Systematics and biogeography of the Styphelieae (Epacridoideae, Ericaceae)

Thesis submitted by

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STATEMENT ON THE CONTRIBUTION OF OTHERS

The chapters of this thesis are also manuscripts that have been published, submitted or are in preparation for submission. Several researchers have made contributions to these manuscripts as follow:

Chapter 2: Two articles have been produced from this chapter, 1) Hislop M., **Puente-Lelièvre, C.** and Crayn D.M. (2012). *Leucopogon extremus* (Styphelieae, Styphelioideae*, Ericaceae), a remarkable new species that expands the morphological circumscription of *Leucopogon sens. str*. *Australian Systematic Botany* 25, 202–209; and 2) Solving the puzzle: multigene phylogeny of the *Styphelia-Astroloma* clade (Styphelieae, Epacridoideae, Ericaceae), which is ready for submission. For this chapter M. Hislop provided plant tissue samples, morphological data, and assistance during the filed trips; E.A. Brown provided assistance during the field trips and morphological data; M. Harrington assisted in the data analyses, D. M. Crayn contributed with morphological data and discussion, and M. Kuzmina generated part of the data.

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Some of the Scanning Electron Microscopy images presented in Chapter 4 were supplied by C. Quinn and A. Wilson.

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* Epacridoideae

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GENERAL ABSTRACT

Considerable work has been undertaken over the last two decades toward a phylogenetic classification of the epacrids (Epacridoideae, Ericaceae). Generic level relationships have been resolved in all major clades, except for the *Styphelia-Astroloma* clade (tribe Styphelieae). With the aim of providing a foundation for a forthcoming generic revision of this clade, phylogenetic relationships and historical biogeographic patterns were investigated using parsimony, maximum likelihood, Bayesian inference and Bayesian relaxed-clock analyses of four chloroplast DNA markers (*rbc*L, *mat*K, *trn*H-*psb*A, *atp*B-*rbc*L) and the nuclear-encoded ribosomal Internal Transcribed Spacer (ITS). With the aim of identifying new morphological synapomorphies to support the phylogenetic groups, a representative pollen survey within the Styphelieae, broadly sampling the *Styphelia-Astroloma* clade, was carried out to document the diversity of pollen types and morphology. These characters were optimised on the estimated molecular phylogeny to investigate their evolutionary patterns in the clade. Finally, to better understand the genetic variation at shallow phylogenetic branches and the possible factors driving diversification in the *Styphelia-Astroloma* clade, genetic structure in the *Leucopogon conostephioides* species complex was investigated using NeighborNet, Bayesian clustering, and Neighbor joining and parsimony phylogenetic analyses of Amplified Fragment Length Polymorphism (AFLP) data.

The monophyly of the *Styphelia-Astroloma* clade is strongly supported. Within this clade twelve well-supported lineages were resolved: Group I (*Astroloma sensu stricto (s.s.),* Group II and III (*Styphelia sensu lato (s.l.)*), Group IV (*Leucopogon rotundifolius* + *L. cuneifolius*), Group V (*Leucopogon s.l. pro parte (p. p.)*), Group VI (*Styphelia s. s.*), Group VII (*Leucopogon s.l. p. p.*), Group VIII (*Leucopogon conostephioides* complex), Group IX ('*Stomarrhena'*), Group X (*Leucopogon s.l. p. p.*), Group XI (*Leucopogon blepharolepis* + *L.* sp. Moore River), and Group XII (New Caledonian *Styphelia s. l.*). On the basis of these results, the genus *Stenanthera* is reinstated, and *Astroloma baxteri* A.. ex DC. and *Leucopogon melaleucoides* A.Cunn. ex DC. are transferred to the genera *Brachyloma* Sond. and Cunn *Acrothamnus* Quinn respectively.

The improved resolution of the phylogenetic relationships within Styphelieae provided the background for historical biogeographical studies. The origins and evolutionary relationships of the New Zealand Styphelieae were investigated because they epitomise the controversies on the biogeographic history of the New Zealand biota. *Cyathodophyllum novaezelandieae* (early Miocene, 20-23 million years (Ma)) constitutes evidence for the antiquity of the Styphelieae in New Zealand. Yet the extant species in the tribe are thought to be very closely related to or conspecific with Australian taxa, which suggests recent trans-Tasman origins. The results of parsimony, maximum likelihood and Bayesian phylogenetic analyses indicate that the sister taxa for each of the extant species of New Zealand Styphelieae is from Tasmania or the east coast of mainland Australia; except for *Acrothamnus colensoi* for which its sister is from New Guinea.

Bayesian relaxed-clock analyses using direct and secondary fossil calibration methods suggest that all of the New Zealand lineages diverged from their non-New Zealand sisters within the last 7 Ma. Time discontinuity between *C. novae-zelandiae* and the origins of the extant New Zealand lineages indicates that the fossil and extant Styphelieae in New Zealand are not related. The relative dating analysis showed that to accept this relationship, it would be necessary to accept that the Styphelieae arose in the early-mid Mesozoic (210-120 Ma), which contradicts multiple lines of evidence on the age of angiosperms. Therefore, the results do not support the hypothesis that Styphelieae have been continuously present in New Zealand since the early Miocene. Instead they suggest a historical biogeographical scenario in which the lineage to which *C. novae-zelandiae* belongs became extinct in New Zealand, and the extant New Zealand Styphelieae are derived from Australian lineages that recolonised (presumably by long distance dispersal) no earlier than the late Miocene to Pliocene.

Three different types of pollen were found in the representative pollen survey: 1) pseudomonads, present in all the species sampled within the *Styphelia-Astroloma* clade as well as in *Monotoca, Oligarrhena* and *Leucopogon s.s.*; 2) A-type (permanent tetras with variable sterility), observed in *Acrothamnus, Acrotriche, Conostephium, Leptecophylla, Pentachondra involucrata, Stenanthera* and *Needhamiella pumilo;* and 3) T-type (regular tetrads), present in *Brachyloma, Lissanthe,* and *Pentacondra pumila*. Pollen type records for the tribes Epacrideae, Cosmelieae, Prionoteae and Richeeae consist of regular tetrads. True regular monads were not recorded in Styphelieae.

Pseudomonads are universally distributed in the *Styphelia-Astroloma* clade.The taxonomic utility of pollen type in the clade is therefore limited. Conversely, pollen morphological characters such as exine ornamentation, number of pores and size of the mature tetrads show a variation that is consistent with the phylogenetic groups and seem promising to support a genus-level phylogenetic classification of the *Styphelia-Astroloma* clade. Moreover, these characters are potentially useful for a more accurate identification of pollen fossils in the Epacridoideae.

The phylogenetic analyses heightened further questions about the taxonomic significance of the morphological and the genetic diversity within the phylogenetic groups (I - XII). One of the groups that required additional examination was the *Leucopogon conostephioides* complex (group VIII).

Leucopogon conostephioides is a broadly circumscribed species with a wide distribution in Western Australia. The pattern of variation in the *L. conostephioides* complex is more consistent with the presence of several currently unrecognised, segregate taxa rather than with a single, highly variable species. NeighbourNet, Bayesian clustering and Neighbor joining and parsimony phylogenetic analyses of AFLP data from 52 individuals revealed four distinctive genetic groups that correspond to the four putative taxa sampled (*Leucopogon conostephioides, L.* sp. 'short style', *L.* sp. 'Biffid Eneabba' and *L.* sp. 'Cockleshell Gully'). Hence, the genetic differentiation is congruent with the morphological variation observed in the species

complex. While some individuals presented genetic admixture, the lack of morphological intermediates and of individuals appearing at the intersection of two splits on the NeighbourNet analysis suggest that this is due to retention of ancestral genetic elements rather than ongoing gene flow between populations. Potential reproductive barriers contributing to the genetic isolation are modifications in floral morphology, disparities in flowering times and edaphic isolation as a consequence of different soil type preferences. Both morphology and genetic structure within the *L. conostephioides* complex indicate that these groups are evolutionarily distinct and they should be recognised as different taxa.

