scope of this study due to the extensive time required to develop and complete the molecular techniques used in the current study, but it is recommended to enable a more comprehensive subgeneric classification of the genus.

Table 6.2 Summary of taxonomic outcomes for Chinnock's (2007) sections of *Eremophila* as interpreted from the nuclear ribosomal (nrDNA) phylogeny.

Section	Clade(s)	Retain	Remove	Comments
1. <i>Platycarpus</i>	Five, Nine			Both species from sect. <i>Platycarpus</i> (<i>E. compressa</i> , <i>E. saligna</i>) are placed in different parts of the phylogeny, there is no molecular evidence that sect. <i>Platycarpus</i> should be maintained.
2. <i>Pholidiopsis</i>	Three, Four			Species from sect. <i>Pholidiopsis</i> form a well-supported lineage in clade four. Although a single specimen is placed separately to all other members (<i>E. deserti</i> , clade three) the section is still largely supported.
3. <i>Crustaceae</i>	Six	*With revision		All species from sect. <i>Crustaceae</i> are positioned in clade six, but nested amongst these species are also species from sections <i>Arenariae</i> , <i>Pulchrisepalae</i> , <i>Eremophila</i> and <i>Contortae</i> . As it stands, sect. <i>Contortae</i> requires re-circumscription, either to include additional species or restricted and split into additional sections.
4. <i>Chamaepogonia</i>	One			Both samples of the single species in this section (<i>E. debilis</i>) are placed in a separate, well supported lineage (clade one), supporting the monophyly and maintenance of sect. <i>Chamaepogonia</i> .
5. <i>Australophilae</i>	Six, Seven, Eight, Ten, Eleven,			Species from sect. <i>Australophilae</i> are placed in multiple clades throughout the phylogeny (strongly polyphyletic). Characters used to define this section are unlikely to be synapomorphic. To achieve monophyly, sect. <i>Australophilae</i> would be best dissolved and species absorbed in other sections to which they are most closely aligned.
6. <i>Duttonia</i>	Two, Four			<i>Eremophila gibbifolia</i> (the type) and <i>E. barbarta</i> are sister in clade two, while <i>E. crassifolia</i> and <i>E. behriana</i> are separate and sister in clade four. As it stands, this section is not monophyletic.
7. <i>Arenariae</i>	Six, Thirteen			Four of the five species sampled from sect. <i>Arenariae</i> , including the type, are placed in clade six, amongst species from sect. <i>Crustaceae</i> , but do not form a single clade. The fifth species (<i>E. spuria</i>) is nested amongst species from sect. <i>Eremaeae</i> in clade thirteen. Based on this placement, <i>E. spuria</i> would be best absorbed into sect. <i>Eremaeae</i> and the remainder of sect. <i>Arenariae</i> merged with sect. <i>Crustaceae</i> .
8. <i>Eremaeae</i>	Seven, Eleven, Thirteen, Fourteen	*With revision		Species of sect. <i>Eremaeae</i> are split between clades thirteen and fourteen. The bulk of species almost exclusively make up clade thirteen, while the section type, <i>E. freelingii</i> , is placed with seven other species from sect. <i>Eremaeae</i> as well as species from sections <i>Pulchrisepalae</i> , <i>Eremophila</i> and <i>Eriocalyx</i> in clade fourteen. While not exclusively monophyletic, sect. <i>Eremaeae</i> could be retained with revision.
9. <i>Carnosae</i>	Four			The single species in sect. <i>Carnosae</i> (<i>E. pentaptera</i>) is placed in clade four with monotypic genus <i>Bontia</i> and <i>E. polyclada</i> (sect. <i>Platytilus</i>). Placement of <i>E. pentaptera</i> as sister to a separate genus, its long branch length and unique morphology indicate it could be retained as a unique, monotypic section.
10. <i>Platytilus</i>	Seven			Two samples of the single species (<i>E. macdonnellii</i>) in sect. <i>Platytilus</i> form a distinct, well supported lineage as part of the basal trichotomy in clade seven, supporting the retention of this section.
11. <i>Eremophila</i>	Fourteen			The majority of species from sect. <i>Eremophila</i> are placed in clade fourteen, with species from other sections (<i>Eremaeae</i> , <i>Pulchrisepalae</i>) and will need revision.
12. <i>Platytilus</i>	Four			Both species (<i>E. polyclada</i> , <i>E. bignoniiflora</i>) are placed in the same lineage of clade four, but are more closely related to other species of <i>Eremophila</i> or genus <i>Bontia</i> than each other.

13. <i>Pholidia</i>	Seven	*With revision		All species from sect. <i>Pholidia</i> are placed in clade seven with strong support, but do not form a clade, being placed, with support, among species of sections <i>Decussatae</i> (all), <i>Sentis</i> (all), <i>Australophilae</i> (<i>E. caerullea</i> and <i>E. densifolia</i>) and <i>Eremaeae</i> (<i>E. attenuata</i>). Section <i>Pholidia</i> (which has equal priority to sect. <i>Sentis</i>) could be expanded to include these additional species.
14. <i>Sentis</i>	Seven			The two species in sect. <i>Sentis</i> (<i>E. divaricata</i> , <i>E. christophorii</i>) are nested in clade seven amongst species from sect. <i>Pholidia</i> , and not-monophyletic relative to one another. They could be placed in an expanded sect. <i>Pholidia</i> (see above).
15. <i>Decussatae</i>	Seven			The four (of five) species from sect. <i>Decussatae</i> sampled in this analysis do not form a clade and are nested in clade seven amongst species from sect. <i>Pholidia</i> .
16. <i>Hygrophanae</i>	Nine			All six species in sect. <i>Hygrophanae</i> form a well-supported, distinct lineage, supporting the maintenance of this section.
17. <i>Contortae</i>	Six, Eleven			Both species of sect. <i>Contortae</i> (<i>E. macgillvrayi</i> , <i>E. pterocarpa</i>) are placed in separate clades of the phylogeny, supporting the dissolution of this section.
18. <i>Eriocalyx</i>	Eleven, Fourteen	*With revision		The majority of species in this large section are well supported as a distinct lineage. The limits of sect. <i>Eriocalyx</i> would need revision, to potentially include additional species from sections <i>Campanulatae</i> , <i>Australophilae</i> , <i>Eremaeae</i> and <i>Pulchrisepalae</i> .
19. <i>Campanulatae</i>	Eleven			Both species in sect. <i>Campanulatae</i> (<i>E. campanulata</i> , <i>E. humilis</i>) are nested amongst species from sect. <i>Eriocalyx</i> in clade eleven; based on this placement and morphological similarity between the sections it is suggested that sect. <i>Eriocalyx</i> and sect. <i>Campanulatae</i> be merged (sect. <i>Eriocalyx</i> is the older name).
20. <i>Subscissocarpae</i>	Four			<i>Eremophila tetraptera</i> is supported to maintain its monotypic sectional status through separate placement in clade four.
21. <i>Amphichilus</i>	Four, Five, Ten			The species from sect. <i>Amphichilus</i> are scattered throughout the phylogeny, indicating the section would be best dissolved and species re-assigned based on their molecular placement.
22. <i>Stenochilus</i>	Eight, Nine	*With revision		With the exclusion of <i>E. maculata</i> and <i>E. racemosa</i> , all species from sect. <i>Stenochilus</i> , including the type, form a distinct, highly supported lineage. <i>Eremophila maculata</i> and <i>E. racemosa</i> form their own lineage and could be reassigned a new section.
23. <i>Virides</i>	Eight			Species from sect. <i>Virides</i> form a distinct, highly supported lineage in clade eight, supporting their sectional status.
24. <i>Scariosepalae</i>	Five			The two species from sect. <i>Scariosepalae</i> (<i>E. alternifolia</i> , <i>E. purpurescens</i>) form a distinct lineage sister to <i>E. rostrata</i> (sect. <i>Amphichilus</i>) and <i>E. saligna</i> (sect. <i>Platycarpus</i>). The limits of sect. <i>Scariosepalae</i> could be expanded to include these species.
25. <i>Pulchrisepalae</i>	Six, Eleven, Twelve, Fourteen			The species from sect. <i>Pulchrisepalae</i> are scattered across the phylogeny, indicating that species could mostly be assigned to other sections, with sect. <i>Pulchrisepalae</i> potentially dissolved.

6.4 Biogeographic implications of this research

6.4.1 Biogeography of Australia's Eremean zone

The Eremean (arid) zone is the largest biome in Australia (Crisp et al., 2004), comprising c. 70% of the total land area (see Figure 6.3), and it is characterised by low (<250 mm) and unpredictable rainfall. Environments in the Eremean zone contain approximately 10% of Australia's plant species (Barker and Greenslade, 1982), of which *Eremophila*, along with *Acacia*, *Triodia*, Amaranthaceae, Brassicaceae and Goodeniaceae, are significant components (Shmida, 1985). The history and origin of the Australian Eremean zone is complex and somewhat uncertain, in part due to a distinct lack of macrofossils from the region due to unfavourable preservation conditions (Field et al., 2002; Miller et al., 2013). Despite this uncertainty, the evidence available suggests the arid zone is relatively recent in origin, with the fossil and geophysical record indicating cyclical conditions but an inexorable shift in climate towards aridification in the Miocene (25–10 ma) followed by the extreme, fully arid landform formation of central Australia during the Pliocene (1–4 ma) (Crisp et al., 2004; Byrne et al., 2008; Pepper et al., 2011).

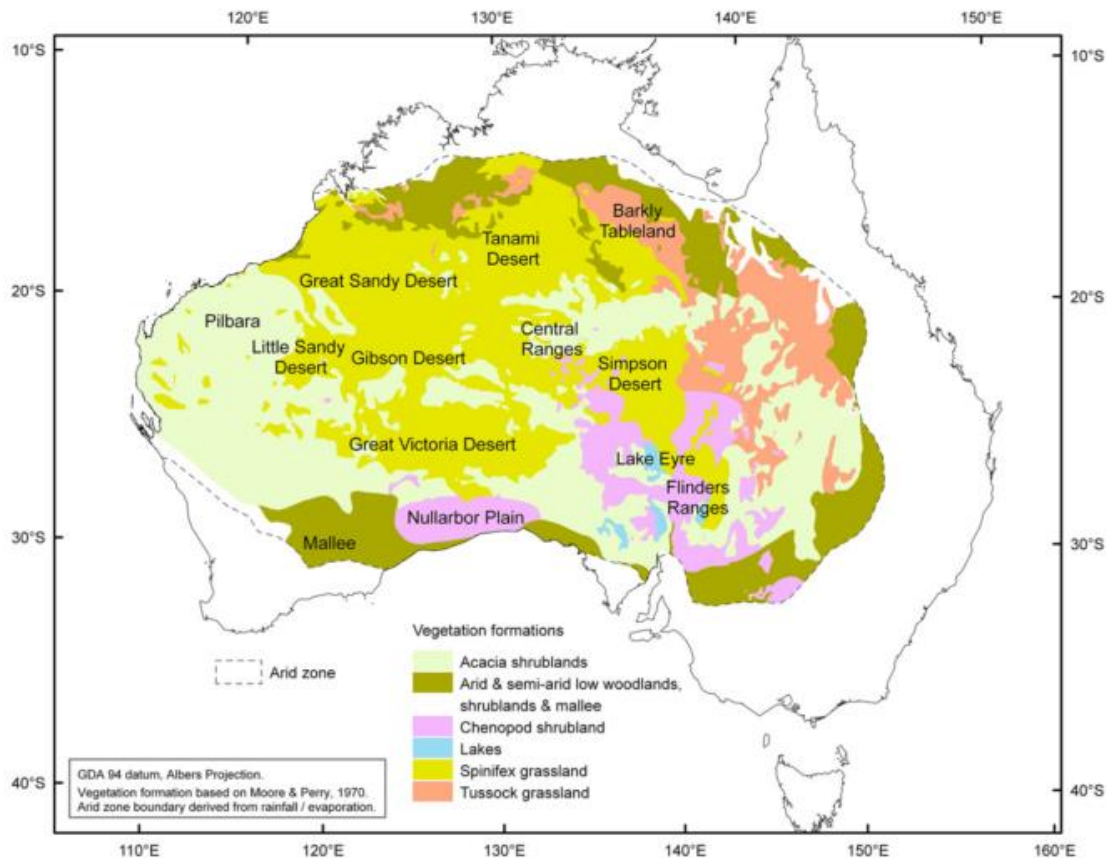


Figure 6.3 Map of the Australian Eremean zone, major deserts and dominant vegetation formations, from Morton et al., 2011.

Formation of the deep ocean passage between Tasmania and Antarctica, which occurred at a similar time to the detachment of South America and Australia from Antarctica (40–30 ma), allowed for the formation of the Antarctic circumpolar current (Wilford and Brown, 1994; Crisp et al., 2004). The circumpolar current increased the latitudinal thermal gradient, leading to a significant change in climate and formation of expansive ice sheets in Antarctica (Crisp et al., 2004). Antarctic glaciation led to a drop in ocean level, and the cyclic onset of cooling and progressive aridification of Australia. While pockets of extreme aridification occurred during the Miocene, widespread aridification and formation of the first stony deserts and sand field formations are very recent (2–4 ma and c. 1 ma respectively) (Fujioka et al., 2005; Fujioka et al., 2009).

The Australian landscape has been heavily shaped by geological and climatic processes of the past 150 million years; ocean inundation, formation of the Antarctic circumpolar current, changes in palaeolatitude, cycles of glacial maxima and Antarctic ice sheet production resulting in aridification, have all contributed to formation of contemporary biomes and patterns of species distribution (Hill, 1994; Byrne et al., 2008).

Approximately 15 ma (mid-Miocene), geological and plant fossil records indicate the Australian landmass exhibited a relatively uniform tropical climate. The area that is now the Australian Eremean zone, was warm and wet, and characterised by rainforest taxa such as species of *Nothofagus*, *Araucaria*, Laurales, flamingos and crocodiles (Alley and Lindsay, 1995; Byrne et al., 2008; Crisp and Cook, 2013).

Based on the relative recency of arid zone formation compared to tropical and temperate biomes, arid zone flora are often predicted to be of a younger age than their mesic sister taxa (Byrne et al., 2008; Catullo and Keogh, 2014). That said, two hypotheses prevail in explaining the biogeography of Australian arid zone taxa. The first assumes *in situ* adaptation of species already distributed in environments that later aridified (this would be evident through the monophyly of extant arid zone taxa). The second hypothesis proposes extant arid zone taxa to have originated in coastal, mesic environments, from where they have diverged and radiated into the arid zone multiple times using multiple pathways (evident through a lack of monophyly in extant arid zone taxa, and sister relationships of arid and mesic taxa) (Crisp et al., 2004; Byrne et al., 2008; Murphy and Crayn 2016). These alternative hypotheses have been explored in a number of Australian arid flora (*Acacia*, Murphy et al., 2003; Ladiges et al., 2006; González-Orozco et al., 2013; *Eucalyptus*, Ladiges et al., 2011; *Nicotiana*, Ladiges et al.,

2011; Clarkson et al., 2017; *Triodia*, Anderson et al., 2016; Goodeniaceae, Jabaily et al., 2014; Amaranthaceae, Kadereit et al., 2005; Cabrera et al., 2010) and faunal taxa (skinks, Chapple and Keogh, 2004; geckos, Pepper et al., 2011; frogs, Catullo and Keogh, 2014; birds and mammals, Cracraft, 1991). The results of these studies have shown patterns of both *in situ* arid speciation and dispersal from taxa in mesic biomes. For example, arid zone members of the *Egernia whitii* group of skinks are proposed to have arisen from a single colonization from a temperate adapted ancestor (Chapple and Keogh, 2004), while in the plant family Goodeniaceae, *Goodenia sensu lato* is hypothesised to have originated in what is now the Eremean zone prior to the onset of aridity then diversified (the majority of extant species now distributed in central Australia occur along water courses and are considered arid avoiders; Jabaily et al., 2014).

6.4.2 Biogeographic history of tribe Myoporeae

Distributed widely throughout the Australian Eremean zone, and with affinities to temperate, tropical and coastal environments, *Eremophila*, and more broadly tribe Myoporeae, are an ideal group to examine the evolutionary history of aridity in Australia. The biogeographic history and distribution of Myoporeae is best considered in the context of the entire family, Scrophulariaceae. Revision of Scrophulariaceae *sensu stricto* (Olmstead et al., 2001; Oxelman et al., 2005) reduced the family to eight tribes; Aptosimeae, Buddlejeae, Hemimerideae, Leucophylleae, Limoselleae, Myoporeae, Scrophularieae and Teedieae. The family has a cosmopolitan distribution, but predominates in the Southern hemisphere, and is thought to be of African origin based on the African distributions of many tribes, particularly those resolved at basal nodes of phylogenies for Scrophulariaceae *sensu stricto* (see Figure 6.4, Tank et al., 2006; Crisp and Cook, 2013). Of the eight tribes, Aptosimeae, Hemimerideae and Teedieae are almost entirely of southern African distribution, while Buddlejeae, Limoselleae and Scrophularieae are distributed globally, but contain sister taxa of African distribution (Tank et al., 2006; Chau et al., 2017; Ito et al., 2017; Scheunert and Heubl, 2017). Tribes Myoporeae and Leucophylleae (distributed in Australia/Pacific and Central America, respectively) are sister and the only two lineages without African representatives (Tank et al., 2006).

A handful of time-calibrated molecular phylogenies focusing on Scrophulariaceae *sensu stricto* have estimated the age of the family, and specific genera of interest, with varied results. An angiosperm-wide analysis completed by Bell et al. (2010) estimated the mean age of Scrophulariaceae to be 51 million years ($\pm 95\%$ hpd 45–57). Navarro-Pérez et al. (2013) time-calibrated a single locus (*ndhF*) chloroplast phylogeny of Lamiales, and more specifically Scrophulariaceae, using five fossils placed externally to Scrophulariaceae. From that analysis, Scrophulariaceae was estimated to be approximately 38 million years old. In contrast, an unpublished analysis by Olmstead (pers comm. 2018) at ordinal rank (Lamiales), detailing all families, estimated the crown age of Scrophulariaceae to be 55 ma (± 11 ma). The variation between these estimates is likely due to differences in calibration points and taxa included in the analyses, which highlights a large degree of uncertainty surrounding the dates. At tribe level there are currently two time-calibrated molecular phylogenies that include tribe Myoporeae. Navarro-Pérez et al. (2013) estimate the divergence time of tribe Myoporeae to be 10.7 ma ($\pm$ c. 5 ma), while Gándara and Sosa (2014) estimate the divergence of Myoporeae and Leucophyllum at c. 15 ma ($\pm$ c. 5 ma).

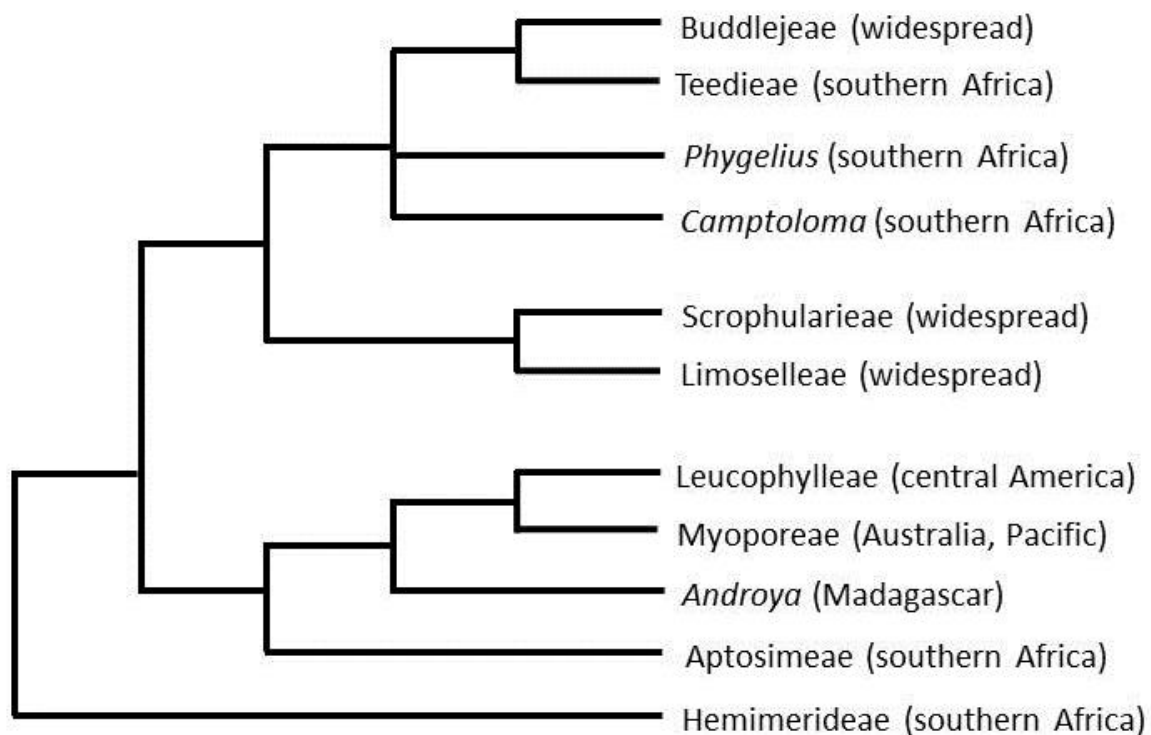


Figure 6.4 Cladogram of relationships between tribes of Scrophulariaceae, adapted from Tank et al., 2006.