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Introductory comments on thesis structure

Each of the data chapters in this thesis has been written as a stand-alone manuscript prepared to conform to the editorial and scientific standards expected of submissions to international peer-reviewed journals. As such, each chapter contains a full introduction relevant to the study, methods and results sections, a full discussion of the significance of the results, and conclusions. Chapter 1 provides an overall introduction to the dissertation, and contains material of a general nature not covered in the individual chapter introductions, as well as the thesis outline and rationale. Chapter 6 is a summary of the research outcomes and how they contributed to the general aim of this thesis.

Chapter 1 General Introduction

1.1 Systematics and biology of the tribe Styphelieae (Epacridoideae, Ericaceae)

The Styphelieae (fleshy-fruited epacrids) is the largest of the seven tribes within the subfamily Epacridoideae Link, Ericaceae Juss. and comprises ~350 species in 22 genera (Kron *et al.* 2002). It is the most widely distributed of the tribes, occurring in Australia, New Zealand, New Caledonia and New Guinea, with outliers in Hawaii and other Pacific islands. Styphelieae are woody plants that range in size and habit from prostrate shrubs to small trees. Their habitat varies from heathlands and sandplains to montane forests. Australia is their centre of diversity where they frequently represent an important component of the native flora.

The extant Styphelieae are diverse and distinctive. Yet fossils are uncommon and their morphological affinities with the extant taxa are often difficult to identify. Besides deposits from the Late Oligocene-Early Miocene found in the South Island of New Zealand (Jordan *et al.* 2010), the Styphelieae fossil record is restricted to south-eastern mainland Australia and Tasmania, and suggests that the tribe had diversified in the Oligocene-Early Miocene and that they had radiated substantially by the beginning of the Pleistocene (ca. 2.6-0.01 million years ago (Ma) (Jordan and Hill 1995, 1996; Jordan *et al.* 2007).

The taxonomic history of the Styphelieae is convoluted. Generic circumscription has been problematic since Robert Brown (1810) first described the family Epacridaceae. Brown recognized 134 species in 24 genera and established two 'sections' (or subfamilies) based on fruit type: Section I – fruit indehiscent-drupe, ovules one per locule; and Section II – fruit a capsule, ovules several per locule. Bartling (1830) formalised Brown's section I as the tribe Styphelieae. Once formalised, the generic concepts within the tribe established by Brown and maintained by Bentham (1868), were adopted by most subsequent authors. These were largely based on variations in floral characters. Conversely, Mueller (1867; 1889) adopted much broader generic concepts and included all the fleshy-fruited genera (*Acrotriche* R.Br., *Astroloma* R.Br., *Cyathodes* Labill., *Cyathopsis* Brongn. and Gris, *Leucopogon* R.Br, *Monotoca* R.Br. and *Pentachondra* R.Br.) in *Styphelia* Sm. (sensu Mueller) His broad concept has been adopted in Malesia (Sleumer 1964) and New Caledonia (Virot 1975), but not in Australia and New Zealand. A more detailed taxonomic history of the subfamily Epacridoideae and the tribe Styphelieae is presented in Powell *et al.* (1997) and Quinn *et al.* (2003).

Cladistic analyses have shown that many of the floral characters upon which these genera are based

are highly homoplasic and fail to accurately delimit the genera (Powell *et al.*1997; Taaffe *et al.* 2001). It is therefore necessary to explore new morphological attributes and assess their potential utility in a phylogenetic framework. New synapomorphies would provide a strong basis for a classification that comprises informative and predictable generic concepts that more accurately describe the morphological diversity and the pattern of well-supported phylogenetic relationships within the clade.

Given that the classification in Styphelieae has relied on characters that fail to clearly delimit genera and is not consistent with the phylogenetic relationships of the taxa, several parsimony analyses of morphological and plastid DNA sequences data have been undertaken with the aim of providing a phylogenetic framework to establish monophyletic genera. These have resulted in phylogenetically-based re-circumscriptions for a number of existing genera and the description of new genera to accommodate novel groups.

Yet, non-monophyletic genera persist in the tribe (Johnson *et al.*2012). These are concentrated in the *Styphelia-Astroloma* clade, a very well supported clade that contains elements currently assigned to *Leucopogon, Styphelia, Astroloma, Croninia* J.M. Powell and *Coleanthera* Stschegl. The poor resolution of relationships inside this clade and the incongruence between the phylogenetic relationships and the morphological patterns observed remain the major barriers to the completion of a phylogenetic classification of Styphelieae. A taxonomy that accurately represents the evolutionary history and the morphological diversity of the tribe is needed to underpin studies on their biology, and for more effective management and conservation strategies of the numerous endangered species in the tribe (e.g. http://florabase.dec.wa.gov.au).

Classification conflicts at the species level also exist in the *Styphelia-Astroloma* clade and pose no less taxonomic turmoil. Investigation of the genetic divergence at population level is required for discerning the taxonomic status of a number of taxa and for gaining a better understanding of the potential ecological and environmental factors driving diversification and the broad patterns of phylogenetic diversity in the *Styphelia-Astroloma* clade.

Previous studies (C. Quinn and A. J. Wilson pers. comm.; C. Puente-Lelièvre unpubl.; Powell *et al.* 1997) indicate that pollen attributes are a promising source of new potential synapomorphies to underpin a phylogenetic classification of the Styphelieae. Unlike the other Ericaceae, Styphelieae show a high diversity in pollen morphology and pollen type. Preliminary surveys (C. Quinn and A. J. Wilson pers. comm.; C. Puente-Lelièvre unpubl.) indicate that the Styphelieae pollen is morphologically very diverse (e.g. in exine ornamentation, nature of apertures, grain size) and that this diversity is generally congruent with the phylogenetic relationships within the tribe.

As in almost all the Ericaceae, the pollen grains in Styphelieae are shed in tetrads (Kron *et al.* 2002). The Styphelieae tetrads however, frequently undergo progressive abortion of the microspores to produce three different pollen types: 1) T-type, regular tetrads, 2) A-type, permanent tetrads comprised of four or fewer functional microspores, and 3) pseudomonads, permanent tetrads with a single fully developed and functional microspore and three aborted ones (Furness 2009; Lemson 2011; Smith-White 1955). Although the ontogeny of the different pollen types has been well studied and shows consistency with the phylogenetic relationships, the pattern of their evolution in the tribe remains unclear. In this dissertation the taxonomic utility and phylogenetic signal of the variation observed in pollen morphology and pollen types within the Styphelieae is assessed.

1.2 Systematics and molecular phylogenetics

Systematics is the study of the biological diversity and of the evolutionary relationships among organisms. Within systematics, taxonomy is a subset that addresses the theory and practise of describing, naming and classifying organisms while phylogenetics investigates the evolutionary relationships. A classification system founded on the criterion of common ancestry and evolutionary relationships is called a phylogenetic classification. The aim of incorporating phylogenetic relationships into a classification is to establish a logical and predictable system that provides biologically meaningful units of classification.

The analysis of DNA sequence data to estimate phylogenies and investigate evolutionary patterns and processes has greatly influenced the field of plant systematics and has been applied at all levels of the taxonomic hierarchy. DNA sequence data are currently the most commonly utilised data source for generating phylogenetic hypotheses for the following reasons: DNA provides a unifying framework for estimating phylogenies because it comprises a set of characters that are common to all organisms; DNA represents a vast pool of potentially phylogenetically informative genotypic characters that can be described by statistical models and which are for the most part independent of the phenotypic characters commonly used in phylogenetic analyses.