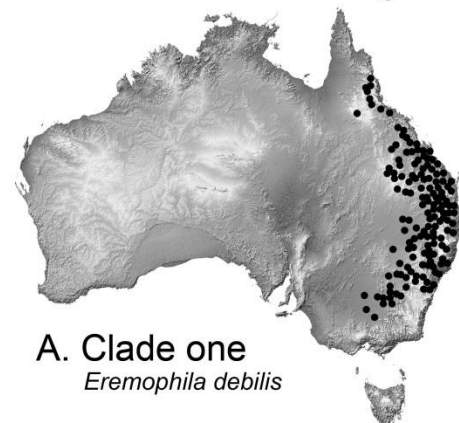
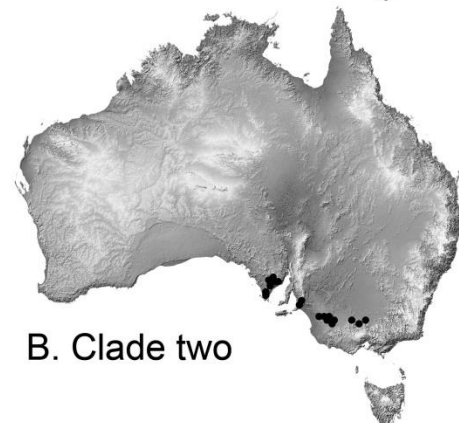
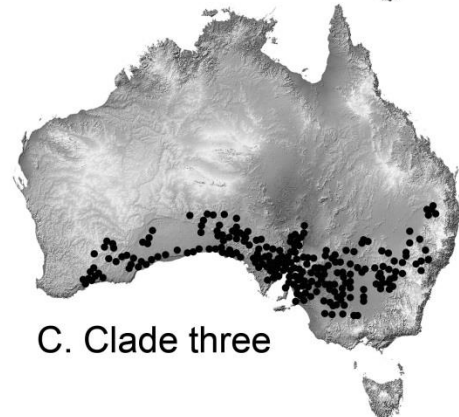
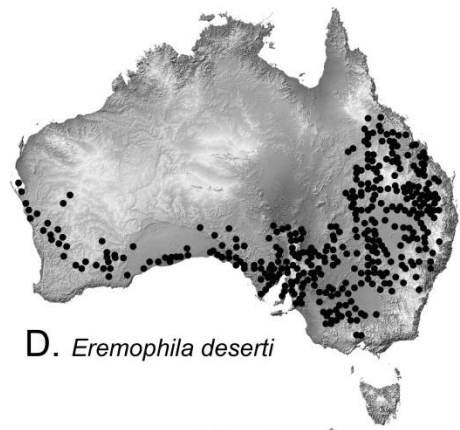
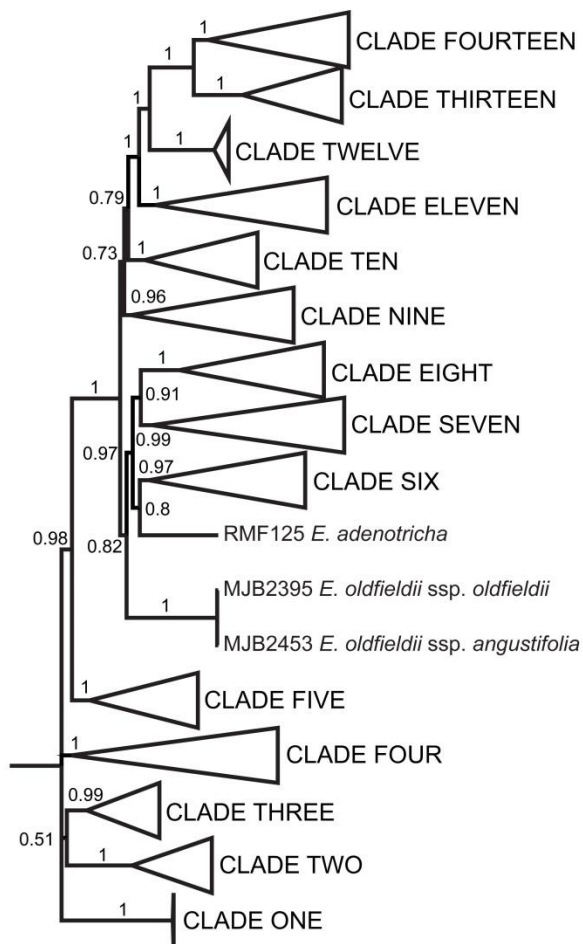
While the time-calibrated studies outlined above allow for an estimate of the ages of specific lineages, it is important to acknowledge the limitations of these methods. Of particular issue is a complete lack of fossils from within the family Scrophulariaceae, meaning nodes are calibrated using age estimates from previous molecular dating studies (secondary calibration) that use fossils located outside of the family (i.e. from other families from within the order Lamiales such as Lamiaceae, Bignoniaceae, Plantaginaceae). Such indirect means of phylogenetic calibration are a possible source of error, as the effect of uncertainty of estimates is multiplied in the divergence dating analyses, leading to questionable accuracy of results (Graur and Martin, 2004; Ho and Phillips, 2009; Schenk, 2016). A second limitation of this research is the use of, often, just a single DNA locus (i.e. *rbcL*) to infer the phylogeny upon which divergence dating studies are based. It is widely acknowledged that incongruence often exists between and within genomes (Doyle, 1992; Maddison and Knowles, 2006), meaning age estimates from such limited reconstructions can be compromised (Pennington et al., 2004). Degrees of incongruence between datasets were reported by Navarro-Pérez et al. (2013) and Gándara and Sosa (2014) and were treated by either excluding or combining datasets.

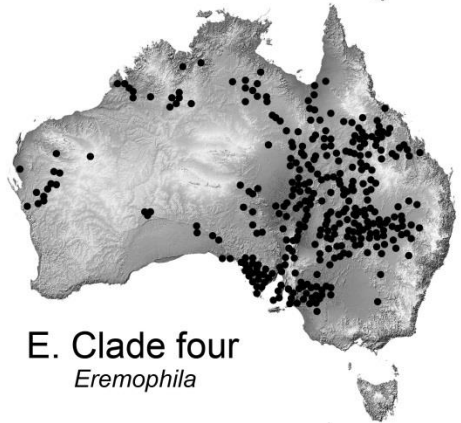
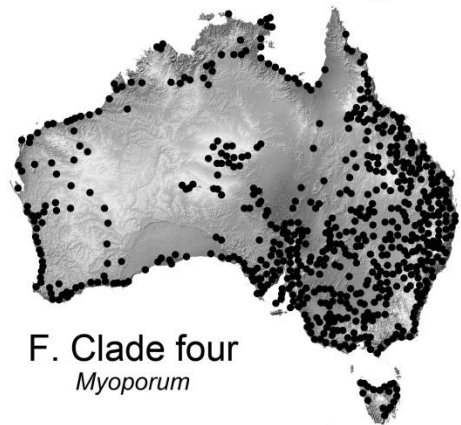
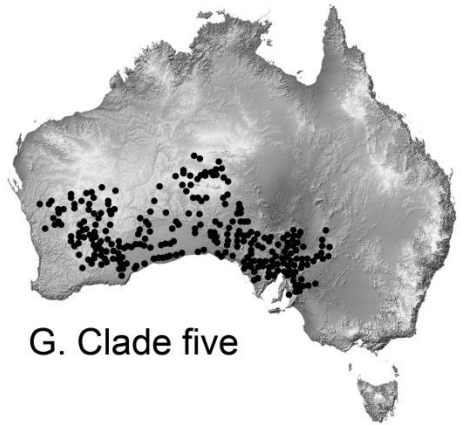
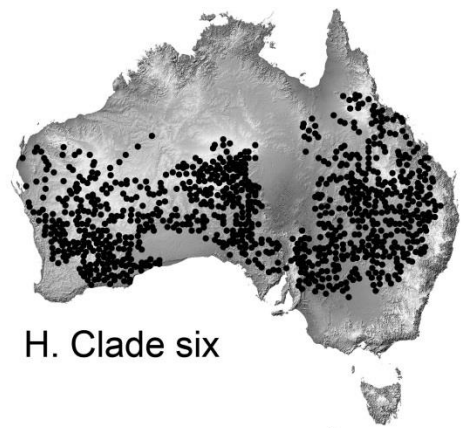
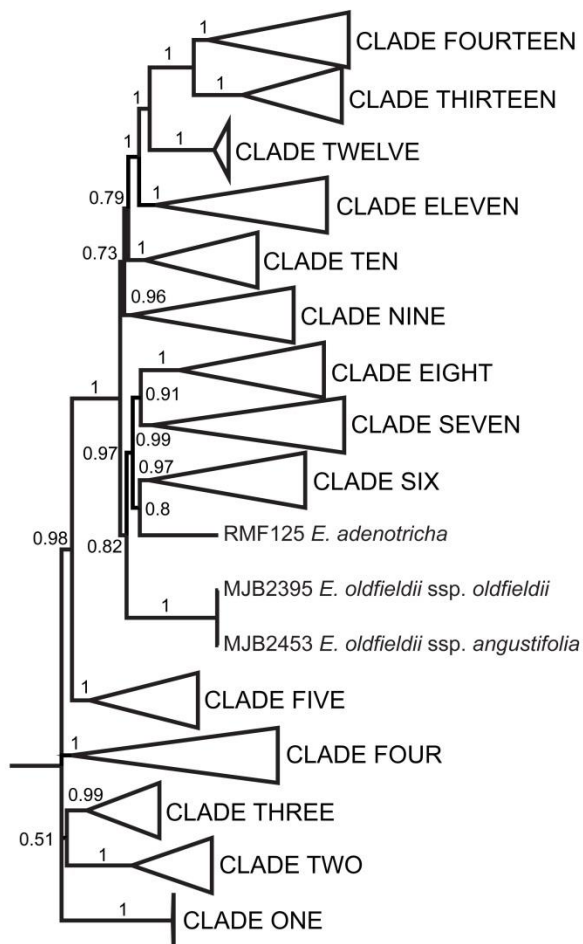
Although the current study comprehensively samples Myoporeae using multiple (chloroplast and nuclear) large datasets, construction of a time-calibrated phylogeny was not attempted for a number of reasons. Widespread incongruence between chloroplast and nuclear phylogenies did not allow for combination of datasets, and a lack of resolution (polytomies) in the nuclear ribosomal phylogeny violates the assumption of a bifurcating pattern of evolution required by Bayesian model tree priors for calibrated analyses (Bromham et al., 2018). In combination with a lack of any new primary calibration points, these issues dissuaded further analysis until a more resolved nuclear phylogeny is available. In the meantime, while it is not possible to estimate the ages of nodes/lineages within Myoporeae, contemporary species distributions can be explored in a phylogenetic context by comparing patterns of distribution between clades of the nrDNA phylogeny (see Figure 6.5).

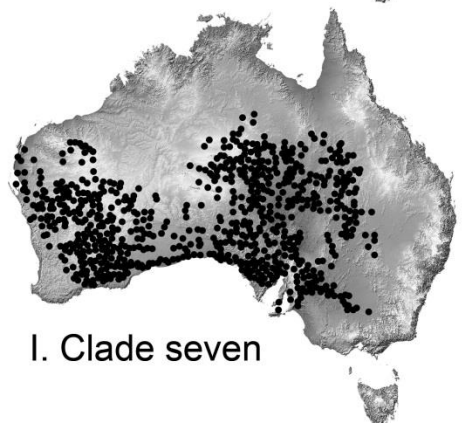
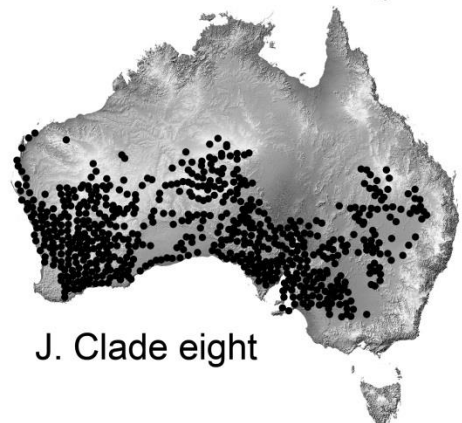
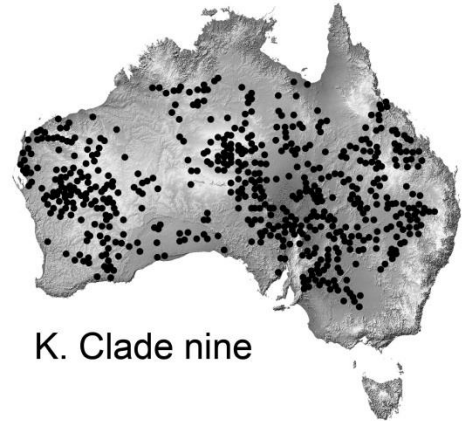
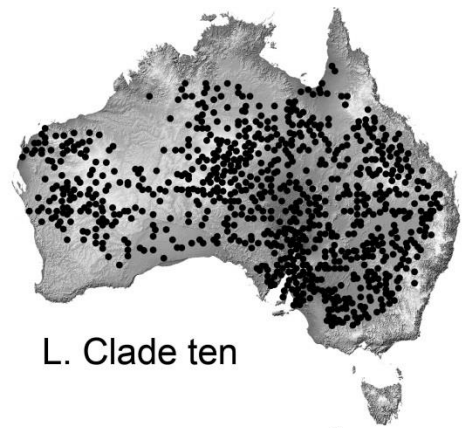
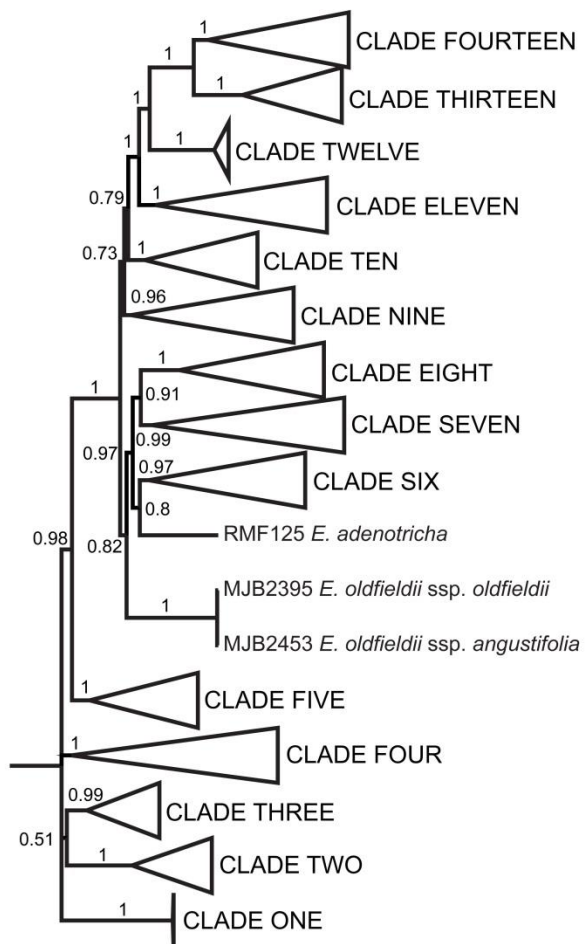
6.4.3 Biogeographic patterns of Australian species in tribe Myoporeae

Looking at the mapped distribution of all Australian Myoporeae does not reveal any marked geographic patterns for the tribe. *Eremophila* and allied genera appear to occur

right across Australia, in all (temperate, sub-tropical, tropical and arid) biomes, absent only from Tasmania and wetter parts of south west Western Australia, southern Victoria and the northern monsoonal tropics. However, by mapping the distributions of each of the fourteen well supported clades identified in the nrDNA phylogenetic analysis, additional insight and lineage specific patterns begin to emerge (see Figure 6.5).







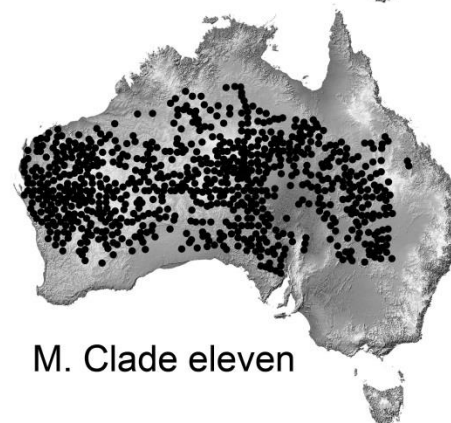
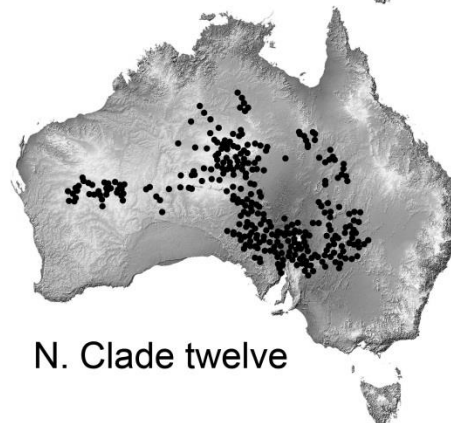
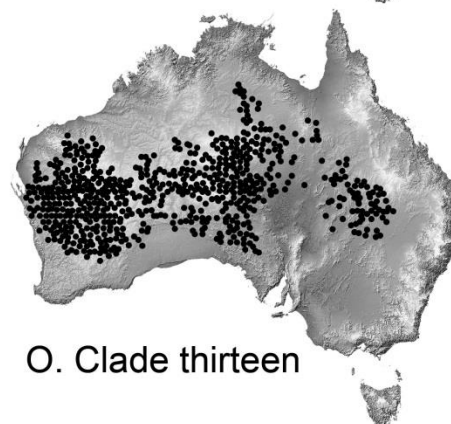
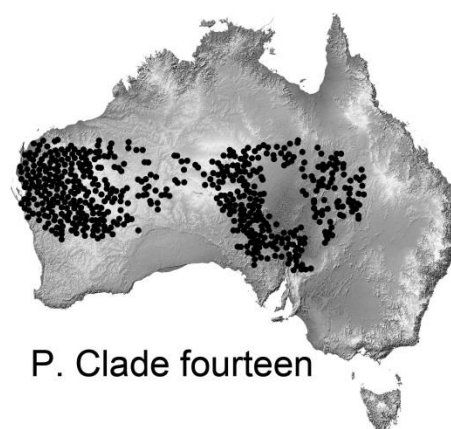
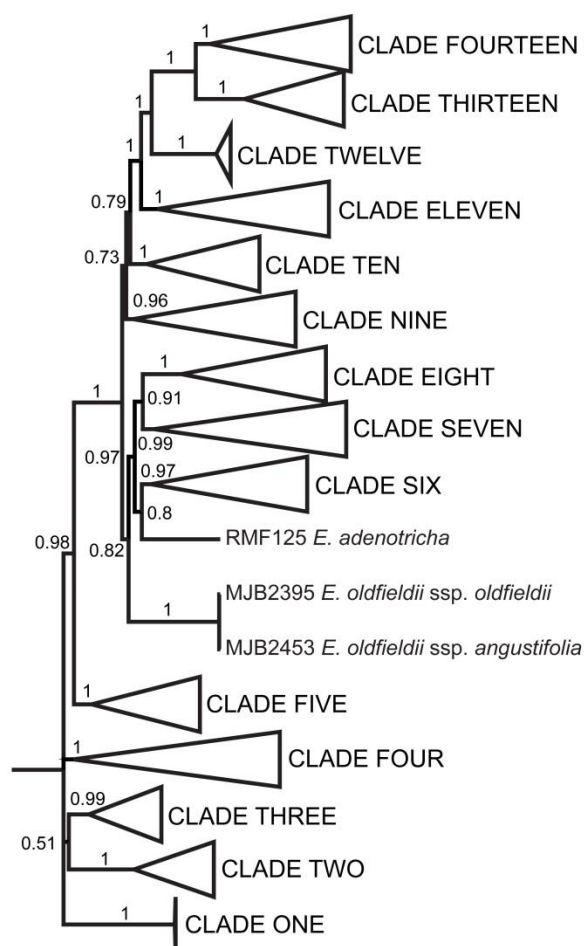


Figure 6.5 Distributions of clades of Australian Myoporeae resolved in the nuclear ribosomal (nrDNA) Bayesian inference analysis. Maps show combined distributions of species placed in each clade, based on species distributions provided by The Australasian Virtual Herbarium (as at January 2018). Distribution of *Eremophila deserti* is mapped independently due to presence of samples in both clades three and four. For Clade four, the distributions of members from *Eremophila* and *Myoporum* are mapped separately.

Four of the clades identified in the basal polytomy of the nrDNA analysis exhibit distributions predominantly outside of the Eremean zone, spanning large tracts of southern and/or eastern Australia. Clade one (Figure 6.5A) represents a single widespread species, *E. debilis*, distributed throughout eastern Australia on a broad range of soil types in temperate, sub-tropical and tropical environments. Clade two (Figure 6.5B) includes two species, *E. gibbifolia* and *E. barbata*, both of which have narrow distributions in temperate regions of South Australia and Victoria. Clade three (Figures 6.5C–D) includes monotypic genus *Glycocystis*, *Myoporum platycarpum*, and a single sample of *E. deserti*. *Glycocystis beckeri* is restricted in distribution to the more temperate south-west of Western Australia (Ravensthorpe) while *M. platycarpum* displays a widespread distribution across southern Australia, from south-east Western Australia to temperate and subtropical regions of New South Wales and Queensland. *Eremophila deserti* (presented separately in Figure 6.5D due to the non-monophyletic placement of samples in both clades three and four) displays a widespread distribution on a range of soil types through temperate and subtropical regions of south-east Queensland, New South Wales, western Victoria and southern South Australia; scattered populations also occur in Western Australia bordering the arid zone. Clade four contains samples of *E. deserti* (Figure 6.5D) and a number of other small or monotypic sections of *Eremophila* (Figure 6.5E, for taxa see Figure 4.3) and all species of *Myoporum*, except *M. platycarpum* (Figure 6.5F). Species of *Eremophila* in clade four are predominantly narrow endemics (*E. fallax*, *E. santalina*, *E. behriana*, *E. crassifolia*, *E. laanii*, *E. tetraptera*, *E. pentaptera*), though a handful of species more widespread (*E. deserti*, *E. polyclada*, *E. bignoniiflora*). Many of the narrow endemics centre around temperate and semi-arid regions of South Australia, while *E. polyclada* and *E. bignoniiflora* occur on floodplains, river and drainage lines in temperate, tropical and arid regions of eastern Australia. *Eremophila bignoniiflora* is one of the only species of *Eremophila* to occur in parts of tropical northern Australia, including the Kimberley Ranges.

Species of *Myoporum* on mainland Australia (Figure 6.5F) are characterised by their temperate coastal distributions. Very few species are found inland in Australia, the exception being the extremely widespread *M. montanum*, which is found across all states and territories (except Tasmania), in temperate and tropical regions of South Australia, New South Wales and Queensland and isolated arid regions of Western

Australia, Northern Territory and South Australia where it is restricted to wetter drainage lines and creek beds.

Of the 29 Australian species in clades one to four of the nrDNA analysis, only a handful enter the Eremean zone. In clade three, *M. platycarpum* and *E. deserti*, both skirt the southern periphery of the arid zone but are most abundant in temperate regions of south-eastern Australia. In clade four, three widespread species (*E. polyclada*, *E. bignoniiflora*, *M. montanum*) and three narrow endemics (*E. laanii*, *E. pentaptera*, *E. tetraaptera*) all occur, at least in part, in regions of the Eremean zone. Interestingly, all of these species are found in less extreme parts of the arid zone with higher water availability; along creek beds, drainage channels and flooding plains. Overall, the pattern of species distribution that emerges at the base of the nrDNA phylogeny, is atypical of tribe Myoporeae more generally. The majority of species are found in temperate environments of the south and east of Australia, while those that are present in the Eremean zone are distributed either on the periphery as part of a more widespread non-arid distribution or as arid avoiders, in less extreme wetter pockets of the arid environment.

Despite *Eremophila* being described as largely an arid lineage, clade five is the first lineage to include species that span the true Eremean zone. Clade five (Figure 6.5G) contains four species, three of which are extremely narrow endemics (*E. saligna*, *E. rostrata*, *E. purpurescens*) while *E. alternifolia* is widespread across southern Australia, in ranges and rocky habitats of the southern deserts. Clades six, seven and eight are similar in distribution, each extending from the south into the Eremean zone but notably excluded from the northern deserts. Clade six (Figure 6.5H) is made up predominantly of species restricted in distribution, with a small handful more widespread in distribution (*E. sturtii*, *E. mitchellii*, *E. oppositifolia*). As a clade, species distributions span almost all longitudes of Australia from east to west, though latitudinally they predominantly span from the south to centre of the continent, with a notable absence of species in the northern deserts and Lake Eyre/Simpson desert regions. Clade seven (Figure 6.5I) includes predominantly narrow endemics, with a few widespread species (*E. scoparia*, *E. macdonnellii*). Clade eight (Figure 6.5J) is large (27 species) and made up predominantly of narrow endemics, with just two species that exhibit widespread distributions (*E. serrulata* and *E. glabra*). Species distributions in clade eight span Australia from east to west, though are restricted to southern

temperate and arid regions, with little to no presence in the northern deserts (Great Sandy Desert, Tanami Desert, Barkly Tableland and Simpson Desert). Overall, clades five, six, seven and eight are characterised in distribution by their dominance in the southern desert regions of Australia. Each clade displays some southern temperate elements, though extend into the central ranges and/or central eastern deserts but are consistently absent from the sandy environments of the Simpson Desert and Lake Eyre region.

Clades nine and ten share a number of features; both contain a small number of species of which one is extremely widespread in distribution. Clade nine (Figure 6.5K) contains nine species, eight of which exhibit narrow distributions (sect. *Hygrophanae*, *E. compressa*, *E. racemosa*), and a single species (*E. maculata*) that is extremely widespread across much of Australia, preferring drainage lines and river flats subject to flooding across the Eremean zone. Clade ten (Figure 6.5L) contains just three species: *E. perglandulosa* and *E. sp. 'Tropicana'*, both of which are extremely narrow in distribution, and *E. longifolia*, one of the most widespread species of the genus, occurring in a range of climates and soil types (rocky ranges to sand dunes/planes) across Australia. The distributions of both *E. maculata* and *E. longifolia* are unique for the tribe, not just because of their widespread nature across temperate, sub-tropical and arid biomes, but also their ability to grow on such a wide range of substrates, including the sandy dune fields of the Simpson Desert, and to a lesser extent, the Gibson Desert. As with the remainder of the tribe, both species are largely absent from the Great Sandy Desert in the north west of the continent.

The nrDNA clades eleven, twelve, thirteen and fourteen show consistent and unique patterns of distribution not seen in other clades of the phylogeny. Species in all four clades are entirely Eremean in distribution, lacking the temperate and/or subtropical components of all other lineages. Clade eleven (Figure 6.5M) is large (39 species), with the majority of species exhibiting narrow species distributions restricted exclusively to the Eremean zone. Just two species in the clade (*E. forrestii*, *E. latrobei*) display widespread distributions in a variety of habitats and soil types though still exclusively in the arid zone. Clade twelve (Figure 6.5N) contains just two species, *E. duttonii* and *E. linearis*. *Eremophila linearis* is restricted in distribution to central Western Australia, while *E. duttonii* is widespread throughout central Australia, with the notable exclusion of the Lake Eyre/Simpson desert region. Clade thirteen (Figure 6.5O) is large (34

species) and made up almost entirely of species from sect. *Eremaeae*. True to name, this section occurs exclusively in the Eremean zone, mostly in the form of narrow endemics, though with a small number of widespread species (*E. gilesii*, and to a lesser extent *E. glutinosa* and *E. exilifolia*). There is a notable absence of species in northern desert regions (i.e. Great Sandy Desert, Tanami Desert), and through the Lake Eyre/Simpson Desert region and across the Nullarbor Plain. Clade fourteen (Figure 6.5P) is large (28 species), with almost all species restricted in distribution, and just a few (*E. freelingii*, *E. platyclayx*) more widespread. All species in clade fourteen are limited exclusively to the Eremean zone, though are absent from all sandy desert regions, including the Great Sandy, Gibson, Great Victoria, and Simpson Desert regions.

The pattern of distribution is particularly interesting in clades eleven to fourteen in the context of the nrDNA phylogeny. Relationships within these clades are poorly resolved, with many short branches and a general lack of support for internal nodes (see Figures 4.8 – 4.11). This lack of support, short branches and morphological consistency between species of each of the four clades is interpreted to be an indication of potentially recent, and rapid radiations of each of these lineages, leading to the large numbers of morphologically distinct, yet genetically very similar species in sections *Eriocalyx* (clade eleven), *Eremaeae* (clade thirteen) and *Eremaeae/Eremophila* (clade fourteen). Being the only lineages distributed exclusively in the Eremean zone lends further support to the hypothesis of rapid radiation, with the recent evolution of Australia's arid zone potentially aligning with the recent expansion and speciation of these lineages in the arid zone, as hypothesised for a number of Australian arid taxa, i.e. Amaranthaceae and Brassicaceae (Crisp et al., 2004, Byrne et al., 2008).

While a well-resolved, time-calibrated phylogeny would add temporal information to our predictions of the evolutionary history and biogeography of Myoporeae, a number of distinct patterns are particularly evident when looking at the contemporary species distributions of the group based on well-resolved clades of the nrDNA analysis (see Chapter Four). Based on these patterns a number of hypotheses are outlined below.

1) Tribe Myoporeae is ancestrally temperate/mesic.

Four of the clades in the basal polytomy of the nrDNA analysis exhibit species distributions predominately spanning temperate and sub-tropical biomes of southern and eastern Australia. The handful of species in these clades that do occur in the

Eremean zone are present around the edge of a larger distribution, or occur in less extreme parts of the environment, such as along flood plains and drainage lines (arid avoiders). This pattern of a broad, temperate, south/eastern distribution of taxa in basal parts of the phylogeny opposes earlier theories for the group, which suggested Myoporeae to have originated in south-west Western Australia based on patterns of endemism (very few endemic species occur in Eastern Australia), and preliminary cytological patterns (more diploids in the west, and polyploids in the east) (Barlow, 1971; Chinnock, 2007). The assumption that centres of species diversity are synonymous with a centre of origin is problematic, with many processes such as increased speciation, increased migration and/or decreased extinction all associated with high rates of species diversity (Lancaster and Kay, 2013). In plants, rapid diversification is often associated with ecological opportunities created by major geological/environmental changes (Rieseberg and Willis, 2007). In the case of Myoporeae, it is likely that the evolution of the Eremean zone provided an ecological opportunity allowing for extensive speciation.

While ancestral Myoporeae were potentially of temperate distribution including southern and eastern Australia, it seems plausible that contemporary species exhibiting such distributions may well have been spread more widely across the continent prior to the onset of extreme aridification c. 6 ma. Around the time tribe Myoporeae is estimated to have diverged from central American sister tribe Leucophylleae (15 ma), fossil records indicate a warm wet climate Australia-wide and particularly in now arid areas (Alley and Lindsay, 1995; Byrne et al., 2008). The presence of some species (i.e. *M. montanum*, *E. polyclada*, *E. bignoniiflora*, *E. laanii*) in the periphery and less extreme parts of the arid zone, may be the remnant of species contractions from historically more temperate/mesic environments.

2) Movement of species into the arid zone is likely to have occurred from the south, northwards.

Biogeographic patterns of diversification in Australia's arid zone have been hypothesised for a number of plant and animal taxa; the prevailing hypothesis being that arid-adapted lineages have evolved from mesic/temperate ancestors in the north of Australia (Byrne et al., 2008; Fujita et al., 2010). Australian species of *Nicotiana* are thought to have historically been widespread across the continent, then with the onset

of aridification, moved into the Eremean biome using multiple pathways from the north to south (Ladiges et al., 2011). A similar possible early northern differentiation was found in the *Acacia victoriae*, *A. pyrifolia* and *A. murrayana* species groups (Ladiges et al., 2006). Similar movement of species from the northern monsoonal tropics into the arid zone has been documented in animal groups such as *Uperoleia* toadlets (Catullo and Keogh, 2014), *Egernia* lizards (Chapple and Keogh, 2014), and *Heteronotea* geckos (Fujita et al., 2010). Patterns of dispersal in tribe Myoporeae do not seem to follow this directionality of movement, with extant arid zone groups potentially differentiating from lineages in temperate regions of the south. Moving up the nrDNA phylogeny, the first clades to exhibit true arid zone components (clades five to eight) are distributed through southern and/or eastern temperate regions spanning north into the Central Ranges, but rarely into the northern (Great Sandy, Tanami or Barkly Tableland) deserts. Very few extant species of *Eremophila* span these northern deserts, and those that do (*E. maculata*, *E. longifolia*, *E. latrobei*) are fairly sparse and on the limits of their range. Based on these contemporary patterns of distribution, it seems most likely that movement of Myoporeae into the arid zone occurred from south, northwards.

3) Much of the species diversity observed in exclusively arid Eremophila is likely to be of recent origin.

While a number of clades in the nrDNA analysis include species spanning parts of the Eremean zone, only four clades (eleven to fourteen) are found exclusively in arid environments. Species in these clades are placed in Chinnock's sections *Eriocalyx*, *Eremaeae*, *Eremophila* and *Pulchrisepalae*, these are morphologically distinct, and adapted for truly arid environments (not less extreme pockets of the arid zone). Sections *Eriocalyx* and *Eremophila* are two of Chinnock's most species-rich sections (composed of 36 and 54 species respectively). The short branch lengths and lack of resolution within each of these clades relative to other lineages of the nrDNA phylogeny may indicate that recent and rapid speciation is responsible for the diversity in each clade.

4) Refugia may have played a role in shaping contemporary species distributions and biogeography of tribe Myoporeae

A growing body of work indicates that inland mountain ranges have played significant roles as refugia for flora and fauna alike; shaping extant species distributions, species

diversity, and population genetic structure in the arid zone (Byrne et al., 2008; Pepper et al., 2011). As the environment in central Australia transitioned from wet forest to the fully arid landscape now observed, topographically complex regions such as the Kimberley, Pilbara, Central and Flinders ranges provided more stable habitat from which species may have expanded and contracted in distribution. The Pilbara region has been shown to be of significance as a refuge for grasses (*Triodia*, Anderson et al., 2016) and geckos (*Heteronotia*, Fujita et al., 2010), and it seems plausible that similar refugial scenarios may help to explain patterns of distribution and diversity in *Eremophila*. Regions such as the Central and Flinders Ranges may have been important historically in harbouring species through times of rapid extinction or climate cycling. Clade four of the nrDNA phylogeny in particular, includes a number of Flinders Ranges' endemics, such as *E. fallax* and *E. santalina*, as well as more widespread species that are particularly abundant in the Flinders Ranges (*E. deserti*, *M. montanum*, *M. brevipes*). While population level study was beyond the scope of this research, a focus on intraspecific diversity of species confined to arid ranges in future studies could shed light on the significance of arid mountain refugia in Myoporeae, and arid species more generally.

6.4.4 Biogeographic patterns of non-Australian taxa of Myoporeae

Current age estimates of tribe Myoporeae of c. 15 mya indicate that contemporary species distributions are too young to be the product of Gondwanan vicariance (which is generally considered in lineages dated prior to c. 35 mya, Crisp and Cook, 2013). While many tribal lineages of Scrophulariaceae *sensu stricto* are hypothesised to be of African origin based on extant species distributions (Tank et al., 2006), the disjunct central American and Australian-Pacific distributions of sister tribes Leucophylleae and Myoporeae do not allow for accurate determination of their origin or pathways of dispersal. A hypothesis of dispersal and subsequent radiation in Australia and the Pacific seems as equally plausible as a vicariance scenario whereby the most recent common ancestor of Myoporeae/Leucophylleae was widespread in distribution, with extinctions accounting for the disjunct distributions of both tribes. Regardless of the origin and ancestral dispersal pathway of Myoporeae, it is interesting to note similarities in the distributions of extant tribe members of Myoporeae and other prominent Australian plant groups. Dispersal of species between mainland Australia, New Zealand, Hawaii and other islands of the Pacific is well documented in many

Australian plant groups, for example *Acacia* (Brown et al., 2012), *Astelia* (Birch and Keeley, 2013), *Scaevola*, *Goodenia* (Jabaily et al., 2014), *Melicope* (Harbaugh et al., 2009; Appelhans et al., 2014), Aspleniaceae (Ohlsen et al., 2014) and *Coprosma* (Cantley et al., 2014, 2016; Heads, 2018). In the case of tribe Myoporeae, *Myoporum*, *Bontia* and *Pentacoelium* are geographically allopatric, though all exhibit similar coastal distributions, are salt tolerant and grow in sandy calcareous soils or soils derived from volcanic or sedimentary rock in coastal environments (Kelchner, 2003; Chinnock, 2007). In conjunction with contemporary coastal distributions and the presence of large fleshy fruits adapted for dispersal via sea water (Chinnock 2007), these genera seem well adapted for long distance dispersal via ocean currents, which could explain their unique, widespread distribution.

6.5 The utility of a genome skimming approach in plant systematics

As demonstrated in this study, genome skimming from high throughput sequences has the ability to generate large amounts of genomic datum (from all three plant genomes) for many specimens in parallel, and with a single library preparation process. The concept of genome skimming is relatively recent (Straub et al., 2012), and although the advantages of this approach lay in its ability to be up-scaled, so multiple samples can be sequenced in parallel for a reduced cost, the vast majority of studies using this approach have only included a handful to tens of samples (Malé et al., 2014; Weitemier et al., 2014; Zhang et al., 2015; Turner et al., 2016; Zhang and Chen, 2016; Berger et al., 2017; Gryta et al., 2017). As of January 2017 the total number of complete chloroplast genomes available on GenBank was c. 1400 (Tonti-Filippini et al., 2017), and while a number of studies have included large numbers of these publicly available genomes in analyses (i.e. 53 species of Triticeae, Bernhardt et al., 2017; 250 species of Poaceae, Saarela et al., 2018) the current research is likely the largest of its kind in applying a genome skimming approach to reconstruct hundreds (+300) of individuals, the scale of which would have been previously unachievable with Sanger sequencing.

Success of this particular genome skimming approach can be attributed to a number of features of the protocol and its application, discussed below. Costs of sample preparation were significantly reduced through the use of an in-house library preparation protocol, rather than commercially available kits. The in-house library

preparation protocol (originally designed by Tibbits, 2013) was optimised specifically for tribe Myoporeae and designed to reduce preparation costs to ~\$15-25AUD per sample. Two significant features of our protocol are the use of 'in-house' prepared magnetic beads, for fragment size selection, and incorporation of Y-adapters, for sample identification and DNA molecule competency for the Illumina platform (details of the bead preparation protocol are outlined in Chapter Two). Unlike traditional blunt-end ligation protocols, the ligation of Y-adapters allows for incorporation of two unique identifying indexes with high efficiency (i.e. each arm of the Y-adapter is compatible to a specific set of indexes, meaning two fragments of the same index cannot be attached to one end). This dual indexing allows for up to 384 unique index combinations (based on two sets of 16 and 24 indexes), so up to 384 samples can be sequenced in parallel. Although the set-up, trouble shooting and optimisation of this protocol took a significant amount of time, this is a robust protocol that our lab has successfully applied across a diverse range of plant groups (Scrophulariaceae, Poaceae, Asphodelaceae, Myrtaceae, Rutaceae, Rhamnaceae, Brassicaceae, Orchidaceae, Proteaceae, Codiaceae).

6.5.1 *Practical recommendations for in-house library preparation*

In developing and implementing a novel in-house library preparation protocol, a number of practical recommendations became apparent that may be of use for those interested in taking a similar approach:

- Use of in-house prepared magnetic 'Ampure-like' beads (i.e. those of Rohland Reich, 2012) for DNA size selection and clean-up markedly reduces the cost of sample preparation; however, differences in fragment size recovery mean modifications to protocol ratios are advisable for each batch of beads prepared.
- Input DNA concentration required for this protocol is high (3000 ng per sample) to account for loss during the many (3–4) clean up steps. This input can be reduced to as low as 100 ng for samples with limited amounts of DNA available, but it is recommended that a less stringent ratio of bead size selection is used to reduce fragment loss.
- Some testing on old, and degraded herbarium specimens indicated that this protocol is still robust for these purposes, though as mentioned above, a less stringent ratio of bead size selection is recommended.

- It is advisable to complete multiple, smaller sequencing runs rather than one single large run. This allows for samples to be re-sequenced in the (likely) event that sample pooling results in some samples to be sequenced with less than ideal coverage.
- The proportion of reads obtained for each genome (chloroplast, nuclear, mitochondrial) will depend largely on the size of the nuclear genome. The larger the nuclear genome, the fewer reads obtained from other genomes. This pattern is particularly evident in instances of polyploidy, where the nuclear genome can be magnitudes larger than in diploid individuals and that is another reason to complete multiple sequencing runs for the purposes of re-sampling.

This method of library preparation is easily applied to a range of plant taxa, and has been implemented by a number of labs in Australia. It is worth noting that once the protocol has been established, the rate limiting step is actually the bioinformatic processing of samples. Although a range of computer programs and pipelines have been developed to streamline the processing and assembly of genomes/loci, a large degree of manual handling is still required. This will likely change as the technology matures and software is developed to streamline the process. In the meantime, elements of human decision-making and manual checking are still integral for establishing patterns and screening issues, but take a significant amount of time – particularly for large datasets such as this one.

From a phylogenetic perspective, a technically straight-forward and cost effective genome skimming approach that allows for access to multiple genomes (and thus multiple pathways of evolution), is critical to achieving robust estimates of phylogenetic relationships. While not appropriate for all research questions, this method can conceivably be applied to a broad range of research areas, from improving plant barcoding initiatives (Wilkinson et al., 2017; Krawczyk et al., 2018), accessing datum from historic records such as museum and herbarium collections (Besnard et al., 2014) and improving conservation efforts (Corlett, 2017). By its very nature, genome skimming ‘skims off’ high copy regions of the genome, leaving the vast majority of the nuclear genome, including highly informative low copy nuclear loci, largely untouched. To target these regions alternative methods of sample preparation are required, either to target specific regions of the genome (target enrichment, Mamanova et al., 2010; Lemmon and Lemmon, 2013; Weitemier et al., 2014) or reduce the portion of the

genome sampled (reduced representation libraries, Peterson et al., 2012; Rubin et al., 2012; Ree and Hipp, 2015) – discussed further in the next section.