Molecular data for phylogenetic studies in plants are frequently obtained from chloroplast and nuclear DNA. Chloroplast DNA (cpDNA) is the most extensively used source of data in plant systematics. Among its advantages are its simple genetics (haploid, non-recombinant and usually maternally inherited in angiosperms) and structural stability within cells and within species. Nevertheless, its utility below genus level is restricted given that its intraspecific rate of variation is relatively low in comparison with nuclear markers. As cpDNA is uniparentally transmitted, it only reveals phylogenetic relationships from the maternal side and thus hybridization events cannot be detected.

The nuclear genome, on the other hand, is biparentally inherited and supplies an additional and independent source of data from cpDNA to estimate phylogenies. The nuclear-encoded ribosomal internal transcribed spacer (ITS) is one of the most widely used nuclear markers in plant phylogenetics, in particular at genus level and below as it usually provides a greater number of informative characters than cpDNA loci (Small *et al.* 2004). Other advantages of ITS for phylogenetic reconstruction are the small size (<700 bp in angiosperms), the high number of copies in plant genomes, and the rapid concerted evolution and length conservation among different angiosperms groups, which results in fairly simple sequence amplification (Alvarez and Wendel 2003). Despite its advantages, ITS must be used with caution to estimate molecular phylogenies. Incomplete concerted evolution may result in pseudogenes, paralogous sequences and high levels of homoplasy that can result in misleading estimations of phylogenetic relationships. Also, the secondary structure of ribosomal DNA means that it is subjected to evolutionary constraints related to the maintenance of this structure, which may imply the occurrence of compensatory mutations that violate the assumptions of neutrality and independence of characters.

Although powerful for resolving phylogenetic relationships at family and genus level, the effectiveness of DNA sequence data in resolving relationships at infraspecific levels is limited. A broad range of molecular techniques is now available to investigate phylogenetic relationships at low taxonomic levels, including Random Amplification of Polymorphic DNA (RAPD), microsatellites, Single Nucleotide Polymorphism (SNP), and Amplified Fragment Length Polymorphisms (AFLP). These techniques differ in their applicability and capacity to detect genetic differences, and in the type of data they generate.

Of these techniques, AFLP is the most time and cost efficient, and does not require previous knowledge of the genetics of the study group. Contrary to the single locus approach, the AFLP technique amplifies fragments from across the entire genome, rather than from small regions within the genome, and generates a reproducible and unique fingerprint for each individual. Among the limitations of AFLPs are the risk of homoplasy between fragments of the same size but of different origins, and the lack of sequence knowledge in fragment data. Such convergence in fragment size can lead to inaccurate estimations of the relationships as they are not based on synapomorphic bands. Sufficient character sampling across the genome can overcome these limitations. Despite the dominant nature of AFLPs and the consequent difficulties in estimating allele frequencies, AFLP data can be used in a wider range of analyses, including population genetics, by implementing models to estimate allele frequencies in dominant data assuming Hardy Weinberg equilibrium.

1.3 Applications of molecular phylogenies in evolutionary biology

1.3.1 Historical biogeography

Historical biogeography investigates the patterns of geographic distribution of organisms over long periods of time (millions of years) and the processes that influenced those patterns. Biogeographical patterns can be explained primarily by three processes: 1) vicariance – separation by a geographic barrier of a group of organisms that result in the differentiation of the original species ; 2) dispersal – movement of organisms and subsequent colonization of new locations ; and 3) extinction – disappearance of a lineage or a group of organisms (taxa). Well-resolved molecular phylogenies can provide a powerful and explicit test of these three historical biogeographical hypotheses. Generally, vicariance is associated with concordant phylogenetic patterns among co-distributed clades, and dispersal and extinction are invoked primarily to explain discordance among clades.

Historical biogeography comprises five basic methods: dispersalism, phylogenetic biogeography, panbiogeography, cladistic biogeography, and parsimony analysis of endemicity (PAE). In dispersalism the explanation for the current patterns of distribution is founded on the concept of a centre of origin and subsequent colonization of new areas and speciation. Phylogenetic biogeography is the study of the geographic distribution of a particular clade in the light of its phylogenetic relationships. Panbiogeography consists of plotting taxa distribution on maps, connecting their distribution areas together with lines, and seeking for coincidences to identify generalized distributional tracks, as they indicate the pre-existence of widespread ancestral biota that were fragmented by geological and/or climatic changes. Cladistic biogeography searches for biogeographic congruence among taxon-area cladograms for several monophyletic groups with the aim of providing insights into the geographical history of several independently evolving groups. PAE classifies areas (analogous to taxa) under the criterion of maximum parsimony by their shared taxa (analogous to characters). The suitability of each method depends on the data set and the type of question to be addressed.

1.3.2 Molecular dating of phylogenetic divergence

The use of DNA sequences to estimate divergence times on phylogenetic trees (molecular dating) has become an important field in evolutionary biology, and is particularly valuable for historical biogeographic studies. The underlying principle of molecular dating is that differences in DNA sequences between two species are proportional to the time elapsed since the divergence from their most recent common ancestor. The rates of change in DNA sequences are heterogeneous among the majority of living organisms and are determined by species-specific factors such as generation time, metabolic and mutation

rates, and effective population size. These rates of variation can be estimated with mathematical models of evolution and statistical tests complemented by biological, biochemical and evolutionary knowledge of the molecular sequence data. The most commonly used methods in the literature incorporate the rate of heterogeneity into the dating procedure using rate change models (relaxed molecular clock).

One of the most frequently employed methods in molecular dating is Bayesian evolutionary analysis by sampling trees as implemented in BEAST. This is a flexible package in which an initial tree topology is not mandatory as it can be inferred from the analysis. It also allows for uncorrelated rates of evolution among branches of the tree in which the rate at each branch is not assumed but drawn from an underlying distribution such as exponential or lognormal. Moreover, in BEAST all parameters (whether they are substitutional, demographic or genealogical) can be given informative prior distributions.

Relative time represented by the branch lengths can be transformed (calibrated) into absolute ages (e.g. million of years) using independent information from the phylogenetic tree and its underlying data. While the fossil record is the most widely used source of information for calibration, phylogenetic trees can also be calibrated using geological events, estimates from independent molecular dating studies (secondary calibration), and to a lesser extent, palaeoclimatic information (e.g. Baldwin and Sanderson, 1998).

Molecular dating provides a temporal framework to the directionality of events demonstrated by the topology of phylogenetic trees and allow further inferences on observed distribution patterns. Despite its great popularity and utility, molecular dating has been subject to strong criticism from some quarters. The critics of main concern are the calibration methods, the priors incorporated to the analyses and the assumptions of DNA sequence change rate, which may direct to misleading conclusions, particularly when based on absolute dates. In spite of these weaknesses, molecular dating has proved to be a powerful tool if the interpretations of the results are carefully drawn from estimated age ranges and the limitations of the methods are taken into account.

1.4 Thesis outline and rationale

The general aim of this dissertation is to provide a comprehensive phylogenetic framework of the Styphelieae, focusing on the *Styphelia-Astroloma* clade, and a foundation to improve our understanding on the morphological and genetic diversity at different taxonomic levels, the evolutionary and biogeographical patterns within the tribe. I explored four different methods to achieve this aim: molecular phylogenetics, molecular dating and historical biogeographical analysis, palynology and population genetics.