6.6 Directions for further research

From the work presented in this thesis we now have a sound picture of chloroplast relationships in Myoporeae and an emerging picture of nuclear DNA relationships. To clarify relationships at the basal nodes of the nuclear phylogeny and proceed with the necessary taxonomic revision, additional datum is needed from the nuclear genome. The genome skimming approach implemented here was able to generate datum to assemble the nuclear ribosomal cistron, but did not target low copy regions of the nuclear genome. Despite the long-recognised utility of low copy nuclear markers, these regions of the nuclear genome have typically been difficult to obtain (Sang, 2002; Lemmon and Lemmon, 2013; Zimmer and Wen, 2015). In the past decade, a number of alternative methods that utilize HTS capabilities and more comprehensively target the nuclear genome have become widely available. Reduced Representation Library (RRL) techniques such as RAD-seq and RAPID-seq use restriction enzymes to digest total genomic DNA, with the resultant DNA fragments size selected and sequenced, yielding the reads that flank the enzyme recognition sites (Davey and Blaxter, 2010; Hou et al., 2015; Ree and Hipp, 2015). These methods are cost effective (Baird et al., 2008; Peterson et al., 2012) and do not require prior genomic information (i.e. transcriptomic assembly or a close reference genome) meaning they can be applied broadly across non-model study systems (Ree and Hipp, 2015). Alternatively, targeted enrichment approaches selectively capture target regions of DNA either via PCR-based targeted amplicon sequencing or through baits/probes that hybridize to desired DNA regions (Mamanova et al., 2010; Bodi et al., 2013; Weitemier et al., 2014). Targeted approaches do require prior genomic information for design of the capture probes, which can increase the time and cost inputs, but are advantageous in the reproducibility of the technique and high yield (Lemmon and Lemmon, 2013). Regardless of the approach selected, with additional nuclear sequence datum, it is hoped that a better resolved phylogeny can be reconstructed, allowing for determination in placement of *Eremophila* and all allied genera, a better understanding of species relationships and patterns of distribution, and a sound foundation from which taxonomic decisions can be based.

Aside from further resolution of the tribe-wide phylogeny of Myoporeae, the current research identified a number of other areas of study that warrant future work. These include:

- 1. Further exploration of species level monophyly.** The current study revealed a significant lack of monophyly in a number of species, and species complexes (i.e. *E. glabra*, *E. platycalyx*). Population level sampling is suggested for, though not limited to, all 24 species identified as non-monophyletic species in the nrDNA analysis (see Chapter Four, 4.4.4.2). Aside from confirming the taxonomic limits, deeper sampling of these species from right across their distributions would allow for exploration of hypotheses of introgression and ILS that are likely causing the reticulate patterns of evolution observed.
- 2. Construction of a tribe-wide time calibrated phylogeny.** Despite the lack of resolution at basal nodes of the nrDNA analysis, paired with the lack of primary calibration points within tribe Myoporeae (or even Scrophulariaceae), at the very least a time calibrated phylogeny of Myoporeae would allow for an understanding of relative ages of lineages within the tribe. Interpretation would have to be treated with due caution, but a dated phylogeny could reveal patterns of diversification and biogeography for Myoporeae in the Eremean zone and the surrounding Australia-Pacific region.
- 3. Investigation of hypothesised Eremean refugial mountain ranges.** Eremean mountain refugia are hypothesised as areas of significant species diversity in a number of arid flora and faunal taxa (Byrne et al., 2008; Pepper et al., 2011; Anderson et al., 2016). Species distributed in the Flinders and Central Ranges and surrounds such as *E. fallax*, *E. santalina*, *E. deserti*, *M. montanum*, would be ideal candidates to explore if mountain refugia have shaped contemporary species distributions and patterns of diversity.
- 4. Morphological assessment of phylogenetic relationships.** The current thesis revealed a number of well supported relationships between species and genera previously unrecognised by morphological taxonomy. A morphological study to identify possible synapomorphic characters that unite these groups will be important in the clarification of generic and species boundaries.
- 5. Investigation of polyploidy, and genome size across the tribe.** Prior cytological study of a subset of species from Myoporeae indicates polyploidy and chromosomal

racess are a prevalent feature of the tribe (Barlow, 1971). Now with the added context of a molecular phylogeny, a more comprehensive analysis of species ploidy could shed light on patterns of diversification in Myoporeae and potentially the role of polyploidy in adaptation to extreme (arid) environments.

6. **Chemical screening of Myoporeae.** Although not included in the current thesis, a collaborative investigation of tribe Myoporeae with regards to chemical composition (particularly of diterpenoids and cyanogenic glucosides) is currently underway. The phylogenetic analyses of the current thesis are helping to directly inform the selection of species from across the tribe for chemical analysis, with the aim of uncovering novel compounds for therapeutic use. Both diterpenoids, which are commonly found in the surface resins of *Eremophila* (Ndi et al., 2007; Singab et al., 2013), and cyanogenic glucosides, also have potential ecological importance, with respect to the feeding behaviour of herbivorous animals (Gleadow and Woodrow, 2002; Gleadow and Møller, 2014).
7. **Co-evolution/speciation between *Eremophila* and insect genus *Lasiacantha*.** A number of insect groups are identified to co-occur almost exclusively with species of *Eremophila* (Cassis and Symonds, 2008; Symonds and Cassis, 2014). Research is currently underway to reconstruct the phylogenies of lace-bug genus *Lasiacantha* and other *Eremophila*-associated genera with the aim of comparing phylogenetic signal between the plant:insect groups for identification of patterns of co-speciation.
8. **Evolution of pollinator syndromes.** Floral morphology is incredibly diverse in *Eremophila*, and though lacking comprehensive observational datum, it is estimated that c. 80% of *Eremophila* species are insect-pollinated and the remaining c. 20% bird-pollinated (Chinnock, 2007). Phylogenetic results of the current study indicate that multiple transitions have occurred in the genus between pollination strategies. More comprehensive study of the different pollination systems in *Eremophila* and ancestral state reconstruction of floral characters could allow for better insight into patterns of morphological evolution and diversification in the group.

6.7 Conclusions

The current study has successfully generated the largest and most comprehensive molecular phylogeny of tribe Myoporeae to date, and quite possibly the largest fully

assembled plastome dataset more generally. Perhaps unsurprisingly, the chloroplast and nuclear phylogenies are partially incongruent, which is largely attributed to different evolutionary processes acting on each genome. While relationships in the nuclear phylogeny more closely reflect hypothesised species relationships based on species morphology, many relationships in the chloroplast phylogeny are anomalous, and likely represent the effects of introgressive hybridization and incomplete lineage sorting. A preliminary mitochondrial phylogeny of Myoporeae displays congruence with some parts of both the chloroplast and nuclear phylogenies, but is most similar overall to the nuclear phylogeny, indicating that further work is needed to clarify the mode of mitochondrial inheritance or processes that may affect the mitogenomic signal. Based on the combined signal of all phylogenies, it is clear that significant revision of the generic level classification of Myoporeae is required, Chinnock's (2007) sectional classification is only partially informative from an evolutionary perspective, and species vary in degrees of monophyly detected. As is often the case, while addressing the original core aims the current research has raised a suite of additional questions that require future study. Directions for this proposed future research have been outlined, and it is hoped that this current thesis marks the beginning of a more comprehensive research program on the fascinating group of plants that is tribe Myoporeae.

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Appendices

Appendix One: Members of tribe Myoporeae currently listed under the Australian Government's *Environment Protection and Biodiversity Conservation Act 1999* (as at May 2018).

Vulnerable	Endangered	Critically Endangered
<i>Eremophila denticulata</i> subsp. <i>denticulata</i>	<i>Eremophila denticulata</i> subsp. <i>trisulcata</i>	<i>Eremophila koobabbiensis</i>
<i>Eremophila prostrata</i>	<i>Eremophila glabra</i> subsp. <i>chlorella</i>	<i>Eremophila rostrata</i>
<i>Eremophila vernicosa</i>	<i>Eremophila lactea</i>	<i>Myoporum obscurum</i>
<i>Myoporum cordifolium</i>	<i>Eremophila nivea</i>	
	<i>Eremophila pinnatifida</i>	
	<i>Eremophila resinosa</i>	
	<i>Eremophila scaberula</i>	
	<i>Eremophila subteretifolia</i>	
	<i>Eremophila ternifolia</i>	
	<i>Eremophila verticillata</i>	
	<i>Eremophila virens</i>	
	<i>Eremophila viscida</i>	
	<i>Myoporum turbinatum</i>	

Appendix Two: Members of tribe Myoporeae currently listed as threatened under the Western Australian *Wildlife Conservation (Rare Flora) Notice 2018* (as at May 2018).

Vulnerable	Endangered	Critically Endangered
<i>Eremophila denticulata</i> subsp. <i>denticulata</i>	<i>Eremophila ciliata</i>	<i>Eremophila glabra</i> subsp. Scaddan (C. Turley s.n. 10/11/2005)
<i>Eremophila ternifolia</i>	<i>Eremophila denticulata</i> subsp. <i>trisulcata</i>	<i>Eremophila koobabbiensis</i>
<i>Eremophila vernicosa</i>	<i>Eremophila glabra</i> subsp. <i>chlorella</i>	<i>Eremophila lactea</i>
	<i>Eremophila resinosa</i>	<i>Eremophila nivea</i>
	<i>Eremophila virens</i>	<i>Eremophila pinnatifida</i>
	<i>Eremophila viscida</i>	<i>Eremophila rostrata</i> subsp. <i>rostrata</i>
	<i>Myoporum cordifolium</i>	<i>Eremophila rostrata</i> subsp. <i>trifida</i>
	<i>Myoporum velutinum</i>	<i>Eremophila scaberula</i>
		<i>Eremophila</i> sp. Narrow leaves (J.D. Start D12-150)
		<i>Eremophila subteretifolia</i>
		<i>Eremophila verticillata</i>

Appendix Three: Members of tribe Myoporeae with a Priority listing under the Western Australian *Wildlife Conservation (Rare Flora) Notice 2018* (as at May 2018), indicating that they have not adequately been surveyed for Threatened status.

Priority List Flora
<i>Calamphoreus inflatus</i>
<i>Diocirea acutifolia</i>
<i>Diocirea microphylla</i>
<i>Eremophila adenotricha</i>
<i>Eremophila anomala</i>
<i>Eremophila appressa</i>
<i>Eremophila arachnoides</i> subsp. <i>tenera</i>
<i>Eremophila arguta</i>
<i>Eremophila attenuata</i>
<i>Eremophila aureivisca</i>
<i>Eremophila ballythunnensis</i>
<i>Eremophila capricornica</i>
<i>Eremophila congesta</i>
<i>Eremophila cuneata</i>
<i>Eremophila daddii</i>
<i>Eremophila decussata</i>
<i>Eremophila eversa</i>
<i>Eremophila ferricola</i>
<i>Eremophila glabra</i> subsp. Kokeby (R. Davis 5080)
<i>Eremophila glabra</i> subsp. Morawa (C.A. Gardner 7521)
<i>Eremophila glabra</i> subsp. York (P.G. Wilson 12172 B)
<i>Eremophila graciliflora</i>
<i>Eremophila hamulata</i>
<i>Eremophila humilis</i>
<i>Eremophila laccata</i>
<i>Eremophila lucida</i>
<i>Eremophila maculata</i> subsp. <i>filifolia</i>
<i>Eremophila margarethae</i> subsp. straight sepals (G. Cockerton & B. McLean LCH 31310)
<i>Eremophila oldfieldii</i> subsp. Karara (D. Coultas s.n. PERTH 07341717)
<i>Eremophila perglandulosa</i>
<i>Eremophila pilosa</i>
<i>Eremophila praecox</i>
<i>Eremophila prolata</i>
<i>Eremophila regia</i>
<i>Eremophila resiliens</i>
<i>Eremophila retropila</i>
<i>Eremophila rhegos</i>
<i>Eremophila scrobiculata</i>
<i>Eremophila</i> sp. Beverley (K. Kershaw KK 2438)
<i>Eremophila</i> sp. Hamersley Range (K. Walker KW 136)
<i>Eremophila</i> sp. Ironstone (G. Cockerton & B. McLean LCH 31311)
<i>Eremophila</i> sp. Katjarra South (N. Gibson et al. NG 7149)
<i>Eremophila</i> sp. Meekatharra (D.J. Edinger 4430)
<i>Eremophila</i> sp. Ostrina (M. Officer 164)
<i>Eremophila</i> sp. Rothsay (D. Coultas & J. Kelt s.n. PERTH 08200440)

<i>Eremophila</i> sp. Snowy Mountain (S. van Leeuwen 3737)
<i>Eremophila</i> sp. West Angelas (S. van Leeuwen 4068)
<i>Eremophila splendens</i>
<i>Eremophila spongiorarpa</i>
<i>Eremophila tenella</i>
<i>Eremophila victoriae</i>
<i>Eremophila viscimarginata</i>
<i>Eremophila warnesii</i>
<i>Eremophila yinnetharrensensis</i>
<i>Eremophila brevifolia</i>
<i>Eremophila buirchellii</i>
<i>Eremophila calcicola</i>
<i>Eremophila complanata</i>
<i>Eremophila dendritica</i>
<i>Eremophila jamesiorum</i>
<i>Eremophila mirabilis</i>
<i>Eremophila occidens</i>
<i>Eremophila pallida</i>
<i>Eremophila pusilliflora</i>
<i>Eremophila sargentii</i>
<i>Eremophila</i> sp. Great Victoria Desert (R. Davis 10621)
<i>Eremophila</i> sp. Rudall River (P.G. Wilson 10512)
<i>Eremophila</i> sp. long pedicels (G. Cockerton 1975) PN
<i>Eremophila undulata</i>
<i>Eremophila annosocaulis</i>
<i>Eremophila arachnoides</i> subsp. <i>arachnoides</i>
<i>Eremophila campanulata</i>
<i>Eremophila chamaephila</i>
<i>Eremophila coacta</i>
<i>Eremophila compressa</i>
<i>Eremophila fasciata</i>
<i>Eremophila flaccida</i> subsp. <i>attenuata</i>
<i>Eremophila forrestii</i> subsp. <i>capensis</i>
<i>Eremophila forrestii</i> subsp. <i>viridis</i>
<i>Eremophila gracillima</i>
<i>Eremophila grandiflora</i>
<i>Eremophila lanata</i>
<i>Eremophila magnifica</i> subsp. <i>velutina</i>
<i>Eremophila muelleriana</i>
<i>Eremophila obliquisepala</i>
<i>Eremophila petrophila</i> subsp. <i>densa</i>
<i>Eremophila physocalyx</i>
<i>Eremophila purpurascens</i>
<i>Eremophila rigens</i>
<i>Eremophila rigida</i>
<i>Eremophila shonae</i> subsp. <i>diffusa</i>
<i>Eremophila simulans</i> subsp. <i>megacalyx</i>
<i>Eremophila</i> sp. Byro (R. Wait 6128/97)
<i>Eremophila</i> sp. Murgoo (S.J.J. Davies s.n. 15/8/1960) PN
<i>Eremophila succinea</i>
<i>Eremophila veronica</i>
<i>Eremophila biserrata</i>

<i>Eremophila caerulea</i> subsp. <i>merrallii</i>
<i>Eremophila hillii</i>
<i>Eremophila magnifica</i> subsp. <i>magnifica</i>
<i>Eremophila microtheca</i>
<i>Eremophila parvifolia</i> subsp. <i>parvifolia</i>
<i>Eremophila pungens</i>
<i>Eremophila racemosa</i>
<i>Eremophila serpens</i>
<i>Eremophila veneta</i>
<i>Eremophila youngii</i> subsp. <i>lepidota</i>
<i>Myoporum turbinatum</i>

Appendix Four: Members of tribe Myoporeae currently listed under the *Advisory List of Rare or Threatened Plants in Victoria 2014* (as at 09/05/2018). Species in **bold** were formally listed as threatened under the Victorian state legislation *Flora and Fauna Guarantee Act 1988*.

Vulnerable	Endangered	Rare
<i>Eremophila bignoniiflora</i>	<i>Eremophila debilis</i>	<i>Eremophila crassifolia</i>
<i>Eremophila polyclada</i>	<i>Eremophila sturtii</i>	<i>Eremophila divaricata</i> subsp. <i>divaricata</i>
	<i>Myoporum brevipes</i>	<i>Eremophila gibbifolia</i>
	<i>Myoporum floribundum</i>	<i>Eremophila maculata</i> subsp. <i>maculata</i>
		<i>Eremophila oppositifolia</i> subsp. <i>oppositifolia</i>
		<i>Eremophila scoparia</i>
		<i>Myoporum montanum</i>

Appendix Five: This table documents all described species in tribe Myoporeae, all specimens collected for the current PhD study, and all specimens sequenced and analysed in either Chapter Three (cpDNA analysis), Chapter Four (nrDNA analysis) or Chapter Five (mtDNA analysis). Chinnock's (2007) sectional classification is included for all species of *Eremophila*. Provenance is given for all cultivated specimens (some unknown), and garden location identified by initials; **AALBG** (Australian Arid Lands Botanic Garden, Port Augusta, SA), **ABG** (Adelaide Botanic Garden, Adelaide, SA), **ANBG** (Australian National Botanic Gardens, Canberra, ACT), **BC** (Bob Chinnock, Adelaide, SA), **PH** (Peter Hall, Port Augusta, SA), **RBGV** (Royal Botanic Gardens Victoria), **RD** (Ron Dadd, Goomalling, WA), **RW** (Russell Waite, Riddles Creek, VIC). Species with no collections in the current study are highlighted in grey.

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF211	<i>Eremophila compressa</i> Chinnock	<i>Platycarpus</i>	-32.90429	121.60685	WA: roadside between road and rail lines 8km North Salmon Gums on road to Norseman	x	x	
RMF36	<i>Eremophila saligna</i> (S. Moore) C. Gardner	<i>Platycarpus</i>	-31.2314	121.4603	WA: Coolgardie, 43 km S towards Norseman	x	x	
MJB2384	<i>Eremophila deserti</i> (Cunn. Ex Benth.)	<i>Pholidiopsis</i>	-30.82	117.48	Cultivated: ex Koorda (RD)	x	x	
RMF204	<i>Eremophila deserti</i> (Cunn. Ex Benth.)	<i>Pholidiopsis</i>	-32.94995	123.20631	WA: 3.8km South on Parmango road, then 26km on track heading West NorthWest on way to Mt Newmont (c. 27km West of Balladonia road)	x	x	
RMF228	<i>Eremophila deserti</i> (Cunn. Ex Benth.)	<i>Pholidiopsis</i>	-34.29655	142.2312	VIC: On corner of Woomera and Pumps Ave, East of Red Cliffs	x	x	x
	<i>Eremophila cuneata</i> Chinnock	<i>Pholidiopsis</i>						
RMF243	<i>Eremophila fallax</i> Chinnock	<i>Pholidiopsis</i>	-31.27039	138.87086	SA: Flinders Ranges National Park, side of dried river bed of Wilkawillina Rd	x	x	
RMF246	<i>Eremophila fallax</i> Chinnock	<i>Pholidiopsis</i>	-31.27932	138.57864	SA: Flinders Ranges National Park, located on banks of river at Aroona campsites - Aroona Valley	x	x	x

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
					Rd off Brachina Gorge Rd.			
RMF244	<i>Eremophila fallax</i> Chinnock	<i>Pholidiopsis</i>	-31.27006	138.87057	SA: Flinders Ranges National Park			
RMF279	<i>Eremophila santalina</i> (F. Muell.) F. Muell.	<i>Pholidiopsis</i>	-32.82286	138.0338	SA: Mt Remarkable National Park, side of Battery Ridge Track	x	x	
RMF272	<i>Eremophila santalina</i> (F. Muell.) F. Muell.	<i>Pholidiopsis</i>			Cultivated: unknown location (PH)			
RWG02	<i>Eremophila lactea</i> Chinnock	<i>Crustaceae</i>	-33.21	121.7	Cultivated: ex West Salmon Gums (RW)	x	x	
MJB2380	<i>Eremophila micrantha</i> Chinnock	<i>Crustaceae</i>	-25.48	119.65	Cultivated: ex Ned's Creek Station (RD)	x	x	
RMF10	<i>Eremophila psilocalyx</i> F. Muell.	<i>Crustaceae</i>	-32.9167	123.5	WA: 67 km S of Balladonia towards Mt Ragged	x	x	
RMF197	<i>Eremophila psilocalyx</i> F. Muell.	<i>Crustaceae</i>	-32.02327	120.73831	WA: Found on roadside outside of McDermid Rock			
RMF212	<i>Eremophila succinea</i> Chinnock	<i>Crustaceae</i>	-32.82656	121.19814	WA: 12km South on Peak Charles road from Frank Hahn road in Charles peak National Park	x	x	
RMF192	<i>Eremophila dempsteri</i> F. Muell.	<i>Crustaceae</i>	-32.15891	120.58533	WA: 34km East of 'the breakaways' on the Hyden-Norseman road	x	x	
RMF193	<i>Eremophila dempsteri</i> F. Muell.	<i>Crustaceae</i>	-32.11367	120.6384	WA: 34km East of 'the breakaways' on the Hyden-Norseman road + few extra km up road			
MJB2362	<i>Eremophila ciliata</i> Chinnock	<i>Crustaceae</i>	-33.41	123.4	Cultivated: ex Mt Newmont (RD)	x	x	
RMF201	<i>Eremophila dichroantha</i> Diels	<i>Crustaceae</i>	-32.06511	122.37669	WA: roadside 60.6km East of Norseman on Eyre Hwy	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF199	<i>Eremophila interstans</i> subsp. <i>interstans</i> (S. Moore) Diels	<i>Crustaceae</i>	-32.18836	121.69377	WA: 9km West of Norseman on Tourist road	x	x	x
MJB2381	<i>Eremophila interstans</i> subsp. <i>interstans</i> (S. Moore) Diels	<i>Crustaceae</i>	-30.99	121.13	Cultivated: ex Coolgardie (RD)			
RMF144	<i>Eremophila interstans</i> subsp. <i>virgata</i> (W.V. Fitzg.) Chinnock	<i>Crustaceae</i>	-30.99	121.13	Cultivated: ex Coolgardie (RD)	x	x	
RMF234	<i>Eremophila sturtii</i> R. Br.	<i>Crustaceae</i>	-34.14759	141.30539	VIC: Ned's Corner Station, off Old Mail Rd	x	x	
RMF242	<i>Eremophila sturtii</i> R. Br.	<i>Crustaceae</i>	-31.27385	138.86301	SA: Flinders Ranges National Park, track towards Wilkawillina Gorge			
RMF292	<i>Eremophila sturtii</i> R. Br.	<i>Crustaceae</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens			
RMF12	<i>Eremophila mitchellii</i> Benth.	<i>Crustaceae</i>	-32.75	145.5833	NSW: Yathong Nature Reserve, 2.3 km from Rockholes Road, S along W fence of S.E. corner.		x	
RMF145	<i>Eremophila paisleyi</i> subsp. <i>paisleyi</i> F. Muell.	<i>Crustaceae</i>	-31.04	123.56	Cultivated: ex Zanthus on Transline (RD)	x	x	
RMF274	<i>Eremophila paisleyi</i> subsp. <i>glandulosa</i> Chinnock	<i>Crustaceae</i>			Cultivated: unknown location (PH)		x	
MJB2428	<i>Eremophila falcata</i> Chinnock	<i>Crustaceae</i>	-26.56599	120.00422	WA: Meekatharra-Wiluna Rd 162.5km by road from Meekatharra end (=24.2km by road west of Wiluna)	x	x	
RMF285	<i>Eremophila</i> aff. <i>falcata</i> 'Soloastes' Chinnock	<i>Crustaceae</i>			Cultivated: unknown location (BC)			
MJB2379	<i>Eremophila caperata</i> Chinnock	<i>Crustaceae</i>	-31.32	119.13	Cultivated: ex Moorine Rock (RD)	x	x	x
RMF198	<i>Eremophila caperata</i> Chinnock	<i>Crustaceae</i>	-32.04559	120.83842	WA: 10.1km East of Victoria Rock road on Hyden-Norseman road			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF289	<i>Eremophila debilis</i> (Andrews)	<i>Chamaepogonia</i>	-36.1082	145.8249	VIC: Located on Banks of Boosey Creek, located via track off Burramine Rd	x	x	x
RMF290	<i>Eremophila debilis</i> (Andrews)	<i>Chamaepogonia</i>	-36.1082	145.8249	VIC: Located on Banks of Boosey Creek, located via track off Burramine Rd	x	x	
RMF218	<i>Eremophila chamaephila</i> Diels	<i>Australophilae</i>	-33.5168	120.58369	WA: 17.8km East of Esperance road (South Coast Hwy) on corner of West Point and Rawlinsons roads		x	
MJB2393	<i>Eremophila perglandulosa</i> Chinnock	<i>Australophilae</i>	-30.79	123.46	Cultivated: ex Ponton Creek at the Transline (RD)	x	x	
RMF17	<i>Eremophila weldii</i> F. Muell.	<i>Australophilae</i>	-31.6667	128.9167	WA: Near Eyre Hwy, 2 km E of Eucla	x	x	
RMF254	<i>Eremophila weldii</i> F. Muell.	<i>Australophilae</i>	-33.52283	135.72063	SA: North West of Lock, on side of Tod Hwy towards Kyancutta, between rd and railway line	x	x	
RMF02	<i>Eremophila weldii</i> F. Muell.	<i>Australophilae</i>	-31.6611	129.1358	SA: Several kms from WA/SA border on SA side. Lookout 170.5 km W of Nullarbor Roadhouse.			
RMF126	<i>Eremophila parvifolia</i> subsp. <i>parvifolia</i> J. Black	<i>Australophilae</i>			Cultivated: unknown location (RD)	x	x	
RMF200	<i>Eremophila parvifolia</i> subsp. <i>auricampa</i> Chinnock	<i>Australophilae</i>	-32.1581	121.76448	WA: roadside on hilltop 4.8km North of Norseman on Norseman-Coolgardie road	x	x	x
RMF203	<i>Eremophila oblonga</i> Chinnock	<i>Australophilae</i>	-32.0843	123.0882	WA: 130.5km East of Norseman	x	x	
MJB2461	<i>Eremophila ionantha</i> Diels	<i>Australophilae</i>	-30.95319	121.1434	WA: beside track just west of Coolgardie cemetery, near SW corner of cemetery	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF252	<i>Eremophila praecox</i> Chinnock	<i>Australophilae</i>	-33.12447	136.24911	SA: Eyre Peninsula, West of Kimbla on side of Tola Rd	x	x	
MJB2458	<i>Eremophila pustulata</i> S. Moore	<i>Australophilae</i>	-30.17	120.60627	WA: between Davyhurst and Callion, beside unnamed track on west side of Coolgardie North Road (c 16.5km by road south of Davyhurst and then 400m west of road)	x	x	
MJB2392	<i>Eremophila homoplastica</i> (S. Moore) C. Gardner	<i>Australophilae</i>	-28.18	119.29	Cultivated: ex South of Sandstone (RD)	x	x	
RWG20	<i>Eremophila eversa</i> Chinnock	<i>Australophilae</i>	-29.47	121.82	Cultivated: ex 150km NE Kalgoorlie (RW)	x	x	
MJB2364	<i>Eremophila verticillata</i> Chinnock	<i>Australophilae</i>	-33.1	119.02	Cultivated: ex Newdegate (RD)	x	x	
MJB2358	<i>Eremophila ternifolia</i> Chinnock	<i>Australophilae</i>	-30.89	116.72	Cultivated: ex Wongan Hills (RD)	x	x	
MJB2394	<i>Eremophila veronica</i> (S. Moore) C. Gardner	<i>Australophilae</i>	-30.99	121.13	Cultivated: ex Coolgardie (RD)		x	
RMF132	<i>Eremophila caerulea</i> subsp. <i>merralli</i> (F. Muell. ex Ewart, Jean White & B. Wood)	<i>Australophilae</i>	-31.88	118.15	Cultivated: ex Bruce Rock (RD)	x	x	
MJB2460	<i>Eremophila caerulea</i> (S. Moore) Diels	<i>Australophilae</i>	-30.95319	121.1434	WA: beside track just west of Coolgardie cemetery, near SW corner of cemetery			
RMF191	<i>Eremophila clavata</i> Chinnock	<i>Australophilae</i>	-32.15881	120.58519	WA: 34km East of 'the breakaways' on the Hyden-Norseman road	x	x	
RMF20	<i>Eremophila densifolia</i> subsp. <i>densifolia</i> F. Muell.	<i>Australophilae</i>	-33.6667	120	WA: Ravensthorpe, 12.2 km S along Moir Road.			
RMF216	<i>Eremophila densifolia</i> subsp. <i>densifolia</i> F. Muell.	<i>Australophilae</i>	-32.68531	119.78984	WA: 2.4km North of Carstairs road on Forrestannia road			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF217	<i>Eremophila densifolia</i> subsp. <i>pubiflora</i> Chinnock	<i>Australophilae</i>	-32.62364	119.7429	WA: 10.3km North of Carstairs road on Forrestannia road	x	x	
RMF263	<i>Eremophila densifolia</i> subsp. <i>capitata</i> Chinnock	<i>Australophilae</i>			Cultivated: unknown location (AALBG)		x	
RMF276	<i>Eremophila labrosa</i> Chinnock	<i>Australophilae</i>			Cultivated: unknown location (PH)			
MJB2354	<i>Eremophila nivea</i> Chinnock	<i>Australophilae</i>	-29.53	115.76	Cultivated: ex Three springs (RD)			
RMF133	<i>Eremophila microtheca</i> (F. Muell. ex Benth.)	<i>Australophilae</i>			Cultivated: unknown location (RD)	x	x	
RMF186	<i>Eremophila phillipsii</i> F. Muell.	<i>Australophilae</i>	-32.54483	118.60204	WA: McGann Rock track at end of Reservoir Rd, just before reaching memorial park between Kondinin and Hyden	x	x	
RMF143	<i>Eremophila complanata</i> Chinnock	<i>Australophilae</i>	-30.9	118.66	Cultivated: ex Chiddarcooping Nature Reserve (RD)	x	x	
RMF125	<i>Eremophila adenotricha</i> (F. Muell. ex Benth.)	<i>Australophilae</i>	-31.93	119.1	Cultivated: ex Holleton (RD)	x	x	
RMF11	<i>Eremophila drummondii</i> F. Muell.	<i>Australophilae</i>	-31.2719	120.0025	WA: 66 km E of Southern Cross on Great Eastern Highway	x	x	
MJB2464	<i>Eremophila drummondii</i> F. Muell.	<i>Australophilae</i>	-31.31781	119.05485	WA: Great Eastern Highway 7.2km by road west of Morine Rock			
RMF172	<i>Eremophila rugosa</i> Chinnock	<i>Australophilae</i>	-30.98	121.14	Cultivated: ex Kalgoorlie (RD)	x	x	
RMF187	<i>Eremophila rugosa</i> Chinnock	<i>Australophilae</i>	-32.41673	119.71435	WA: roadside 80.6km East of Hyden on Hyden-Norseman road	x	x	
RMF176	<i>Eremophila papillata</i> Chinnock	<i>Australophilae</i>	-30.82	116.68	Cultivated: ex Wongan Hills (RD)		x	
MJB2363	<i>Eremophila scaberula</i> W.V. Fitzg.	<i>Australophilae</i>	-30.63	116	Cultivated: ex Moora (RD)	x	x	
RMF123	<i>Eremophila sargentii</i> (S.	<i>Australophilae</i>	-30.82	116.68	Cultivated: ex Wongan Hills (RD)		x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
	Moore) Chinnock							
MJB2361	<i>Eremophila vernicosa</i> Chinnock	<i>Australophilae</i>	-30.39	117.12	Cultivated: ex Kalannie (RD)			
MJB2355	<i>Eremophila pinnatifida</i> Chinnock	<i>Australophilae</i>	-30.27	116.65	Cultivated: ex Dalwallinu (RD)			
RMF173	<i>Eremophila brevifolia</i> (Bartling)	<i>Australophilae</i>	-28.399	115.01	Cultivated: ex Chapman Valley (RD)	x	x	x
RMF185	<i>Eremophila lehmanniana</i> (Sonder ex Lehm.) Chinnock	<i>Australophilae</i>	-32.328	117.79542	WA: Corrigin - Quairading Rd, c. 2km from Corrigin - Brookton Rd	x	x	
RMF219	<i>Eremophila lehmanniana</i> (Sonder ex Lehm.) Chinnock	<i>Australophilae</i>	-33.54627	120.63891	WA: 5.6km West on Rawlinson road from Rockhole road	x		
MJB2389	<i>Eremophila occidens</i> Chinnock, G. Paczkowska & A.R. Chapman	<i>Australophilae</i>	-22.1	114	Cultivated: ex Charles Knife Canyon (RD)	x	x	
RMF195	<i>Eremophila hamulata</i> Buirchell & A.P.Br	<i>Australophilae</i>	-32.02324	120.73772	WA: Found in carpark/camping area opposite toilet block at McDermid Rock			
MJB2353	<i>Eremophila koobabbiensis</i> Chinnock	<i>Australophilae</i>	-30.48	116.27	Cultivated: ex Miling (RD)	x	x	
PH01	<i>Eremophila barbata</i> Chinnock	<i>Duttonia</i>			Cultivated: unknown location (PH)	x	x	
RMF258	<i>Eremophila behriana</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-33.91742	136.19766	SA: East of Hincks Conservation Park, North West of Wharminda on Range Rd	x	x	
RMF18	<i>Eremophila behriana</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-34.3583	135.375	SA: Mount Greenly, SE foot			
RMF255	<i>Eremophila behriana</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-33.53115	135.73161	SA: NW of Lock, side of Tod Hwy			
RMF07	<i>Eremophila crassifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-33.4167	136.9167	SA: c. 27 km N of Cowell Causeway	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
TMS14-11	<i>Eremophila crassifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-34.52	141.49	VIC: Murray Sunset NP, Settlement Rd	x	x	
RMF269	<i>Eremophila crassifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>			Cultivated: unknown location (AALBG)			
RMF259	<i>Eremophila gibbifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-33.63334	136.54605	SA: Yeldulknie Conservation Park, located up track on the side of Cleve-Mangalo Rd	x	x	x
MJB2559	<i>Eremophila gibbifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-36.62289	141.74581	VIC: Little Desert National Park	x	x	
RMF19	<i>Eremophila gibbifolia</i> (F. Muell.) F. Muell.	<i>Duttonia</i>	-34.4833	135.8417	SA: Todd River Reservoir Reserve, 0.7 km W of entrance gate, S side of dam			
MJB2422	<i>Eremophila spuria</i> Chinnock	<i>Arenariae</i>	-26.58504	118.60338	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 11km by road from Meekatharra end (intersection with Great Northern Hwy)	x	x	
	<i>Eremophila setacea</i> Chinnock	<i>Arenariae</i>						
MJB2431	<i>Eremophila platythamnos</i> subsp. <i>platythamnos</i> Diels	<i>Arenariae</i>	-26.28864	121.08012	WA: Lorna Glen Station on Granite Peakes Road c18.3km NE or entry to Milrose Station. Sand dune on North side of road	x	x	x
RMF160	<i>Eremophila platythamnos</i> subsp. <i>exotrachys</i> (Kraenzlin) Chinnock	<i>Arenariae</i>	-25.81	123.14	Cultivated: ex 20 km E of Carnegie Station (RD)	x	x	
KK085	<i>Eremophila platythamnos</i> subsp. <i>exotrachys</i> (Kraenzlin) Chinnock	<i>Arenariae</i>	-22.81	127.76	WA: Gibson Desert		x	
RMF114	<i>Eremophila gibsonii</i> F. Muell.	<i>Arenariae</i>			Cultivated: unknown location (RD)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF174	<i>Eremophila arenaria</i> Chinnock	<i>Arenariae</i>			Cultivated: unknown location (RD)	x	x	
MJB2367	<i>Eremophila aureivisca</i> Chinnock	<i>Arenariae</i>	-28.76	124.33	Cultivated: ex Lake Rason (RD)	x	x	
MJB2427	<i>Eremophila enata</i> Chinnock	<i>Eremaeae</i>	-26.54832	119.95721	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 157km by road from Meekatharra end (=29.3km by road west of Wiluna)	x	x	
MJB2429	<i>Eremophila gilesii</i> subsp. <i>gilesii</i> F. Muell.	<i>Eremaeae</i>	-26.55436	120.66073	WA: Granite Peakes Road 9.8km north of Gunbarrel Hwy (Wongawol Road)	x		
MJB2430	<i>Eremophila gilesii</i> subsp. <i>gilesii</i> F. Muell. (broad leaf form)	<i>Eremaeae</i>	-26.55436	120.66073	WA: Granite Peakes Road 9.8km north of Gunbarrel Hwy (Wongawol Road)	x	x	
BB16	<i>Eremophila gilesii</i> subsp. <i>gilesii</i> F. Muell.	<i>Eremaeae</i>	-23.59022	132.51219	NT: Campground, Redbank Gorge, West McDonnell Ranges			
BB35	<i>Eremophila gilesii</i> subsp. <i>variabilis</i> Chinnock	<i>Eremaeae</i>	-24.01503	117.28156	WA: 81.3 km W on Pingandy Rd towards Mt Augustus	x	x	
MJB2481	<i>Eremophila gilesii</i> F. Muell.	<i>Eremaeae</i>	-23.50827	133.85552	NT: Stuart Hwy 3.3km North of intersection with Tanami Rd	x	x	x
MJB2409	<i>Eremophila foliosissima</i> Kraenzlin	<i>Eremaeae</i>	-27.82393	117.92946	WA: Great Northern Hwy c32km north of Mt Magnet	x	x	
RMF84	<i>Eremophila gracillima</i> Chinnock	<i>Eremaeae</i>	-24.65475	118.56202	WA: 5.4km South of Tangadee turnoff towards Milgen Station	x		
RMF64	<i>Eremophila lanceolata</i> Chinnock	<i>Eremaeae</i>	-24.44214	119.67825	WA: 0.24km West on road to Ilgarari off Great Northern Hwy	x	x	
RW2372	<i>Eremophila alatisepala</i> Chinnock	<i>Eremaeae</i>	-23.34944	142.72361	QLD	x	x	
RMF118	<i>Eremophila linsmithii</i> R.D. Henderson	<i>Eremaeae</i>			Cultivated: unknown location (RD)		x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF266	<i>Eremophila linsmithii</i> R.D. Henderson	<i>Eremaeae</i>			Cultivated: unknown location (AALBG)			
MJB2399	<i>Eremophila prolata</i> Chinnock	<i>Eremaeae</i>	-25.69	118.07	Cultivated: ex Yarlaweelor Rd, Mt Padbury Station (RD)	x	x	
BB02	<i>Eremophila freelingii</i> F. Muell.	<i>Eremaeae</i>	-31.21825	139.64822	SA: 178.4 km N of Yunta towards Arkaroola	x	x	
RMF248	<i>Eremophila freelingii</i> F. Muell.	<i>Eremaeae</i>	-31.34296	138.56223	SA: Flinders Ranges National Park, Brachina Gorge Rd, between Brachina East and Teamsters campgrounds	x	x	
TW710	<i>Eremophila freelingii</i> F. Muell.	<i>Eremaeae</i>	-23.7772	133.0733	NT: Ellery Big Hole, east side of the chasm mouth on west facing slope. Tjoritja/West MacDonnell National Park	x	x	
RMF300	<i>Eremophila freelingii</i> F. Muell.	<i>Eremaeae</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens			
RMF61	<i>Eremophila spectabilis</i> subsp. <i>spectabilis</i> C. Gardner	<i>Eremaeae</i>	-24.9467	119.82532	WA: 42.8km East of Great Northern Hwy in road to Marymia Station	x	x	
MJB2426	<i>Eremophila spectabilis</i> subsp. <i>brevis</i> Chinnock	<i>Eremaeae</i>	-26.50627	119.86596	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 147km by road from Meekatharra end (=39.6km by road west-northwest of Wiluna)		x	
MJB2418	<i>Eremophila phyllopoda</i> subsp. <i>phyllopoda</i> Chinnock	<i>Eremaeae</i>	-26.58322	118.46572	WA: near Peace Gorge, west of Meekatharra	x	x	
BB32	<i>Eremophila phyllopoda</i> subsp. <i>obliqua</i> Chinnock	<i>Eremaeae</i>	-23.45561	117.13253	WA: S of Ashburton Downs on the road to Mt Vernon	x	x	
RMF83	<i>Eremophila phyllopoda</i> subsp. <i>obliqua</i> Chinnock	<i>Eremaeae</i>	-24.37546	118.541	WA: 30 km North on Mt Vernon road from Tangadee turnoff			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF78	<i>Eremophila phyllopoda</i> subsp. <i>obliqua</i> Chinnock	<i>Eremaeae</i>	-23.99254	119.74986	WA: c. 62.8km South of Capricorn roadhouse, Great Northern Hwy			
RMF139	<i>Eremophila rigens</i> Chinnock	<i>Eremaeae</i>	-24.2	116.87	WA: Pingandy Rd N of Mt Augustus	x	x	
MJB2387	<i>Eremophila rigens</i> Chinnock	<i>Eremaeae</i>	-23.99	117.52	Cultivated: ex Pingandy (RD)			
BB34	<i>Eremophila rigens</i> Chinnock	<i>Eremaeae</i>	-23.96128	117.41225	WA: 62.6 km W on Pingandy Rd towards Mt Augustus			
RMF103	<i>Eremophila crenulata</i> Chinnock	<i>Eremaeae</i>	-25.48907	114.3763	WA: Pimbee Rd			
MJB2440	<i>Eremophila cuneifolia</i> Kraenzlin	<i>Eremaeae</i>	-26.25232	121.68553	WA: Wangawol Station, track between Lorna Glen Station and Wongawol Road, c 7.6km by road east of the fence on the Eastern boundary of Lorna Glen	x	x	x
RMF79	<i>Eremophila cuneifolia</i> Kraenzlin	<i>Eremaeae</i>	-24.32425	119.54748	WA: located on Ilgararie Station, past homestead on track. 21km West of Great Northern Hwy on road to Tangadee Station.	x	x	
RMF94	<i>Eremophila cuneifolia</i> 'Big leaf' Kraenzlin	<i>Eremaeae</i>	-24.21885	116.53811	WA: c. 40km West from Mount Augustus on road to Cobra		x	
RWG17	<i>Eremophila accrescens</i> Chinnock	<i>Eremaeae</i>	-24.21	116.58	WA: West of Mt Augustus, between Cobra Station	x	x	
RMF177	<i>Eremophila goodwinii</i> subsp. <i>goodwinii</i> F. Muell.	<i>Eremaeae</i>			Cultivated: unknown location (RD)			
RMF178	<i>Eremophila goodwinii</i> subsp. <i>ecapitata</i> Chinnock	<i>Eremaeae</i>			Cultivated: unknown location (RD)	x	x	
RMF15	<i>Eremophila willsii</i> subsp. <i>willsii</i> F. Muell.	<i>Eremaeae</i>	-24.5492	133.5319	NT: Uluru - Kata Tjuta National Park: Talinguru, 1.2 km ENE of Uluru (Ayers Rock)			
BB18	<i>Eremophila willsii</i> subsp.	<i>Eremaeae</i>	-22.83806	131.91492	NT: 41 km N Papunya			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
	<i>willsii</i> F. Muell.							
RMF13	<i>Eremophila willsii</i> subsp. <i>integrifolia</i> (Ewart) Chinnock	<i>Eremaeae</i>	-25.3464	131.0636	NT: Henbury Station; 28 km E of Henbury homestead; heading up red sand dune (from mulga community in swale).	x	x	
RMF267	<i>Eremophila prostrata</i> Chinnock	<i>Eremaeae</i>			Cultivated: unknown location (AALBG)		x	
RMF179	<i>Eremophila elderii</i> F. Muell.	<i>Eremaeae</i>	-24.859	128.02	Cultivated: ex Rawlinson Range (RD)	x	x	
MJB2375	<i>Eremophila canaliculata</i> Chinnock	<i>Eremaeae</i>	-25.08	118.28	Cultivated: ex Vernon Rd North of Milgun (RD)	x	x	
RMF124	<i>Eremophila acrida</i> Chinnock	<i>Eremaeae</i>	-24.859	128.02	Cultivated: ex Rawlinson Range (RD)	x	x	
MJB2410	<i>Eremophila georgei</i> Diels	<i>Eremaeae</i>	-27.40926	117.90519	WA: Great Northern Hwy c2km by road north of Cue, near outcrops on East side of road	x	x	
BB41	<i>Eremophila</i> aff. <i>clarkei</i> 'Kennedy Ranges' Oldfield & F. Muell.	<i>Eremaeae</i>	-24.66297	115.17722	WA: Temple Gorge, Kennedy Range NP, WA	x		
RMF107	<i>Eremophila clarkeii</i> Oldfield & F. Muell.	<i>Eremaeae</i>	-26.64231	114.61106	WA: Butchers Track	x	x	
MJB2403	<i>Eremophila clarkeii</i> Oldfield & F. Muell.	<i>Eremaeae</i>	-29.52933	117.16661	WA: Great Northern Hwy 65.4km by road southwest of Paynes Fnd			
MJB2452	<i>Eremophila metallicorum</i> S. Moore	<i>Eremaeae</i>	-28.69941	122.07719	WA: Laverton - Leonora Road c23.4km SW of intersection with road to Windarra	x	x	
MJB2459	<i>Eremophila granitica</i> S. Moore	<i>Eremaeae</i>	-30.43796	120.81703	WA: Coolgardie North Road c 3km by road NW of turnoff to Credo Homestead	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF52	<i>Eremophila granitica/metallicorum</i> S. Moore	<i>Eremaeae</i>	-29.28745	117.59454	WA: Just off Great Northern Hwy roadside (c. 10km from Paynes Find) - short walk from campsite at Ningham Station			
RMF65	<i>Eremophila appressa</i> Chinnock	<i>Eremaeae</i>	-24.34813	119.6922	WA: Great Northern Hwy, South of Capricorn	x	x	
MJB2439	<i>Eremophila punctata</i> Chinnock	<i>Eremaeae</i>	-26.25219	121.6821	WA: track between Lorna Glen Station and Wongawol Road, c 7.2km by road east of the fence on the Eastern boundary of Lorna Glen	x	x	
RMF87	<i>Eremophila obliquiseppala</i> Chinnock	<i>Eremaeae</i>	-24.97115	118.42377	WA: few km down road from Milgen Station	x	x	
RMF86	<i>Eremophila obliquiseppala</i> Chinnock	<i>Eremaeae</i>	-24.9451	118.45454	WA: Few km West of Milgen travelling towards Mt Augustus (North of Milgen Station)			
MJB2450	<i>Eremophila simulans</i> subsp. <i>simulans</i> Chinnock	<i>Eremaeae</i>	-27.80841	122.28447	WA: Bandy Road c130m South of Swanson Creek	x	x	
RMF171	<i>Eremophila simulans</i> subsp. <i>lapidnesis</i> Chinnock	<i>Eremaeae</i>	-28	119.03	Cultivated: Sandstone (RD)	x		
RMF110	<i>Eremophila simulans</i> subsp. <i>megacalyx</i> Chinnock	<i>Eremaeae</i>	-27.21885	116.3052	WA: South East of Murchison	x	x	
RW9907	<i>Eremophila</i> aff. <i>simulans</i> Chinnock	<i>Eremaeae</i>	-24.58133	117.47606	WA: East Mt Augustus National Park			
RMF134	<i>Eremophila conglomerata</i> Chinnock	<i>Eremaeae</i>	-28	119.03	Cultivated: Sandstone (RD)	x	x	x
RW14041	<i>Eremophila conglomerata</i> Chinnock	<i>Eremaeae</i>	-27.94786	119.29708	WA: North of Sandstone			
MJB2433	<i>Eremophila pungens</i> Chinnock	<i>Eremaeae</i>	-26.14454	121.14867	WA: Lorna Glen Station, breakways 4.7km by track west of Newmarket Bore	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF56	<i>Eremophila</i> aff. <i>pungens</i> 'Meekatharra' Chinnock	<i>Eremaeae</i>	-25.97086	118.9006	WA: side of 'Eye Rock' c. 80km North of Meekatharra and 10km East		x	
RMF69	<i>Eremophila incisa</i> Chinnock	<i>Eremaeae</i>	-24.22421	119.78342	WA: c. 8km NorthEast on Gialong-Mundiwindi road	x	x	
RMF60	<i>Eremophila incisa</i> Chinnock	<i>Eremaeae</i>	-25.27786	119.33559	WA: Great Northern Hwy, c. 80km South of Kumarina on side of road (20mins from previous site)			
MJB2423	<i>Eremophila flabellata</i> Chinnock	<i>Eremaeae</i>	-26.58504	118.60338	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 11km by road from Meekatharra end (intersection with Great Northern Hwy)	x	x	
RMF137	<i>Eremophila attenuata</i> Chinnock	<i>Eremaeae</i>	-29.76647	126.85611	Cultivated: (RD)	x	x	
MJB2414	<i>Eremophila glutinosa</i> Chinnock	<i>Eremaeae</i>	-27.20193	118.01662	WA: Great Northern Hwy 11.3km by road southwest of Tuckanarra		x	
RMF66	<i>Eremophila petrophila</i> subsp. <i>petrophila</i> Chinnock	<i>Eremaeae</i>	-24.34786	119.6922	WA: Great Northern Hwy, South of Capricorn	x	x	
NG6474	<i>Eremophila petrophila</i> subsp. <i>petrophila</i> Chinnock	<i>Eremaeae</i>	-25.06816	120.64433	WA: At entrance of broad valley. Ca. 5.1 km on a bearing of 291 degrees from Mt Methwin, Katjarra (Carnarvon Range), Birriliburru Indigenous Protected Area			
RMF102	<i>Eremophila petrophila</i> subsp. <i>densa</i> Chinnock	<i>Eremaeae</i>	-25.46398	115.18572	WA: 60.6km South West on Pimbee road from Gasgoyne Junction	x	x	
MJB2444	<i>Eremophila hughesii</i>	<i>Eremaeae</i>	-26.02358	121.96667	WA: Gunbarrel Highway, (Carnegie Rd) c 5km by road	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
					northeast of crossing of Charles Wells Creek			
BB84	<i>Eremophila hughesii</i> subsp. <i>hughesii</i> F. Muell.	<i>Eremaeae</i>	-25.04268	128.30277	WA: Warakurna	x	x	
MJB2435	<i>Eremophila pendulina</i> Chinnock	<i>Eremaeae</i>	-26.20009	121.26826	WA: Lorna Glen Station on road to homestead, 5km by Road from junction with Granite Peaks Rd			
MJB2406	<i>Eremophila exilifolia</i> F. Muell.	<i>Eremaeae</i>	-28.26294	117.85965	WA: Great Northern Hwy c123km by road North of Paynes Find (=23.6km by road south of Mt Magnet)		x	
RW13997	<i>Eremophila exilifolia</i> F. Muell.	<i>Eremaeae</i>	-28.00303	118.95242	WA: West of Sandstone			
RWG15	<i>Eremophila shonae</i> subsp. <i>shonae</i> Chinnock	<i>Eremaeae</i>	-26.51	117.65	Cultivated: West of Meekatharra (RW)	x	x	
MJB2449	<i>Eremophila shonae</i> subsp. <i>diffusa</i> Chinnock	<i>Eremaeae</i>	-27.64173	121.63891	WA: North of Banjarn, c 1km by road southwest of Jacks Well	x	x	
MJB2442	<i>Eremophila annosocaula</i> Chinnock	<i>Eremaeae</i>	-26.27867	121.83372	WA: Wangawol Station, track between Lorna Glen Station and Wongawol Road, c 23.5km by road east of the fence on the Eastern boundary of Lorna Glen	x	x	
MJB2373	<i>Eremophila coacta</i> Chinnock	<i>Eremaeae</i>	-23.36	117.07	Cultivated: ex Ashburton Downs (RD)			
RMF120	<i>Eremophila battii</i> F. Muell.	<i>Eremaeae</i>	-26.59	120.22	Cultivated: ex Wiluna (RD)	x	x	
RMF181	<i>Eremophila battii</i> 'Granite Peaks' F. Muell.	<i>Eremaeae</i>	-25.67	121.3	Cultivated: S of Granite Peaks Station (RD)	x	x	
MJB2438	<i>Eremophila spinescens</i> Chinnock	<i>Eremaeae</i>	-26.22714	121.445	WA: Lorna Glen Station on road to homestead, 24.3km by Road from junction with Granite Peaks			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
					Rd			
MJB2398	<i>Eremophila magnifica</i> subsp. <i>magnifica</i> Chinnock	<i>Eremaeae</i>	-22.25	117.98	Cultivated: ex Karajini N.P. (RD)	x	x	
BB28	<i>Eremophila magnifica</i> Chinnock	<i>Eremaeae</i>	-22.70586	117.74814	WA: Mt Nameless, near Tom Price			
MJB2386	<i>Eremophila spongiorcarpa</i> Chinnock	<i>Eremaeae</i>	-22.44	119.92	Cultivated: ex Roy Hill (Station) (RD)	x		
RMF182	<i>Eremophila arguta</i> Chinnock	<i>Eremaeae</i>	-24.42	117.07	Cultivated: ex E of Mt Augustus on Woodlands Road (RD)	x	x	
MJB2382	<i>Eremophila pallida</i> Chinnock	<i>Eremaeae</i>	-23.5	126.37	Cultivated: ex Gibson Desert, Gary Hwy (RD)	x	x	
RWG06	<i>Eremophila viscimarginata</i> Chinnock	<i>Eremaeae</i>	-24.69	125.85	Cultivated: ex base of Mt Beadle (RW)	x	x	
	<i>Eremophila jamesiorum</i> Buirchell & A.P.Br.	<i>Eremaeae</i>						
	<i>Eremophila laccata</i> Buirchell & A.P.Br.	<i>Eremaeae</i>						
	<i>Eremophila scrobiculata</i> Buirchell & A.P.Br.	<i>Eremaeae</i>						
	<i>Eremophila victoriae</i> Buirchell & A.P.Br.	<i>Eremaeae</i>						
RW2320	<i>Eremophila woodiae</i> Edginton	<i>Eremaeae</i>	-23.50639	147.74	QLD		x	
RMF119	<i>Eremophila pentaptera</i> J. Black	<i>Carnosae</i>			Cultivated: unknown location (RD)	x	x	
RMF265	<i>Eremophila macdonnellii</i> (broad leaf) F. Muell.	<i>Platycalyx</i>			Cultivated: unknown location (AALBG)	x	x	
RMF293	<i>Eremophila macdonnellii</i> F. Muell.	<i>Platycalyx</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens	x		x