Firstly, in **Chapter 2**, I present an estimate of the molecular phylogeny of the Styphelieae based on a comprehensive sample of taxa, in which I identify the main lineages in the *Styphelia-Astroloma* clade and

their relationships through parsimony and Bayesian analyses of DNA sequence data from four chloroplast markers (*rbc*L, *mat*K, *trn*H-*psb*A, and *atp*B-*rbc*L) and the nuclear-encoded ITS region. Based on the phylogenetic hypotheses of relationships, I discuss the morphological generalities of these lineages.

In **Chapter 3** I use parsimony historical biogeographical and Bayesian relaxed-clock analyses with relative dating, direct and secondary fossil calibration methods to investigate the origins and tempo of evolution of the extant Styphelieae in New Zealand on the basis of the estimated molecular phylogeny presented in Chapter 2.

Chapter 4 consists of a representative pollen survey within the Styphelieae, broadly sampling the *Styphelia-Astroloma* clade, in which the diversity of pollen types and pollen morphological characters is documented, and their homology is tested against the molecular estimate of the phylogeny from Chapter 2. The purpose of this survey was to identify new morphological synapomorphies that support the groups identified in Chapter 2.

The aim of **Chapter 5** is to complement the broad appreciation of the phylogenetic diversity in the *Styphelia-Astroloma* clade and to better understand the genetic divergence at shallow branches of the phylogeny, its taxonomic implications, and the possible diversification drivers within the clade. This is a preliminary study in which the level of genetic structure in the *Leucopogon conostephioides* species complex (Group VIII) is estimated using AFLPs. I discuss the potential taxonomic implications of the results in the light of preliminary field and morphological observations, and I propose hypotheses regarding the factors driving the diversification processes in the group that merit further investigation.

Chapter 6 provides a synthesis of the research outcomes and a summary of the directions for further investigation.

Chapter 2 Solving the puzzle: Multigene phylogeny of the *Styphelia-Astroloma* clade (Styphelieae, Epacridoideae, Ericaceae)

ABSTRACT

The Styphelieae (fleshy-fruited epacrids) is the largest of the seven tribes within the subfamily Epacridoideae Link, Ericaceae Juss. Recent molecular phylogenetic work has resulted in the recircumscription of some genera and the erection of new ones, but several non-monophyletic genera remain. Most of the remaining taxonomic problems are concentrated in the *Styphelia-Astroloma* clade, a very well supported clade that contains elements currently assigned to *Leucopogon* R.Br., *Styphelia* Sm., *Astroloma* R.Br., *Croninia* J.M. Powell and *Coleanthera* Stschegl. In order to address these taxonomic problems, Parsimony and Bayesian analyses of sequence data from four chloroplast markers (*rbc*L, *mat*K, *trn*H-*psb*A, and *atp*B-*rbc*L), and the Internal Transcribed Spacer (ITS) were undertaken across 207 taxa yielding 856 parsimony informative characters. The results corroborate the polyphyly of the genera *Astroloma, Styphelia* and *Leucopogon.* Twelve robust groups were identified: Group I (*Astroloma sensu stricto (s.s.)*), Group II (Western *Styphelia sensu lato (s.l.)*), Group III (Western *Styphelia s.l.*), Group IV (*Leucopogon. rotundifolius* + *L. cuneifolius*), Group V (*Leucopogon s.l. pro parte (p.p.)*), Group VI (*Styphelia s.s.,*), Group VII (*Leucopogon s.l. p. p.*), Group VIII (*Leucopogon. conostephioides* complex), Group IX (*Stomarrhena*), Group X (*Leucopogon s.l. p. p.*), Group XI (*Leucopogon. blepharolepis* + *L.* sp. Moore River), and Group XII (New Caledonian *Styphelia s.l.*). The composition and relationships of these clades and their morphological generalities are discussed. New combinations are made for *Astroloma baxteri* A.Cunn. ex DC. and *Leucopogon melaleucoides* A.Cunn. ex DC., which are transferred to the genera *Brachyloma* Sond. and *Acrothamnus* Quinn respectively. The genus *Stenanthera* R.Br. is reinstated for *Astroloma conostephioides* (Sond) Benth. and *A. pinifolia* (R.Br.) Benth.

2.1 Introduction

The Styphelieae (fleshy-fruited epacrids) comprises ~350 species in 22 genera and is the largest and most widely distributed of the seven tribes within the subfamily Epacridoideae Link, Ericaceae Juss. Members of this tribe are woody plants ranging in size and habit from prostrate shrubs to small trees. Their habitat varies from heathlands and sandplains to montane forests (Kron *et al.* 2002). They are highly diverse and abundant in Australia, where they frequently represent an important component of the native flora, but they are also present in New Zealand, New Caledonia and New Guinea, with outliers in Hawaii and other Pacific islands.

The taxonomic history of the Styphelieae is complex. Generic circumscription has been problematic since Robert Brown (1810) first described the family Epacridaceae (Quinn *et al.* 2000). Brown recognized 134 species in 24 genera and established two sections based on fruit type: Section I - fruit indehiscent-drupe, ovules one per locule; Section II - fruit a capsule, ovules several per locule. Bartling (1830) formalised Brown's section I as the tribe Styphelieae. Once formalised, the generic concepts within the tribe established by Brown and maintained by Bentham (1868), were adopted by most subsequent authors. These were largely based on variations in floral characters. Conversely, Mueller (1867; 1889) adopted much broader generic concepts and included all the fleshy-fruited genera (*Acrotriche* R.Br.*, Astroloma* R.Br.*, Cyathodes* Labill., *Cyathopsis* Brongn. and Gris, *Leucopogon* R.Br, *Monotoca* R.Br. and *Pentachondra* R.Br.) in *Styphelia* Sm. Mueller's broad concepts were adopted in Malesia (Sleumer, 1964) and New Caledonia (Virot, 1975), but not in Australia and New Zealand. A more detailed taxonomic history of the subfamily Epacridoideae and the tribe Styphelieae is presented in Powell *et al.* (1997) and Quinn *et al.* (2003).

Relative to that of Mueller, Brown and Bentham's multi-generic classifications appeared to better reflect the morphological diversity of the tribe. Nonetheless, cladistic analyses have shown that many of the floral morphological characters upon which these classifications are based on are highly homoplasic (Powell *et al.* 1997; Taaffe *et al.* 2001). As a result, generic limits in Styphelieae are unclear and the current classification is not consistent with the phylogenetic relationships of the taxa.

Several parsimony analyses of morphological and plastid DNA sequences data have been undertaken in order to provide a general phylogenetic framework and establish monophyletic genera in the Styphelieae (Powell *et al.* 1997; Crayn and Quinn 2000; Taaffe *et al.* 2001; Quinn *et al.* 2003; Albrecht *et al.* 2010). These have enabled more detailed revisionary work on a clade-by-clade basis and resulted in more rigorous, phylogenetically-based circumscriptions for a number of existing genera, e.g. *Androstoma* Hook.f.,

Cyathopsis Brongn. and Gris (Quinn *et al.* 2005), *Lissanthe* R.Br**.** (Crayn *et al.* 2003), and *Monotoca* R.Br. (Albrecht *et al.* 2010). Additionally, the new genera *Acrothamnus* Quinn, *Agiortia* Quinn (Quinn *et al.* 2005), *Dielsiodoxa* Albr**.** and *Montitega* C.M.Weiller (Albrecht *et al.* 2010**)** have been described to accommodate novel groups.

Yet, the systematics of Styphelieae continues to be problematic as non-monophyletic genera persist in the tribe (Johnson *et al.* 2012). These remaining problems are concentrated in the *Styphelia-Astroloma* clade, a well supported clade that contains elements currently assigned to *Leucopogon*, *Styphelia, Astroloma, Croninia* J.M. Powell and *Coleanthera* Stschegl. (Quinn *et al.* 2003). The poor resolution inside this clade and the incongruence between the phylogenetic relationships and the observed morphological patterns remain the major barriers to the completion of a phylogenetic classification of Styphelieae.