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
DQ444239	<i>Eremophila macdonnellii</i> F. Muell.	<i>Platycalyx</i>					x	
RMF284	<i>Eremophila arbuscula</i> Chinnock	<i>Eremophila</i>	-26.5	143.81	Cultivated: unknown location (BC)		x	
RMF270	<i>Eremophila arbuscula</i> Chinnock	<i>Eremophila</i>			Cultivated: unknown location (AALBG)			
RMF226	<i>Eremophila oppositifolia</i> subsp. <i>oppositifolia</i> R. Br.	<i>Eremophila</i>	-34.27234	142.18491	VIC: Calder Hwy, South of Mildura			
RMF230	<i>Eremophila oppositifolia</i> subsp. <i>oppositifolia</i> R. Br.	<i>Eremophila</i>	-34.41359	141.4272	VIC: Yarrara Nature Conservation Reserve			
MJB2401	<i>Eremophila oppositifolia</i> subsp. <i>angustifolia</i> (S. Moore) Chinnock	<i>Eremophila</i>	-29.70754	117.0847	WA: Great Northern Hwy, 89km by road SW of Paynes Find (7.75km by road northeast of intersection of Great Northern Hwy and Goodlands Rd)	x	x	
RMF277	<i>Eremophila oppositifolia</i> subsp. <i>rubra</i> (White & Francis) Chinnock	<i>Eremophila</i>			Cultivated: unknown location (AALBG)		x	
MJB2368	<i>Eremophila reticulata</i> Chinnock	<i>Eremophila</i>	-24.32	117.23	Cultivated: ex Mt Augustus (RD)		x	
MJB2390	<i>Eremophila cryptothrix</i> Chinnock	<i>Eremophila</i>	-23.16	117.63	Cultivated: ex N-W Parabadoo (RD)	x	x	
BB06	<i>Eremophila rotundifolia</i> F. Muell.	<i>Eremophila</i>	-29.57689	135.07367	72.6 km S of Coober Pedy on the Stuart Hwy, South Australia		x	
RMF271	<i>Eremophila rotundifolia</i> F. Muell.	<i>Eremophila</i>			Cultivated: Olive Pink Botanic Garden NT			
DQ444240	<i>Eremophila rotundifolia</i> F. Muell.	<i>Eremophila</i>					x	
MJB2415	<i>Eremophila spathulata</i> W.V. Fitzg.	<i>Eremophila</i>	-27.04921	118.17319	WA: Great Northern Hwy c12.4km by road northeast of Tuckanarra	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2385	<i>Eremophila rigida</i> Chinnock	<i>Eremophila</i>	-24.94	118.7	Cultivated: ex Mingah Springs (RD)	x	x	
RMF100	<i>Eremophila tietkensis</i> F. Muell.	<i>Eremophila</i>	-24.678	115.25732	WA: 1.9km along Kennedy Range National Park road	x	x	
RMF74	<i>Eremophila tietkensis</i> 'Hamersby' F. Muell.	<i>Eremophila</i>	-23.04108	118.85095	WA: Mount Robinson Gorge, 106.9km North West of Newman. Right hand side of walking track before gorge closes in.	x	x	
BB17	<i>Eremophila tietkensis</i> F. Muell.	<i>Eremophila</i>	-23.91603	128.74172	WA: Sandy Blight Road	x	x	
RMF98	<i>Eremophila tietkensis</i> OR <i>platycalyx</i> sp. 'Gascoyne'	<i>Eremophila</i>	-25.22272	115.90216	WA: 84km South of Yinnetharra homestead		x	
RMF121	<i>Eremophila mirabilis</i> Chinnock	<i>Eremophila</i>	-29.35	121.29	Cultivated: ex Niagra Dam Nature Reserve (RD)		x	
MJB2400	<i>Eremophila platycalyx</i> subsp. <i>platycalyx</i> F. Muell.	<i>Eremophila</i>	-26.51	118.5	Cultivated: ex Meekatharra granites (RD)	x	x	
RMF105	<i>Eremophila platycalyx</i> subsp. <i>platycalyx</i> F. Muell.	<i>Eremophila</i>	-26.28749	114.41776	WA: South of Carbla		x	
RMF80	<i>Eremophila platycalyx</i> subsp. <i>pardolata</i> Chinnock	<i>Eremophila</i>	-24.25978	119.40621	WA: 39.9km West on Ilgararie - Tangadee road from Great Northern Hwy			
RMF158	<i>Eremophila platycalyx</i> 'Wongawol' F. Muell	<i>Eremophila</i>			Cultivated: ex Wongawol creek (RD)			
RMF169	<i>Eremophila platycalyx</i> 'Leonora' F. Muell.	<i>Eremophila</i>	-28.85	121.64	Cultivated: ex Leonora (RD)		x	
RMF85	<i>Eremophila platycalyx</i> 'Milgen' F. Muell.	<i>Eremophila</i>	-24.94533	118.45538	WA: Few km West of Milgen travelling towards Mt Augustus (North of Milgen Station)		x	
RMF76	<i>Eremophila platycalyx</i> 'Eastern' F. Muell	<i>Eremophila</i>	-23.3629	119.72612	WA: Radio Hill lookout, Newman			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF63	<i>Eremophila platycalyx</i> 'Ilgarari' F. Muell	<i>Eremophila</i>	-24.44248	119.67854	WA: 0.24km West on road to Ilgarari off Great Northern Hwy			
RMF50	<i>Eremophila platycalyx</i> 'Yalgoo' F. Muell.	<i>Eremophila</i>	-29.31962	117.38321	WA: c. 30km South of Paynes Find, just off main roadside	x	x	
RMF54	<i>Eremophila platycalyx</i> 'Granite' F. Muell.	<i>Eremophila</i>	-26.57933	118.47971	WA: c. 3km West of Meekatharra at Peace Gorge	x	x	
MJB2413	<i>Eremophila macmillaniana</i> C. Gardner	<i>Eremophila</i>	-27.20172	118.01705	WA: Great Northern Hwy 11.3km by road southwest of Tuckanarra	x	x	
RMF21	<i>Eremophila polyclada</i> (F. Muell.) F. Muell.	<i>Platychilus</i>	-28.9206	145.7167	QLD: 50 km N of Enngonia on Mitchell Highway	x	x	
RMF232	<i>Eremophila polyclada</i> (F. Muell.) F. Muell.	<i>Platychilus</i>	-34.13445	141.31102	VIC: Ned's Corner Station	x	x	
RMF236	<i>Eremophila bignoniiflora</i> (Benth.) F. Muell.	<i>Platychilus</i>	-34.13842	141.33371	VIC: Ned's Corner Station, located on river bank East of Homestead	x	x	
RMF162	<i>Eremophila arachnoides</i> subsp. <i>arachnoides</i> Chinnock	<i>Pholidia</i>			Cultivated: unknown location (RD)	x	x	
RMF147	<i>Eremophila arachnoides</i> subsp. <i>tenera</i> Chinnock	<i>Pholidia</i>			Cultivated: unknown location (RD)	x	x	x
RMF23	<i>Eremophila dalyana</i> F. Muell.	<i>Pholidia</i>	-27.7322	142.9833	QLD: 92 km W of Thargomindah towards Jackson.	x	x	
RMF113	<i>Eremophila stenophylla</i> Chinnock	<i>Pholidia</i>			Cultivated: unknown location (RD)	x	x	
MJB2411	<i>Eremophila pantonii</i> F. Muell.	<i>Pholidia</i>	-27.27829	117.97772	WA: Great Northern Hwy c19km by road northeast of Cue, (c 1km by road south of Nallan Creek crossing)		x	
RWG08	<i>Eremophila verrucosa</i> subsp. <i>verrucosa</i> Chinnock	<i>Pholidia</i>	-27.95	134.05	SA: East of Cadney Park - Stuart Hwy	x	x	
MJB2402	<i>Eremophila scoparia</i> (R. Br.)	<i>Pholidia</i>	-29.52997	117.16625	WA: Great Northern Hwy 65.4km by road southwest of Paynes Fnd	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF194	<i>Eremophila scoparia</i> (R. Br.)	<i>Pholidia</i>	-32.03243	120.74231	WA: 55km East of 'the breakaways' on Hyden-Norseman road - Few meters into entrance of McDermid Rock	x	x	
TMS14-13	<i>Eremophila scoparia</i> (R. Br.)	<i>Pholidia</i>	-34.79	141.63	VIC: Murray Sunset NP, underbool Rd	x	x	
RMF231	<i>Eremophila scoparia</i> (R. Br.)	<i>Pholidia</i>	-34.38153	141.33385	VIC: Meringur, Meringur North Rd travelling towards Sturt Hwy	x	x	
RMF249	<i>Eremophila scoparia</i> (R. Br.)	<i>Pholidia</i>	-31.33969	138.55145	SA: Flinders Ranges National Park, Brachina Gorge Rd, opposite Teamsters grave	x	x	x
MJB2441	<i>Eremophila youngii</i> subsp. <i>youngii</i> F. Muell.	<i>Pholidia</i>	-26.25773	121.75411	WA: Wangawol Station, track between Lorna Glen Station and Wongawol Road, c 15km by road east of the fence on the Eastern boundary of Lorna Glen	x	x	
RMF73	<i>Eremophila youngii</i> subsp. <i>lepidota</i> Chinnock	<i>Pholidia</i>	-22.69531	119.94966	WA: 84.2km North of Newman on Marble Bar road	x	x	
RMF16	<i>Eremophila divaricata</i> subsp. <i>divaricata</i> (F. Muell.) F. Muell.	<i>Sentis</i>	-29.8497	144.1325	NSW: Nocoleche Homestead. 50 m N of Paroo River Bank	x	x	
RMF227	<i>Eremophila divaricata</i> subsp. <i>divaricata</i> (F. Muell.) F. Muell.	<i>Sentis</i>	-34.28471	142.21725	VIC: Kings Billabong Wildlife Reserve, approximately 200m into reserve, beside lake	x	x	
RMF22	<i>Eremophila divaricata</i> subsp. <i>divaricata</i> (F. Muell.) F. Muell.	<i>Sentis</i>	-29.8497	144.1325	NSW: Nocoleche Homestead. 50 m N of Paroo River Bank			
RMF235	<i>Eremophila divaricata</i> subsp. <i>divaricata</i> (F. Muell.) F. Muell.	<i>Sentis</i>	-34.13708	141.32263	VIC: Ned's Corner Station			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF273	<i>Eremophila divaricata</i> subsp. <i>callewatta</i> Chinnock	<i>Sentis</i>			Cultivated: unknown location (PH)			
RMF08	<i>Eremophila christophorii</i> F. Muell.	<i>Sentis</i>			Cultivated: unknown location (ANBG)	x	x	
TMS 16-30	<i>Eremophila christophorii</i> F. Muell.	<i>Sentis</i>	-24.01	132.87	NT: Ellery Creek	x	x	
RMF299	<i>Eremophila christophorii</i> F. Muell.	<i>Sentis</i>			NT: Olive Pink Botanic Gardens			
RMF150	<i>Eremophila decussata</i> Chinnock	<i>Decussatae</i>			Cultivated: unknown location (RD)	x	x	
MJB2424	<i>Eremophila malacoides</i> Chinnock	<i>Decussatae</i>	-26.49111	119.78154	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 137km by road from Meekatharra end (=48.5km by road northwest of Wiluna)	x	x	
MJB2352	<i>Eremophila resinosa</i> (Endl.) F. Muell.	<i>Decussatae</i>	-31.29	118.7	Cultivated: ex Westonia (RD)			
RMF148	<i>Eremophila delisseri</i> F. Muell.	<i>Decussatae</i>			Cultivated: unknown location (RD)	x	x	
RMF149	<i>Eremophila dendritica</i> Chinnock	<i>Decussatae</i>	-30.79	125.37	Cultivated: ex Rawllina (RD)	x	x	
BB07	<i>Eremophila dendritica</i> Chinnock	<i>Decussatae</i>	-31.31736	125.42597	39 km S of Rawlina on way to Cocklebiddy, Western Australia			
RMF159	<i>Eremophila mackinlayi</i> subsp. <i>mackinlayi</i> F. Muell.	<i>Hygrophanae</i>	-28.07	117.75	Cultivated: ex Mt Magnet (RD)	x	x	x
RMF09	<i>Eremophila mackinlayi</i> subsp. <i>spatulata</i> Chinnock	<i>Hygrophanae</i>	-28.3672	116.4614	WA: 26.4 km W of Yalgoo on Geraldton - Mount Magnet Road.	x	x	
RMF106	<i>Eremophila strongylophylla</i> F. Muell.	<i>Hygrophanae</i>	-26.54748	114.51396	WA: on roadside 15.7km South of the Overland Roadhouse	x	x	
MJB2454	<i>Eremophila hygrophana</i> Chinnock	<i>Hygrophanae</i>	-28.88918	121.37331	WA: Laverton - Leonora Road c4.2km east of Leonora (=4.2km east of intersection with	x	x	x

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
					Godlfield Hwy)			
RMF115	<i>Eremophila ovata</i> Chinnock	<i>Hygrophanae</i>			Cultivated: unknown location (RD)	x	x	
MJB2378	<i>Eremophila fasciata</i> Chinnock	<i>Hygrophanae</i>	-26.88	118.66	Cultivated: ex Mt Yagahong sth of Meekathara (RD)	x	x	
MJB2396	<i>Eremophila warnesii</i> Chinnock	<i>Hygrophanae</i>	-25.58	117.98	Cultivated: ex Yarlaweelor - Mt Clere (RD)		x	
RMF183	<i>Eremophila macgillivrayi</i> J. Black	<i>Contortae</i>			Cultivated: unknown location (RD)	x		
RMF104	<i>Eremophila pterocarpa</i> subsp. <i>pterocarpa</i> W.V. Fitzg.	<i>Contortae</i>	-25.57502	114.2225	WA: Pimbee Rd	x	x	
NG6475	<i>Eremophila pterocarpa</i> subsp. <i>acicularis</i> Chinnock	<i>Contortae</i>	-25.32736	120.86552	WA: South of track between Blue Hills and Well5 (Canning Stock Route), ca. 19.2km on a bearing of 107 degrees from Trig Point M6, Katjarra (Carnarvon Range), Birriliburru Indigenous Protected Area		x	
RMF57	<i>Eremophila pterocarpa</i> subsp. <i>acicularis</i> Chinnock	<i>Contortae</i>	-25.97085	118.90038	WA: side of 'Eye Rock' c. 80km North of Meekatharra and 10km East			
RMF05	<i>Eremophila bowmanii</i> subsp. <i>bowmanii</i> F. Muell.	<i>Eriocalyx</i>	-30.6075	145.7986	NSW: Gundabooka National Park, 10.9 km from park access road towards Mulgowan art site.		x	
RMF01	<i>Eremophila bowmanii</i> subsp. <i>latifolia</i> (L.S. Smith) Chinnock	<i>Eriocalyx</i>	-29.9167	144.1	NSW: Far west plains; 30 km from Wanaaring South towards Wilcannia.	x	x	
RMF152	<i>Eremophila bowmanii</i> subsp. <i>nutans</i> Chinnock	<i>Eriocalyx</i>			Cultivated: unknown location (RD)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2405	<i>Eremophila forrestii</i> subsp. <i>forrestii</i> F. Muell.	<i>Eriocalyx</i>	-28.94095	117.82689	WA: Great Northern Hwy 41.45km by road southwest of Paynes Fnd		x	
RMF108	<i>Eremophila forrestii</i> subsp. <i>forrestii</i> F. Muell.	<i>Eriocalyx</i>	-26.66038	114.84635	WA: Butchers Track			
RMF142	<i>Eremophila forrestii</i> subsp. <i>capensis</i> Chinnock	<i>Eriocalyx</i>	-22.11	114.04	WA: Charles Knife Canyon, Exmouth	x	x	
RMF167	<i>Eremophila forrestii</i> subsp. <i>viridis</i> Chinnock	<i>Eriocalyx</i>	-21.64	115.12	Cultivated: ex E of Onslow (RD)	x	x	
MJB2455	<i>Eremophila glandulifera</i> Chinnock	<i>Eriocalyx</i>	-28.88918	121.37331	WA: Laverton - Leonora Road c4.2km east of Leonora (=4.2km east of intersection with Godlfield Hwy)	x	x	
MJB2407	<i>Eremophila punicea</i> S. Moore	<i>Eriocalyx</i>	-28.26294	117.85965	WA: Great Northern Hwy c123km by road North of Paynes Find (=23.6km by road south of Mt Magnet)	x	x	
MJB2421	<i>Eremophila latrobei</i> subsp. <i>latrobei</i> F. Muell.	<i>Eriocalyx</i>	-26.58322	118.46572	WA: near Peace Gorge, west of Meekatharra	x	x	
NG6477	<i>Eremophila latrobei</i> subsp. <i>latrobei</i> F. Muell.	<i>Eriocalyx</i>	-25.06827	120.6555	WA: In valley, ca. 4.1 km on a bearing of 297 degrees from Mt Methwin, Katjarra (Carnarvon Range), Birrilburru Indigenous Protected Area [plot C111]		x	
RMF72	<i>Eremophila latrobei</i> subsp. <i>filiformis</i> Chinnock	<i>Eriocalyx</i>	-22.78621	120.00291	WA: 71.2km North of Newman on Marble Bar road	x	x	
RMF268	<i>Eremophila latrobei</i> subsp. <i>glabra</i> (L.S. Smith) Chinnock	<i>Eriocalyx</i>			Cultivated: unknown location (AALBG)	x	x	x
MJB2482	<i>Eremophila latrobei</i> F. Muell.	<i>Eriocalyx</i>	-23.50798	133.85527	NT: Stuart Hwy 3.3km North of intersection with Tanami Rd			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2487	<i>Eremophila latrobei</i> F. Muell.	<i>Eriocalyx</i>	-22.08883	133.42733	NT: 5km North of Ti Tree, on Stuart Hwy West of Rd			
RMF165	<i>Eremophila compacta</i> subsp. <i>compacta</i> S. Moore	<i>Eriocalyx</i>			Cultivated: unknown location (RD)		x	
RMF135	<i>Eremophila compacta</i> subsp. <i>fecunda</i> Chinnock	<i>Eriocalyx</i>	-26.37	117.34	Cultivated: ex Mileura Station (RD)	x	x	
RMF101	<i>Eremophila</i> aff. <i>compacta</i> 'Kennedy Ranges' S. Moore	<i>Eriocalyx</i>	-24.66147	115.17414	WA: found on ringroad to Honeycomb gorge at Kennedy Ranges	x	x	
MJB2370	<i>Eremophila conferta</i> Chinnock	<i>Eriocalyx</i>	-24.2	116.44	Cultivated: ex Cobra (RD)	x	x	
RMF93	<i>Eremophila conferta</i> Chinnock	<i>Eriocalyx</i>	-24.32825	116.87273	WA: At Mount Augustus, on walk to Kokte Gorge	x	x	
RMF97	<i>Eremophila rhegos</i> Chinnock	<i>Eriocalyx</i>	-24.7399	116.06875	WA: 16km South of Yinnetharra homestead	x	x	
RMF77	<i>Eremophila</i> aff. <i>rhegos</i> Chinnock	<i>Eriocalyx</i>	-23.73387	119.72642	WA: c. 33km South of Capricorn roadhouse, Great Northern Hwy	x	x	
RMF130	<i>Eremophila congesta</i> Chinnock	<i>Eriocalyx</i>	-26.37	120.24	WA: Mt Alice, 30 km N of Wiluna	x	x	x
MJB2436	<i>Eremophila citrina</i> Chinnock	<i>Eriocalyx</i>	-26.20009	121.26826	WA: Lorna Glen Station on road to homestead, 5km by Road from junction with Granite Peaks Rd		x	
	<i>Eremophila pilosa</i> Chinnock	<i>Eriocalyx</i>						
RMF59	<i>Eremophila demissa</i> Chinnock	<i>Eriocalyx</i>	-25.48275	119.26962	WA: Great Northern Hwy, 24km North of Doolgunna turnoff	x	x	
MJB2416	<i>Eremophila jucunda</i> subsp. <i>jucunda</i> Chinnock	<i>Eriocalyx</i>	-27.04921	118.17319	WA: Great Northern Hwy c12.4km by road northeast of Tuckanarra	x	x	
RMF75	<i>Eremophila jucunda</i> subsp. <i>pulcherrima</i> Chinnock	<i>Eriocalyx</i>	-23.04178	118.85008	WA: Mount Robinson Gorge, 106.9km North West of Newman.	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2419	<i>Eremophila retropila</i> Chinnock	<i>Eriocalyx</i>	-26.58322	118.46572	WA: near Peace Gorge, west of Meekatharra	x	x	
MJB2425	<i>Eremophila margarethae</i> S. Moore	<i>Eriocalyx</i>	-26.49111	119.78154	WA: Meekatharra-Wiluna Rd (Goldfields Hwy), 137km by road from Meekatharra end (=48.5km by road northwest of Wiluna)	x	x	
RWG01	<i>Eremophila anomala</i> Chinnock	<i>Eriocalyx</i>	-26.27	119.74	WA: North of Paroo (west of Wiluna)	x	x	
RMF55	<i>Eremophila</i> aff. <i>anomala</i> 'Meekatharra' Chinnock	<i>Eriocalyx</i>	-25.97079	118.90061	WA: side of 'Eye Rock' c. 87km North of Meekatharra	x	x	
MJB2372	<i>Eremophila muelleriana</i> C. Gardner	<i>Eriocalyx</i>	-25.11	117.58	Cultivated: ex Mt Clere (RD)	x	x	
MJB2371	<i>Eremophila eriocalyx</i> F. Muell.	<i>Eriocalyx</i>	-27.74	115.81	Cultivated: ex Woolgorang (RD)	x	x	
RMF51	<i>Eremophila eriocalyx</i> F. Muell.	<i>Eriocalyx</i>	-29.28615	117.59457	WA: Just off Great Northern Hwy roadside (c. 10km from Paynes Find) - short walk from campsite at Ningham Station			
BB83	<i>Eremophila revoluta</i> Chinnock	<i>Eriocalyx</i>	-26.77639	125.55833	WA	x	x	
RMF68	<i>Eremophila caespitosa</i> Chinnock	<i>Eriocalyx</i>	-24.22388	119.78333	WA: c. 8km NorthEast on Gigalong-Mundiwindi road	x	x	
RMF128	<i>Eremophila obovata</i> subsp. <i>obovata</i> L.S. Smith	<i>Eriocalyx</i>			Cultivated: unknown location (RD)	x	x	
RMF127	<i>Eremophila obovata</i> subsp. <i>glabriuscula</i> (L.S. Smith) Chinnock	<i>Eriocalyx</i>			Cultivated: unknown location (RD)	x	x	
RW2416	<i>Eremophila hispida</i> Chinnock	<i>Eriocalyx</i>	-23.10181	142.83644	QLD	x	x	
RW2216	<i>Eremophila cordatisepala</i> L.S. Smith	<i>Eriocalyx</i>			Cultivated: Unknown location (RW)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2397	<i>Eremophila maitlandii</i> F. Muell. ex Benth.	<i>Eriocalyx</i>	-24.9	113.67	Cultivated: ex Dairy Creek (Station) (RD)	x	x	
RMF99	<i>Eremophila recurva</i> Chinnock	<i>Eriocalyx</i>	-25.26767	115.56365	WA: 30km West of Dairy Creek turnoff on Gascoyne Rd	x	x	
RMF141	<i>Eremophila physocalyx</i> Chinnock	<i>Eriocalyx</i>	-32.2	121.76	Cultivated: ex Norseman (RD)	x	x	
MJB2417	<i>Eremophila lachnocalyx</i> C. Gardner	<i>Eriocalyx</i>	-26.58322	118.46572	WA: near Peace Gorge, west of Meekatharra	x	x	
RMF62	<i>Eremophila lanata</i> Chinnock	<i>Eriocalyx</i>	-24.97008	119.85807	WA: 46.9km East of Great Northern Hwy towards Marymia Station	x		
RMF164	<i>Eremophila tenella</i> Chinnock	<i>Eriocalyx</i>	-21.9	122.1	Cultivated: ex Christmas Pool, S of Telfer (RD)	x	x	
BB48a	<i>Eremophila ballythunnensis</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>	-26.071	115.7495	WA: 145 km E of Northwest Coastal Hwy on Woodleigh Rd	x	x	
RMF96	<i>Eremophila yinnetharrensensis</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>	-24.71546	116.1329	WA: Found on track between Cobra and Dairy Hill, 8.5km South of Yinnetharra	x	x	
RMF92	<i>Eremophila buirchellii</i> A.P.Br.	<i>Eriocalyx</i>	-24.32963	116.86642	WA: North side of Mt Augustus on West side of Kokte Gully	x	x	
	<i>Eremophila capricornica</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>						
	<i>Eremophila pusilliflora</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>						
	<i>Eremophila regia</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>						
	<i>Eremophila resiliens</i> Buirchell & A.P.Br.	<i>Eriocalyx</i>						
MJB2448	<i>Eremophila campanulata</i> Chinnock	<i>Campanulatae</i>	-26.58209	122.80328	WA: 7.5km by road south of Prenti Downs homestead on road to cosmo Newberry	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF81	<i>Eremophila humilis</i> Chinnock	<i>Campanulatae</i>	-24.61329	118.60305	WA: 20 km East of Mt Vernon road on road to Tangadee	x	x	
RMF175	<i>Eremophila tetraptera</i> C.T. White	<i>Subscissocarpae</i>			Cultivated: unknown location (RD)	x	x	
RMF109	<i>Eremophila laanii</i> F. Muell.	<i>Amphichilus</i>	-26.83023	115.44857	WA: 93km East of NW Coastal Hwy on Butchers track	x	x	
MJB2412	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-27.27829	117.97772	WA: Great Northern Hwy c19km by road northeast of Cue, (c 1km by road south of Nallan Creek crossing)		x	
RMF225	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-34.28999	142.1859	VIC: North of Red Cliffs, side of Calder Hwy next to railway lines	x	x	
RMF241	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-31.3024	138.83626	SA: Flinders Ranges National Park, on Wilkawillina Rd towards gorge	x	x	
RMF297	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens	x	x	
NGW9739	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-35.10856	143.34767	VIC: Murray Valley Highway at Wood Wood			
RMF229	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-34.40555	141.42712	VIC: Corner Millewa Rd and Yarrara S Rd			
MJB2480	<i>Eremophila longifolia</i> (R. Br.) F. Muell.	<i>Amphichilus</i>	-23.60628	133.87946	NT: c. 11km North of Alice Springs on Stuart Hwy			
	<i>Eremophila graciliflora</i> F. Muell.	<i>Amphichilus</i>						
MJB2357	<i>Eremophila rostrata</i> subsp. <i>rostrata</i> Chinnock	<i>Amphichilus</i>	-27.42	117.89	Cultivated: ex Cue (RD)			
MJB2351	<i>Eremophila rostrata</i> subsp. <i>trifida</i> Chinnock	<i>Amphichilus</i>	-29.46	116.48	Cultivated: ex Perenjori (RD)	x	x	
RMF233	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F.	<i>Stenochilus</i>	-34.13435	141.31055	VIC: Ned's Corner Station		x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
	Muell.							
RMF24	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-32.2167	137.5167	SA: c. 40 km NNW of Port Augusta along Stuart Highway			
TMS14-08	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-34.3	142.23	VIC: Red Cliffs Scenic Reserve	x	x	
TMS14-12	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-34.78	141.63	VIC: Murray Sunset NP, underbool Rd			
TMS14-14	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-34.78	141.63	VIC: Murray Sunset NP, underbool Rd			
TMS14-15	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-34.78	141.63	VIC: Murray Sunset NP, Settlement Rd			
MJB2484	<i>Eremophila maculata</i> subsp. <i>maculata</i> (Ker Gawler) F. Muell.	<i>Stenochilus</i>	-23.3011	133.7875	NT: Stuart Hwy, North of Alice Springs	x	x	x
MJB2462	<i>Eremophila maculata</i> subsp. <i>brevifolia</i> (Benth) Chinnock	<i>Stenochilus</i>	-30.95319	121.1434	WA: beside track just west of Coolgardie cemetery, near SW corner of cemetery	x	x	
MJB2443	<i>Eremophila maculata</i> subsp. <i>brevifolia</i> (Benth) Chinnock	<i>Stenochilus</i>	-26.05096	121.94071	WA: Gunbarrel Highway, 8.6km by road northeast of turnoff to Wongawol homestead (= c1km North of crossing of Charles Wells Creek)			
RMF166	<i>Eremophila maculata</i> subsp. <i>filifolia</i> Chinnock	<i>Stenochilus</i>	-21.09	119.83	Cultivated: ex Marble Bar (RD)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF189	<i>Eremophila racemosa</i> (Endl.) F. Muell.	<i>Stenochilus</i>	-32.35143	119.74735	WA: roadside 7.2km North of crossroads, Marvel Loch road	x	x	x
RMF06	<i>Eremophila calorhabdos</i> Diels	<i>Stenochilus</i>	-33.2253	123.1211	WA: Mt Buraminya	x	x	
RMF213	<i>Eremophila calorhabdos</i> Diels	<i>Stenochilus</i>	-32.9413	120.34617	WA: roadside 100.2km West of Peak Charles turnoff on Norseman - Lake King road		x	
MJB2350	<i>Eremophila denticulata</i> subsp. <i>denticulata</i> F. Muell.	<i>Stenochilus</i>	-33.89	119.85	Cultivated: ex Fitzgerald N.P (RD)	x	x	
MJB2365	<i>Eremophila denticulata</i> subsp. <i>trisulcata</i> Chinnock	<i>Stenochilus</i>	-33.44	123.46	Cultivated: ex Mt Ragged (RD)	x	x	
RMF163	<i>Eremophila splendens</i> Chinnock	<i>Stenochilus</i>	-26.27	113.89	Cultivated: ex Shark Bay (RD)	x	x	
RMF188	<i>Eremophila biserrata</i> Chinnock	<i>Stenochilus</i>	-32.4673	119.75875	WA: roadside c. 6km South Forrestania road off Norseman - Hyden road	x	x	
RWG07	<i>Eremophila serpens</i> Chinnock	<i>Stenochilus</i>	-33.09	119.37	Cultivated: ex West of Lake King (RW)	x	x	
MJB2437	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-26.22714	121.445	WA: Lorna Glen Station on road to homestead, 24.3km by Road from junction with Granite Peaks Rd	x	x	
RMF251	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-33.03468	136.79251	SA: Eyre Hwy, between Iron Knob and Kimba	x	x	
MJB2391	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-30.96	119.11	Cultivated: ex Evaston Rd, Bullfinch WA (RD)			
RMF221	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-35.12598	142.04156	VIC: Malley Hwy, ~27km West Ouyen			
RMF222	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-35.17408	141.83281	VIC: Malley Hwy, ~49km West Ouyen on way to Linga			