The aim of this study was to provide a comprehensive phylogenetic framework of the *Styphelia-Astroloma* clade as the basis of a phylogenetic generic classification, through phylogenetic analyses of DNA sequence data from the chloroplast markers *rbc*L, *mat*K, *trn*H-*psb*A, and *atp*B-*rbc*L, and the nuclear-encoded ribosomal Internal Transcribed Spacer (ITS).

2.2 Methods

2.2.1 Sampling

Two hundred and seven taxa (including 56 putative new taxa) of the ca. 350 recognized species from 18 genera in the tribe Styphelieae were sampled: *Acrothamnus* (4 taxa), *Acrotriche* (4), *Agiortia* (1), *Astroloma* (34)*, Brachyloma* (10) *Coleanthera* (1), *Conostephium* (3), *Croninia* (1), *Cyathopsis* (1), *Leptecophylla* (3), *Leucopogon* (108)*, Lissanthe* (6), *Melichrus* (4), *Monotoca* (2), *Montitega* (1), *Pentachondra* (3), *Planocarpa* (2), *Styphelia* (19). The sampling strategy was intended to broadly survey the tribe and densely sample taxa from the non-monophyletic genera *Leucopogon, Astroloma* and *Styphelia* that were expected to fall within the *Styphelia-Astroloma* clade based on previous studies and morphological observations. Taxa from mainland Australia were densely sampled as the highest diversity occurs there. Taxa from New Caledonia (*Styphelia*), Tasmania (*Pentachondra, Leptecophylla*), and New Zealand (*Leucopogon, Leptecophylla*) were also included to represent the range of morphological variation and geographical distribution. Material from 2-3 different populations was collected for 5% of the taxa with the purpose of evaluating intraspecific variation in widely distributed species and verifying sample identity. Eleven species from the tribes Epacrideae (*Epacris*, *Rupicola*, *Lysinema*), Richeeae (*Dracophyllum*, *Richea*), Cosmelieae (*Cosmelia*, *Andersonia*), Oligarrheneae (*Oligarrhena*, *Needhamiella*), and Prionoteae (*Prionotes*) were included as the outgroup (all genera represented by a single species except *Dracophyllum* which was

represented by two species). The trees from all the analyses were rooted on Prionoteae as previous analyses indicate this tribe is sister to all other taxa in the subfamily (Kron *et al.* 2002). GenBank accession numbers and taxa sampled are listed in Appendix 2.1.

2.2.2 DNA extraction, amplification and sequencing

For newly collected samples, total genomic DNA was extracted from silica dried leaf material at the Australian Genome Research Facility (AGRF). Tissue samples (25–50 mg) were ground to a fine powder by bead milling with 3 mm tungsten carbide beads in a TissueLyser II (30 Hz, 2 x 60 s pulses; Qiagen Pty Ltd, Doncaster, Australia). DNA extraction was performed using the Nucleospin Plant II system (Machery-Nagel GmbH and Co, Düren, Germany) according to the manufacturer's instructions using the SDS buffer set option (PL2/3). Four chloroplast regions (*rbc*L, *mat*K, *trn*H-*psb*A, *atp*B-*rbc*L) and the nuclear-encoded ITS were amplified using the PCR primers and protocols reported in **Table 2.1**. PCR products were cleaned using Exo-SAP-IT (USB Corporation, Cleveland, Ohio, USA). DNA was bidirectionally sequenced on an AB3730xl 96-capillary sequencer at the AGRF. Several *rbcL* and *matK* sequences were generated at the Biodiversity Institute of Ontario, University of Guelph, Canada, as part of the barcode of life data system (BOLD). Protocols can be found in www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification-Plants.pdf, www.dnabarcoding.ca/CCDB_DOCS/CCDB_PrimerSets-Plants.pdf. Sequences were automatically aligned using MAFFT (Katoh *et al.* 2002) and manually adjusted in Geneious Pro 5.6.2 software (Drummond *et al.* 2010).

2.2.3 Phylogenetic analyses

Each marker dataset was analysed individually as well as in two combinations: concatenated chloroplast DNA markers (cpDNA) only and cpDNA concatenated with the nuclear ribosomal DNA (nrDNA) marker ITS. Missing data were scored as ambiguous. Phylogenetic trees were estimated using Maximum Parsimony (MP) and Bayesian inference (BI).

Parsimony analyses were performed with PAUP* Version 4.0b10 (Swofford, 2002). Characters were unordered and equally weighted, and gaps were treated as missing data. Heuristic searches were performed with tree bisection-reconstruction (TBR) branch swapping, MULTREES option selected, 1000 random addition sequence (RAS) replicates saving maximum 100 trees per replicate to identify the most parsimonious trees (MPTs). The MPTs were used as starting trees for a second search, using TBR and MULTREES options, and saving a maximum of 10,000 trees. Relative clade support was estimated using jackknife (1000 replicates, holding a maximum of 100 in each replicate, 33% character deletion, 'Jac'

resampling emulated).

Bayesian inference analyses were executed in MrBayes v3.2 (Ronquist *et al.* 2012). The most appropriate nucleotide substitution model parameters for each partition were determined using AIC (Akaike, 1974) in jModeltest (Posada, 2008). A separate model was applied to each partition (Table 2.1). Four Markov Chain Monte Carlo (MCMC) searches (nruns=4, nchains=4) starting from random trees were run independently for five million generations with a tree sampled every 100 generations. To ensure that the runs converged on a stationary distribution, analyses were run until the average standard deviation of split frequencies was <0.01. The first 25% of trees were discarded from each run as the burn-in. A maximum clade credibility tree was calculated from both runs with posterior probability values (PP) plotted. Trees were viewed and exported using Figtree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3 Results

2.3.1 Chloroplast DNA data

The cpDNA dataset included 124 sequences obtained from GenBank and 576 newly generated sequences: 199 *rbc*L, 240 *trn*H-*psb*A, 25 *atp*B-*rbc*L and 112 *mat*K. Aligned length, number of parsimony-informative characters (and percent), and percent missing data (not including alignment gaps) for each DNA region are given in **Table 2.2**.

No conflicts among the topologies from the analyses of the single marker partitions were identified (i.e. no conflicting branches received jackknife >90%, PP ≥ 0.95, trees not shown); thus only the results of the analyses of the combined cpDNA dataset are reported. Results from MP and BI analyses show general congruence in the topology. Parsimony analysis found at least 10.000 MPTs of 2479 steps (Appendix 2.2). The different markers were complementary in providing informative variation at different levels of the phylogeny: *rbc*L and *mat*K helped define the major clades whereas *trn*H-*psb*A and *atp*β-*rbc*L were more variable and provided resolution within these groups.

2.3.2 Nuclear ribosomal DNA data

ITS sequences were obtained for 133 taxa: 12 from GenBank and 121 newly generated. Aligned length, number (and percent) of parsimony-informative characters, and percent missing data (not including alignment gaps) are given in Table 2.2. Results from MP and BI analyses were also broadly congruent for topology. Parsimony analysis found at least 10.000 MPTs of 1397 steps (Appendix 2.3).

Table 2.1 DNA regions, evolutionary model (AIC criterion), primer sequences and PCR protocols. AIC = Akaike Information Criterion.

DNA region	Evolutionary model (AIC)	Primers sequences	PCR protocol	Reference
*rbc*L	GTR+I+G	rbcLa-F: ATGTCACCACAAACAGAGACTAAAGC rbcLa-R: GTAAAATCAAGTCCACCRCG	94°C, 4:00+ 35x (94°C, 0:30- 55°C, 0:30- 72°C, 1:00)+ 72°C 10:00	Levin *et al.* 2003; Kress and Erickson, 2007
*mat*K	GTR+G	MatK-1RKIM-f: ACCCAGTCCATCTGGAAATCTTGGTTC MatK-3FKIM-r: CGTACAGTACTTTTGTGTTTACGAG	94°C, 1:00+ 35x(94°C, 0:30-52°C, 0:20-72°C, 0:50)+72°C, 5:00	Ki-Joong Kim, unpublished.
*trn*H-*psb*A	GTR+G	psbA3_f: GTTATGCATGAACGTAATGCTC trnHf_05: CGCGCATGGTGGATTCACAATCC	98°C, 0:45+ 35x(98°C, 0:10-64°C, 0:30-72°C, 0:40) 72°C, 10:00	Sang, Crawford, and Stuessy, 1997; Tate and Simpson, 2003;
*atp*B-*rbc*L	GTR+G	377: GTGGAAACCCCGGGACGAGAAGTAGT 2607: ACTCGGAATGCTGCTAAGA	95°C, 4:00+ 35x(95°C, 0:30-50°C, 1:00-72°C, 1:00) 72°C, 5:00	Crayn and Quinn, 2000
ITS	GTR+I+G	ITS5F: GGAAGTAAAAGTCGTAACAAGG ITS4R: TCCTCCGCTTATTGATATGC	95°C, 2:00+ 30x(95°C, 0:30-55°C, 0:30-72°C, 1:00) 72°C, 5:00	White *et al.*1990

The resulting trees were well resolved and supported in the shallow branches. Deeper branches and relationships between the primary groups within the *Styphelia-Astroloma* clade received lower support. No conflict with the topologies generated from cpDNA data was found (i.e. no conflicting branches received jackknife≥ 90%, PP ≥ 0.95). Therefore, the nrDNA and cpDNA datasets were combined in a single analysis.

Table 2.2 DNA region, aligned length, number of potentially parsimony-informative characters including alignment gaps (and %), and number of missing taxa (and %). Missing data were scored as ambiguous.

DNA region	Aligned length (bp)	Informative characters (%)	# Missing taxa (%)
*rbc*L	559	41 (7.4)	54 (23.8)
*mat*K	833	149 (17.4)	66 (29.1)
*trn*H-*psb*A	933	89 (17.1)	3 (1.32)
*atp*B-*rbc*L	1447	201 (18.4)	112 (49.3)
ITS	782	378 (48.3)	94 (41.4)
Combined	4554	858	

2.3.3 Combined analyses

Analyses of the concatenated cpDNA and nrDNA data showed significant improvement in the resolution of phylogenetic relationships and support over the single marker partition analyses and combined plastid analyses. The monophyly of the Styphelieae recieves only moderate support (81/0.90) and may be influenced by the fact that not all markers (e.g. *trn*H-*psb*A, *rbc*L) provided resolution and support at that level of the phylogeny. Within the tribe there is no substantial topological disagreement with previously published phylogenies (Powell *et al.* 1997; Quinn *et al.* 2003; Taaffe *et al.* 2001). The results are consistent with the monophyly of the genera *Conostephium, Melichrus, Leptecophylla, Lissanthe,* and *Acrotriche* as currently recognized. *Acrothamnus* is paraphyletic with respect to *Leucopogon melaleucoides*, and *Brachyloma* is monophyletic only if incorporating *Astroloma baxteri*.

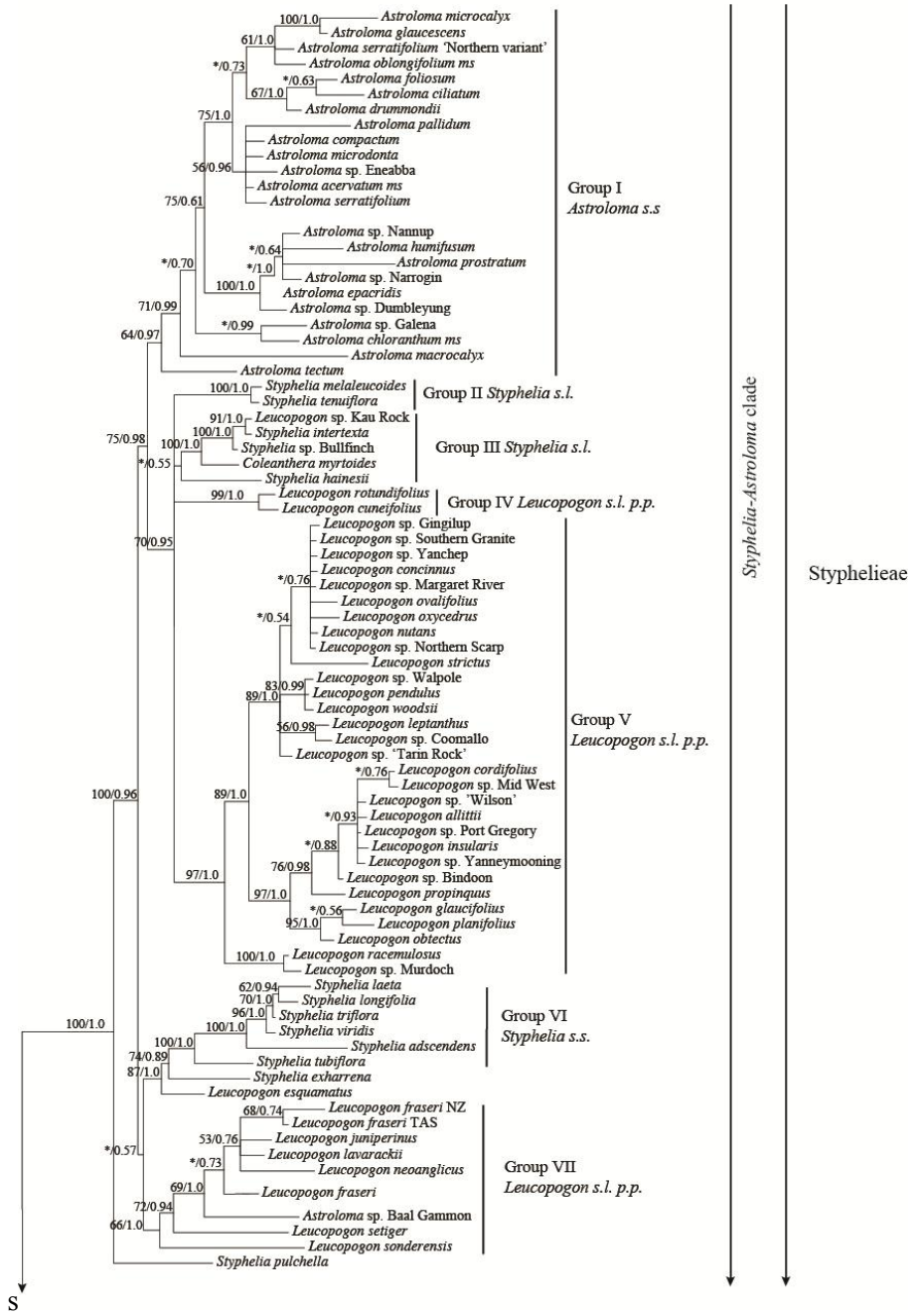
The *Styphelia-Astroloma* clade receives strong support (98/1.0) and comprises several lineages composed of species currently assigned to *Astroloma, Leucopogon* and *Styphelia* (Figure **2.1**). Its sister relationship with the clade that contains *Brachyloma, Conostephium, Leucopogon s.s., Melichrus, Monotoca, Montitega* and *Stenathera* is poorly supported (52/0.54) but is consistently present in the majority of the analyses.