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RMF223	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-35.1738	141.83304	VIC: Malley Hwy, ~49km West Ouyen on way to Linga			
RMF239	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-34.25739	139.82678	SA: Sturt Hwy, between Waikerie and Blanchetown			
RMF301	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-23.7	133.88	NT: Olive Pink Botanic Garden			
BB81	<i>Eremophila glabra</i> subsp. <i>glabra</i> (R. Br.) Ostenf.	<i>Stenochilus</i>	-27.47758	124.30667	WA	x		x
RMF215	<i>Eremophila glabra</i> subsp. <i>albicans</i> (Bartling) Chinnock	<i>Stenochilus</i>	-33.15895	120.09927	WA: 11km South SouthEast of Lake King - Norseman road along vermin fence road. 32km East of Lake King		x	
RMF154	<i>Eremophila glabra</i> subsp. <i>albicans</i> (Bartling) Chinnock	<i>Stenochilus</i>			Cultivated: unknown location (RD)			
RWG05	<i>Eremophila glabra</i> subsp. <i>psammophora</i> Chinnock	<i>Stenochilus</i>	-26.14	113.16	Cultivated: ex West of Steep Point (RW)	x	x	
RMF153	<i>Eremophila glabra</i> subsp. <i>carnosa</i> Chinnock	<i>Stenochilus</i>	-28.19	114.25	Cultivated: ex Port Gregory (RD)	x	x	
RMF122	<i>Eremophila glabra</i> subsp. <i>chlorella</i> (Gandoger) Chinnock	<i>Stenochilus</i>	-32.02	115.92	Cultivated: ex Cannington (RD)	x	x	
RWG04	<i>Eremophila glabra</i> subsp. <i>murrayana</i> Chinnock	<i>Stenochilus</i>	-34.79	142.06	Cultivated: ex 50km West of Hattah (RW)	x	x	
RWG03	<i>Eremophila glabra</i> subsp. <i>tomentosa</i> Chinnock	<i>Stenochilus</i>	-27.82	114.14	Cultivated: ex South of Kalbarri (RW)	x	x	
MJB2377	<i>Eremophila glabra</i> subsp. <i>elegans</i> Chinnock	<i>Stenochilus</i>	-29.23	116.07	Cultivated: ex Morawa Lake Jones Rd (RD)			
RMF287	<i>Eremophila glabra</i> subsp. <i>elegans</i> Chinnock	<i>Stenochilus</i>			Cultivated: unknown location (BC)			
RMF151	<i>Eremophila glabra</i> subsp. <i>verrucosa</i> Chinnock	<i>Stenochilus</i>	-29.42	121.89	Cultivated: ex Lake Raeside W of Menzies (RD)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF53	<i>Eremophila glabra</i> 'Paynes Find' (R. Br.) Ostenf.	<i>Stenochilus</i>	-29.2907	117.59231	WA: Just off Great Northern Hwy roadside (c. 10km from Paynes Find) - 50m down track from campsite at Ningham Station	x	x	
RMF184	<i>Eremophila glabra</i> 'York' (R. Br.) Ostenf.	<i>Stenochilus</i>	-31.94689	116.97558	WA: Green Hills church, East of York on York-Quairading road	x	x	
RW	<i>Eremophila glabra</i> 'Coral Bay' (R. Br.) Ostenf.	<i>Stenochilus</i>	-23.14178	113.77192	WA	x	x	x
MJB2456	<i>Eremophila decipiens</i> subsp. <i>decipiens</i> Ostenf.	<i>Stenochilus</i>	-29.727	120.82164	WA: Evanston-Menzies Road c 20.5km by road WSW of Goldfields Highway (menzies)	x	x	
RMF190	<i>Eremophila decipiens</i> subsp. <i>decipiens</i> Ostenf.	<i>Stenochilus</i>	-32.34968	119.74717	WA: roadside c. 200m from RMF189 (7.2km North of crossroads, Marvel Loch road)	x	x	
PH02	<i>Eremophila decipiens</i> subsp. <i>linearifolia</i> (S. Moore) Chinnock	<i>Stenochilus</i>			Cultivated: unknown location (PH)	x	x	
MJB2356	<i>Eremophila subteretifolia</i> Chinnock	<i>Stenochilus</i>	-32.82	119.52	Cultivated: ex Lake Barley (RD)	x	x	
RMF04	<i>Eremophila veneta</i> Chinnock	<i>Stenochilus</i>			Cultivated: unknown location (ANBG)	x	x	
MJB2369	<i>Eremophila hillii</i> E.A. Shaw	<i>Stenochilus</i>			Cultivated: unknown location (RD)	x	x	
BB07.5	<i>Eremophila hillii</i> E.A. Shaw	<i>Stenochilus</i>	-31.31736	125.42597	WA: 39 km S of Rawlina on way to Cocklebidy	x	x	
BB08	<i>Eremophila hillii</i> E.A. Shaw	<i>Stenochilus</i>	-31.02214	125.32358	WA: 1.9 km S of Transline road near Rawlina on road to Cocklebidy	x	x	
RMF03	<i>Eremophila subfloccosa</i> subsp. <i>subfloccosa</i> Benth.	<i>Stenochilus</i>	-31.7794	117.4839	WA: Charles Gardner Nature Reserve, S of Tammin.	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF205	<i>Eremophila subfloccosa</i> subsp. <i>glandulosa</i> Chinnock	<i>Stenochilus</i>	-33.24094	123.22465	WA: 6.1km on road to mine off Parmango road		x	
RMF253	<i>Eremophila subfloccosa</i> subsp. <i>lanata</i> Chinnock	<i>Stenochilus</i>	-33.2052	136.0592	SA: 43.3km West of Kimba on Eyre Hwy, towards Kyancutta	x	x	x
RMF140	<i>Eremophila calcicola</i> R.W. Davis	<i>Stenochilus</i>	-33.699	122.6	WA: Parmango Rd east of Condinup	x	x	
RMF196	<i>Eremophila gibbosa</i> Chinnock	<i>Virides</i>	-32.02489	120.73406	WA: Found at the base of McDermid Rock, left of entrance walkway.	x	x	
MJB2359	<i>Eremophila virens</i> C. Gardner	<i>Virides</i>	-30.92	118.23	Cultivated: ex Muckinbudin (RD)	x	x	
RMF146	<i>Eremophila undulata</i> Chinnock	<i>Virides</i>			Cultivated: unknown location (RD)	x	x	
MJB2404	<i>Eremophila serrulata</i> (Cunn. ex A. DC.) Druce	<i>Virides</i>	-28.94095	117.82689	WA: Great Northern Hwy 41.45km by road southwest of Paynes Fnd	x	x	
RMF261	<i>Eremophila serrulata</i> (Cunn. ex A. DC.) Druce	<i>Virides</i>			Cultivated: unknown location (AALBG)	x	x	
RMF157	<i>Eremophila ferricola</i> Buirchell & A.P.Br.	<i>Virides</i>	-28.12	115.61	Cultivated: ex Tallering Peak N of Mullewa (RD)			
MJB2434	<i>Eremophila alternifolia</i> R.Br.	<i>Scariosepalae</i>	-26.14361	121.2885	WA: Lorna Glen Station near intersection of Lorna Glen Road and Wiluna-Granite Peak Road (called Granite Peak-Lake Violet Rd on some maps)	x	x	
RMF247	<i>Eremophila alternifolia</i> R.Br.	<i>Scariosepalae</i>	-31.34098	138.56705	SA: Flinders Ranges National Park, on Brachina Gorge Rd between Brachina East and Teamsters campgrounds	x	x	
RMF282	<i>Eremophila alternifolia</i> 'Broadleaf' R. Br.	<i>Scariosepalae</i>			Cultivated: unknown location (ABG)			

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RMF202	<i>Eremophila alternifolia</i> 'Broadleaf' R. Br.	<i>Scariosepalae</i>	-32.02849	122.86559	WA: 109km East of Norseman	x	x	
RMF168	<i>Eremophila purpurascens</i> Chinnock	<i>Scariosepalae</i>	-32.2	121.68	Cultivated: ex Norseman (RD)	x	x	
MJB2451	<i>Eremophila abietina</i> subsp. <i>abietina</i> Kraenzlin	<i>Pulchrisepalae</i>	-28.48833	122.42218	WA: Bandy Road 5.6km north of intersection with Great Central Road	x	x	
MJB2366	<i>Eremophila abietina</i> subsp. <i>ciliata</i> Chinnock	<i>Pulchrisepalae</i>	-28.76	124.33	Cultivated: ex Lake Rason (RD)	x	x	
RMF70	<i>Eremophila fraserii</i> subsp. <i>fraserii</i> F. Muell.	<i>Pulchrisepalae</i>	-24.26112	119.71781	WA: South of Capricorn	x	x	
RMF88	<i>Eremophila fraserii</i> subsp. <i>fraserii</i> F. Muell.	<i>Pulchrisepalae</i>	-25.11716	118.28693	WA: Arpilles Hills, South West of Milgen Station		x	
MJB2408	<i>Eremophila fraserii</i> subsp. <i>fraserii</i> F. Muell.	<i>Pulchrisepalae</i>	-28.18623	117.87362	WA: Great Northern Hwy c132km by road North of Paynes Find (=14.6km by road south of Mt Magnet)			
RMF90	<i>Eremophila fraserii</i> subsp. <i>parva</i> Chinnock	<i>Pulchrisepalae</i>	-25.17587	117.68446	WA: Mount Clere Rd, Meekatharra	x	x	
MJB2447	<i>Eremophila galeata</i> Chinnock	<i>Pulchrisepalae</i>	-26.5822	123.05959	WA: Carnegie - Prenti Downs Road c 22.2km by road southwest of Wharton Bore		x	
RMF58	<i>Eremophila galeata</i> Chinnock	<i>Pulchrisepalae</i>	-25.97263	118.89314	WA: located c. 3km along track from Eye Rock		x	
MJB2445	<i>Eremophila ramiflora</i> Dell	<i>Pulchrisepalae</i>	-25.85671	122.5485	WA: between Gunbarrel Highway (Carnegie Road) and Nooloo Breakway		x	
RMF67	<i>Eremophila flaccida</i> subsp. <i>flaccida</i> Chinnock	<i>Pulchrisepalae</i>	-24.26702	119.71123	WA: c. 24km North East on Gialong-Mundiwindi road	x	x	x
RMF161	<i>Eremophila flaccida</i> subsp. <i>attenuata</i> Chinnock	<i>Pulchrisepalae</i>	-25.18	115.49	WA: East of Gascoyne Junction	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
MJB2446	<i>Eremophila miniata</i> Gardner	<i>Pulchrisepalae</i>	-26.53575	123.18797	WA: Carnegie - Prenti Downs Road near southeast corner of Lake Carnegie, c6.3km by road SSE of Wharton Bore	x	x	
MJB2388	<i>Eremophila miniata</i> Gardner	<i>Pulchrisepalae</i>	-30.46	117.5	Cultivated: ex Mollerin (RD)	x	x	
RMF129	<i>Eremophila neglecta</i> J. Black	<i>Pulchrisepalae</i>			Cultivated: unknown location (RD)	x	x	
RMF296	<i>Eremophila neglecta</i> J. Black	<i>Pulchrisepalae</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens			
MJB2360	<i>Eremophila viscida</i> Endl.	<i>Pulchrisepalae</i>	-30.7	116.87	Cultivated: ex Kundot (RD)	x	x	x
RMF292	<i>Eremophila lucida</i> Chinnock	<i>Pulchrisepalae</i>	-32.27932	120.26237	NT: Olive Pink Botanic Garden	x	x	
MJB2376	<i>Eremophila lucida</i> Chinnock	<i>Pulchrisepalae</i>	-32.38	119.77	Cultivated: ex East of Lake Cronin (RD)			
MJB2395	<i>Eremophila oldfieldii</i> subsp. <i>oldfieldii</i> F. Muell.	<i>Pulchrisepalae</i>	-30.29	116.68	Cultivated: ex Dalwallinu (RD)	x	x	
MJB2453	<i>Eremophila oldfieldii</i> subsp. <i>angustifolia</i> (S. Moore) Chinnock	<i>Pulchrisepalae</i>	-28.7434	122.02129	WA: Laverton - Leonora Road c31km SW of intersection with road to Windarra	x	x	
MJB2420	<i>Eremophila linearis</i> Chinnock	<i>Pulchrisepalae</i>	-26.58322	118.46572	WA: near Peace Gorge, west of Meekatharra	x	x	
MJB2483	<i>Eremophila duttonii</i> F. Muell.	<i>Pulchrisepalae</i>	-23.30118	133.78698	NT: c. 50km North of Alice Springs on West side of Stuart Hwy	x	x	x
RMF136	<i>Eremophila duttonii</i> F. Muell.	<i>Pulchrisepalae</i>			Cultivated: unknown location (RD)		x	
RMF250	<i>Eremophila duttonii</i> F. Muell.	<i>Pulchrisepalae</i>	-31.3384	138.52936	SA: Flinders Ranges National Park, found at Western entrance on Brachina Gorge Rd (opposite sign)		x	
BB01	<i>Eremophila duttonii</i> F. Muell.	<i>Pulchrisepalae</i>	-31.8245	139.46397	SA: 92.2 km N of Yunta towards Arkaroola			

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF294	<i>Eremophila duttonii</i> F. Muell.	<i>Pulchrisepalae</i>	-23.7	133.88	NT: Olive Pink Botanic Gardens			
MJB2374	<i>Eremophila grandiflora</i> A.P. Br & Buirchell	<i>Pulchrisepalae</i>	-29.09	117.19	Cultivated: ex Warriear (RD)		x	
RMF116	<i>Eremophila</i> sp. 'Tropicana'	(unknown)	-29.56	124.375	Cultivated: (RD)	x	x	
RMF117	<i>Eremophila</i> sp. 'Rock'	(unknown)			Cultivated: unknown location (RD)	x	x	
RMF49	<i>Eremophila</i> sp. 'Wubin North'	(unknown)	-30.09924	116.62247	WA: 1km North of Wubin, off road to cemetery.	x	x	
RMF89	<i>Eremophila</i> sp. 'Yarlarweelor'	(unknown)	-25.295	118.06142	WA: 33km West on Milgen - Yarlarweelor rd from Milgen	x	x	
BB50	<i>Eremophila</i> sp. 'Murgoo'	(unknown)	-26.73422	115.8965	WA: 66 km S of Woodleigh Rd turnoff towards Murchison Settlement	x	x	
BB55	<i>Eremophila pensilis</i> MS	(unknown)	-25.49556	122.65833	WA: 31.5 km E of Glenayle-Carnegie Rd , 1.5 km east of Tungarra Tank	x	x	
RMF82	<i>Eremophila</i> sp.	(unknown)	-24.61331	118.60313	WA: 20 km East of Mt Vernon road on road to Tangadee		x	
RMF31	<i>Myoporum montanum</i> R. Br.		-27.615	152.9069	QLD: Woogaroo Creek, Goodna.	x	x	
	<i>Myoporum tenuifolium</i> Forster f.							
RMF46	<i>Myoporum boninense</i> subsp. <i>australe</i> Chinnock		-35.70108	150.19981	NSW: Batemans Bay region, Cullendulla Creek Nature Reserve, access from parking lot North end of Myamba Parade	x	x	
RMF281	<i>Myoporum insulare</i> R. Br.		-33.15676	138.06808	SA: North of Port Pirie on side of Augusta (Wakefield) Hwy - almost directly opposite BP petrol station	x	x	
	<i>Myoporum crassifolium</i>							

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	Forster f.							
RMF45	<i>Myoporum acuminatum</i> R. Br.		-35.70231	150.18958	NSW: Batemans Bay region, Mundarra Way, roadside before Batemans Bay Public School	x	x	
MO2	<i>Myoporum obscurum</i> Endl.		-29.01497	167.9462	Norfolk Island	x	x	
MC	<i>Myoporum laetum</i> Forster f.		-40.385	175.617	NZ: Massey University Campus, Palmerston North	x	x	
WP	<i>Myoporum laetum</i> Forster f.		-40.382	175.595	NZ: Waitoetoe Park, Palmerston North	x	x	
RMF29	<i>Myoporum caprarioides</i> Benth.		-34.6833	117.95	WA: Stirling district; 4 km SE of Porongorup.	x	x	
	<i>Myoporum oppositifolium</i> R. Br.							
	<i>Myoporum viscosum</i> R. Br.							
RMF32	<i>Myoporum petiolatum</i> Chinnock		-38.4167	144.1333	VIC: Between Anglesea and Urquharts Bluff, Great Ocean Rd. (near Hutt Gully, 71-1/2 miles [115.5 km] from Melbourne).	x	x	
RMF280	<i>Myoporum petiolatum</i> Chinnock		-32.82301	138.18399	SA: Mt Remarkable National Park, walking track to Cathedral Rock	x	x	
RMF209	<i>Myoporum tetrandrum</i> (Labill.) Domin.		-33.79922	121.96305	WA: East side of Mullet Lake Reserve, Merrivale road towards Esperance	x	x	
	<i>Myoporum velutinum</i> Chinnock							
RMF38	<i>Myoporum betcheanum</i> subsp. <i>betcheanum</i> L. Smith		-28.05	152.3833	QLD: QMO/QDD: Great Dividing Range, Mt Cordeaux, c. 1.5 km along track to summit	x	x	x
RMF25	<i>Myoporum parvifolium</i> R. Br.		-33.3667	136.6333	SA: 78 miles [126 km] from Port Lincoln towards Cowell	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF260	<i>Myoporum brevipes</i> Benth.		-33.65805	136.98543	SA: North East of Cowell towards Lucky Bay, on side of sandy roadside pit	x	x	
	<i>Myoporum mauritianum</i> A. DC.							
MeyPap	<i>Myoporum wilderi</i> Skottsb.		21.461	-157.991	Hawaii	x	x	
	<i>Myoporum rapense</i> F. Brown							
Mey3093	<i>Myoporum stokesii</i> F. Brown		21.461	-157.991	Hawaii	x	x	
	<i>Myoporum rimatarense</i> F. Brown							
NTBG061301	<i>Myoporum sandwicense</i> subsp. <i>sandwicense</i> A. Gray		21.461	-157.991	Hawaii	x	x	
NTBG070621	<i>Myoporum sandwicense</i> subsp. <i>sandwicense</i> A. Gray		21.461	-157.991	Hawaii	x	x	
Gal120	<i>Myoporum sandwicense</i> subsp. <i>sandwicense</i> A. Gray		21.461	-157.991	Hawaii	x	x	
Gal118	<i>Myoporum sandwicense</i> subsp. <i>lanaiense</i> (Webster) Chinnock		21.461	-157.991	Hawaii	x	x	x
NagGan	<i>Myoporum sandwicense</i> subsp. <i>lanaiense</i> (Webster) Chinnock		21.461	-157.991	Hawaii			
GalBou110	<i>Myoporum sandwicense</i> subsp. ' <i>Papa</i> ' A. Gray		21.461	-157.991	Hawaii	x	x	
Nag2344	<i>Myoporum sandwicense</i> subsp. ' <i>St John</i> ' A. Gray		21.461	-157.991	Hawaii			
GalBou109	<i>Myoporum sandwicense</i>		21.461	-157.991	Hawaii	x	x	
Nag1869	<i>Myoporum sandwicense</i>		21.461	-157.991	Hawaii			
Hulu	<i>Myoporum degeneri</i> (Webster) Degener & I.		21.461	-157.991	Hawaii	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
	Degener							
	<i>Myoporum stellatum</i> (Webster) Degener & I. Degener							
RMF42	<i>Myoporum bateae</i> F. Muell.		-35.77214	150.169	NSW: Batemans Bay region, Mogo Trig Rd c. 2km from Princes Hwy	x	x	
RMF291	<i>Myoporum floribundum</i> A. Cunn. ex Benth.		-37.08	148.41	Cultivated: (RBGV)	x	x	
RMF35	<i>Myoporum platycarpum</i> subsp. <i>platycarpum</i> R. Br.		-33.1833	137.0333	SA: 60.7 km from Kimba toward Whyalla.	x	x	
	<i>Myoporum turbinatum</i> Chinnock							
RWG16	<i>Myoporum cordifolium</i> (F. Muell.) Druce				Cultivated: unknown location (RW)	x	x	
Mey2053	<i>Myoporum</i> sp.				Unknown location	x	x	
RMF214	<i>Glycocystis beckeri</i> (F. Muell.) Chinnock		-32.95107	120.32957	WA: roadside 102km West of Peak Charles turnoff on Norseman - Lake King road	x	x	
FTBG64271	<i>Bontia daphnoides</i> L.		25.67469	- 80.276301	Cultivated: Fairchild Tropical Botanic Garden (Florida)	x	x	
MSTANG	<i>Pentacoelium bontioides</i> Sieb. & Zucc.		22.91693	120.20098	Kaohsiung City, Taiwan, Hunei District	x	x	x
RMF220	<i>Calamphoreus inflatus</i> (C. Gardner) Chinnock		-33.1	119.66	Cultivated: WA, Kings Park	x	x	
RMF14	<i>Diocirea acutifolia</i> Chinnock		-31.8	121.7	WA: 48km from Norseman along Eyre Highway towards Coolgardie	x	x	
MJB2383	<i>Diocirea microphylla</i> Chinnock		-31.01	120.9	Cultivated: ex Bullabulling (RD)	x	x	

Sample Number	Taxon	Section	Latitude	Longitude	Location	cpDNA study	nrDNA study	mtDNA study
RMF111	<i>Diocirea ternata</i> Chinnock		-31.68	123.16	Cultivated: 76 km east of Karoone Dam on Transline (RD)	x	x	
RMF131	<i>Diocirea violacea</i> Chinnock				Cultivated: unknown location (RD)	x	x	x
RMF210	<i>Diocirea violacea</i> Chinnock		-32.93161	121.61952	WA: Just out of Salmon Gums between road and rail line. 5.8km North Salmon Gums on road between Esperance and Norseman		x	
RMF275	<i>Leucophyllum frutescens</i> I.M. Johnst				Cultivated: unknown location (PH)	x	x	
RMF288	<i>Leucophyllum frutescens</i> I.M. Johnst				Cultivated: unknown location (BC)	x	x	x
RMF286	<i>Leucophyllum zygophyllum</i> I.M. Johnst				Cultivated: unknown location (BC)	x	x	