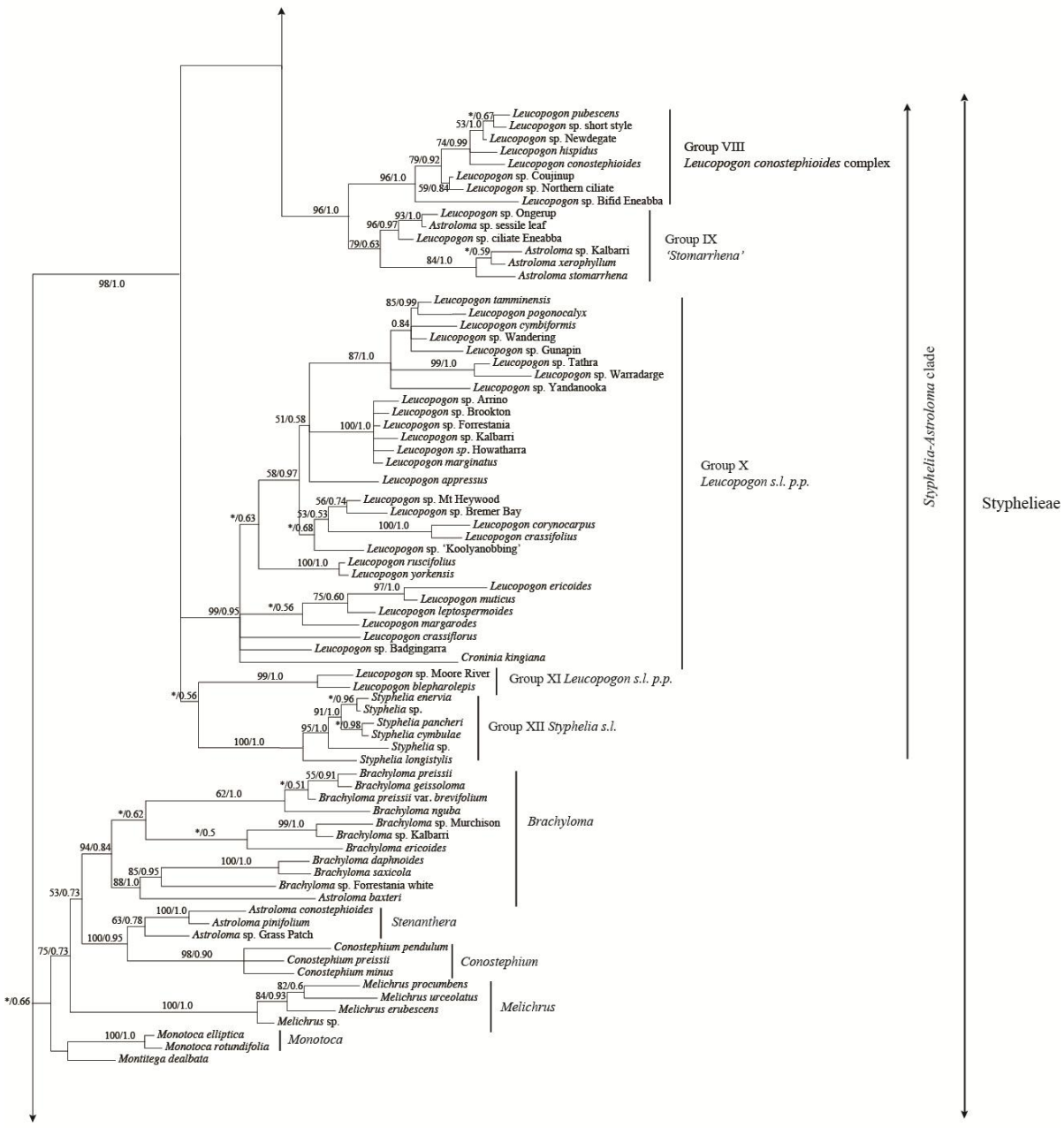
2.4 Discussion

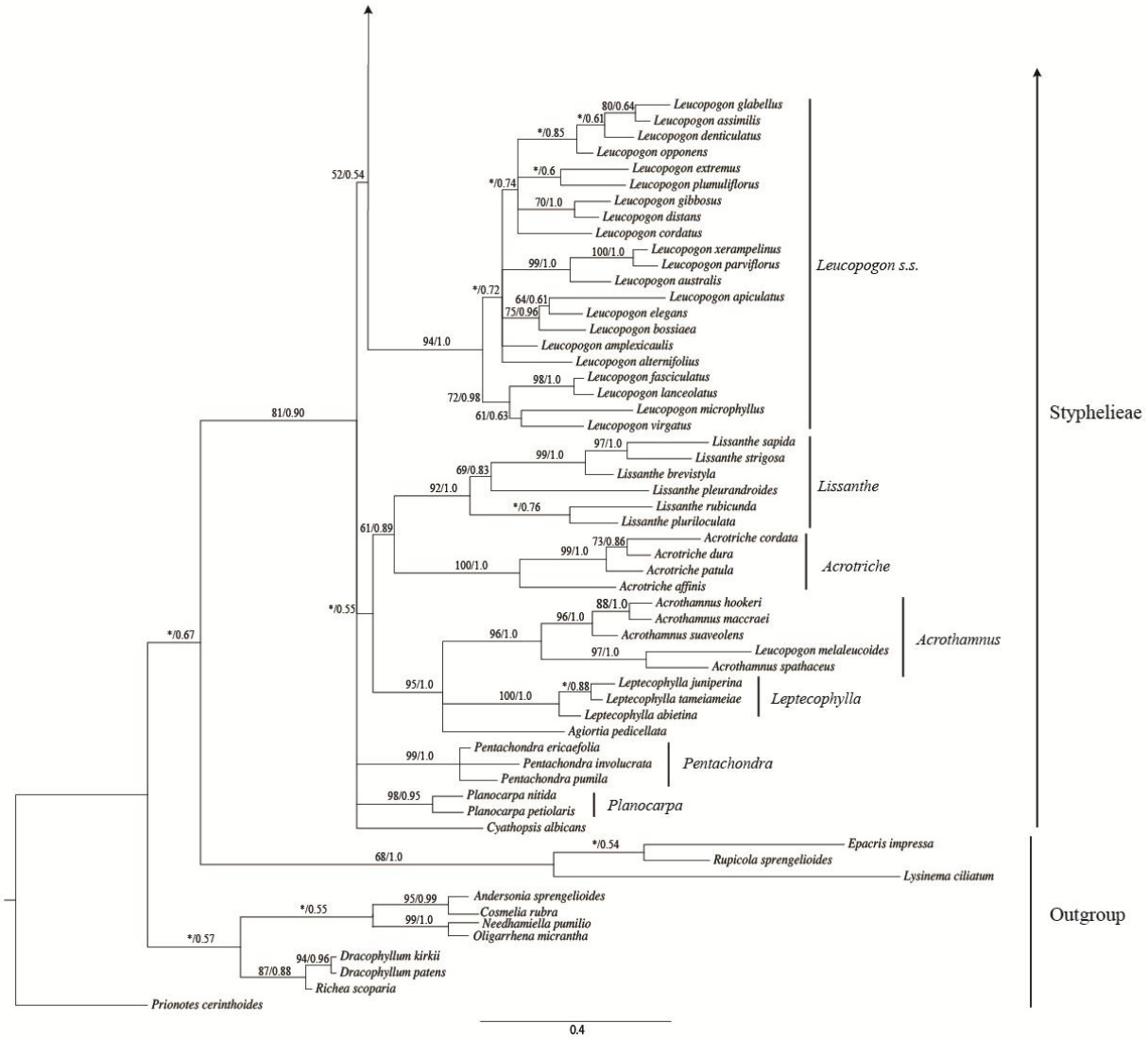
This study improves considerably our knowledge of genus-level relationships and clade composition within the Styphelieae relative to previous parsimony analyses of chloroplast markers only (Quinn *et al.* 2003; Taaffe *et al.* 2001). The results from the analyses of the greatly expanded dataset presented here are congruent with the monophyly of the long accepted generic circumscriptions of *Conostephium, Melichrus, and Pentachondra,* the recently revised circumscriptions of *Acrotriche* (Quinn *et al.* 2005)*, Lissanthe* (Crayn *et al.* 2003; 2005)*, Monotoca* (Albrecht *et al.* 2010) and the more recently segregated genera *Acrothamnus* (Quinn *et al.* 2005)*, Dielsiodoxa* (Albrecht *et al.* 2010), *Lepecophylla,* and *Planocarpa* (Weiller, 1996; 1999). As previously identified, generic limits in *Leucopogon, Astroloma and Styphelia* need to be redefined to accurately reflect their phylogenetic relationships and morphological heterogeneity.

A large portion of the diversity in Styphelieae is within the *Styphelia-Astroloma* clade (at least 200 of the ~350 species), which includes five genera with clear morphological discrepancies: *Astroloma, Coleanthera, Croninia, Leucopogon* and *Styphelia*. Even though the phylogenetic topology is generally not at odds with the morphological patterns observed in the clade, some of the lineages can not currently be clearly diagnosed by any morphological character. For practical purposes, the lineages inside the *Styphelia-Astroloma* clade are accommodated here in twelve groups: Group I (*Astroloma sensu stricto (s.s.)*), Group II (western Australian *Styphelia sensu lato (s.l.)*), Group III (western Australian *Styphelia s.l.*), Group IV (*L. rotundifolius* + *L. cuneifolius*), Group V (*Leucopogon s.l. pro parte (p.p.)*), Group VI (*Styphelia s.s.,*), Group VII (*Leucopogon s.l. p. p.*), Group VIII (*L. conostephioides* complex), Group IX (*Stomarrhena*), Group X (*Leucopogon s.l. p. p.*), Group XI (*L. blepharolepis* + *L.* sp. Moore River), and Group XII (New Caledonian *Styphelia s.l.*). These groups are discussed below on a genus-by-genus basis.

Figure 2.1 Maximum clade credibility tree from Bayesian analysis of the combined chloroplast regions *rbc*L, *mat*K, *trn*H-*psb*A, and *atp*B-*rbc*L, and the nuclear-encoded ribosomal Internal Transcribed Spacer (ITS). Branch support values are to the left of nodes in the following order: MP Jacknife/BI posterior probability * Branch collapses in the parsimony strict consensus tree. NZ: New Zealand; TAS: Tasmania.




*/0.67
Leucopogon pubescens
53/1.0
Leucopogon sp. short style
Leucopogon sp. Newdegate
74/0.99
Leucopogon hispidus
79/0.92
Leucopogon conostephioides
96/1.0
Leucopogon sp. Coujinup
59/0.84
Leucopogon sp. Northern ciliate
Leucopogon sp. Bifid Eneabba
Group VIII
Leucopogon conostephioides complex
96/1.0
93/1.0
Leucopogon sp. Ongerup
96/0.97
Astroloma sp. sessile leaf
Leucopogon sp. ciliate Eneabba
79/0.63
*/0.59
Astroloma sp. Kalbarri
84/1.0
Astroloma xerophyllum
Astroloma stomarrhena
Group IX
'Stomarrhena'
98/1.0
85/0.99
Leucopogon tamminensis
Leucopogon pogonocalyx
0.84
Leucopogon cymbiformis
Leucopogon sp. Wandering
Leucopogon sp. Gunapin
87/1.0
99/1.0
Leucopogon sp. Tathra
Leucopogon sp. Warradarge
Leucopogon sp. Yandanooka
Leucopogon sp. Arrino
Leucopogon sp. Brookton
51/0.58
100/1.0
Leucopogon sp. Forrestania
Leucopogon sp. Kalbarri
Leucopogon sp. Howatharra
Leucopogon marginatus
58/0.97
Leucopogon appressus
Group X
Leucopogon s.l. p.p.
56/0.74
Leucopogon sp. Mt Heywood
Leucopogon sp. Bremer Bay
*/0.63
53/0.53
*/0.68
100/1.0
Leucopogon corynocarpus
Leucopogon crassifolius
Leucopogon sp. 'Koolyanobbing'
100/1.0
Leucopogon ruscifolius
Leucopogon yorkensis
97/1.0
Leucopogon ericoides
75/0.60
Leucopogon muticus
99/0.95
*/0.56
Leucopogon leptospermoides
Leucopogon margarodes
Leucopogon crassiflorus
Leucopogon sp. Badgingarra
Croninia kingiana
99/1.0
Leucopogon sp. Moore River
Leucopogon blepharolepis
Group XI Leucopogon s.l. p.p.
*/0.56
*/0.96
Styphelia enervia
Styphelia sp.
91/1.0
*/0.98
Styphelia pancheri
95/1.0
Styphelia cymbulae
100/1.0
Styphelia sp.
Styphelia longistylis
Group XII Styphelia s.l.
Stylphelia-Astroloma clade
Styphelieae
55/0.91
Brachyloma preissii
*/0.51
Brachyloma geissoloma
62/1.0
Brachyloma preissii var. brevifolium
Brachyloma nguba
*/0.62
99/1.0
Brachyloma sp. Murchison
*/0.5
Brachyloma sp. Kalbarri
Brachyloma ericoides
94/0.84
100/1.0
Brachyloma daphnoides
85/0.95
Brachyloma saxicola
88/1.0
Brachyloma sp. Forrestania white
Astroloma baxteri
Brachyloma
53/0.73
100/1.0
Astroloma conostephioides
63/0.78
Astroloma pinifolium
Astroloma sp. Grass Patch
Stenanthera
100/0.95
Conostephium pendulum
98/0.90
Conostephium preissii
Conostephium minus
Conostephium
75/0.73
82/0.6
Melichrus procumbens
84/0.93
Melichrus urceolatus
*/0.66
100/1.0
Melichrus erubescens
Melichrus sp.
Melichrus
100/1.0
Monotoca elliptica
Monotoca rotundifolia
Monotoca
Montitega dealbata



2.4.1 Morphology and topology

- ***Astroloma and segregates***

First described by Brown (1810), the original concept of *Astroloma* was homogeneous and is shown here to be monophyletic (Group I (64/0.97)). Bentham (1868) expanded the circumscription of *Astroloma* significantly to include *Stomarrhena* DC. (*A. xerophyllum, A. serratifolium*) and most elements of *Stenanthera* R.Br. (*A. baxteri, A. conostephioides, A.*

pinifolium, A. ciliatum) and this concept has remained essentially unchanged. Cladistic analyses of morphological data and plastid DNA sequences have indicated however that *Astroloma* sensu Bentham is polyphyletic (Powell *et al.* 1997; Quinn *et al.* 2003; Streiber, 1999). Three separate lineages of *Astroloma* are identified in this study and two further species are shown to be nested within other genera. The largest grouping of taxa (Group I) includes the type species*, A. humifusum* (Sleumer, 1964). Thus, Group I is named here *Astroloma s.s.* This is a well-defined group morphologically and can be distinguished by the following character combination: filaments linear or narrowly elliptic in section; anthers partially included within the corolla tube; corolla variously coloured (shades of red, pink or orange, to cream and light green, but never white), corolla lobes erect in basal two thirds to three quarters, spreading or recurved above or rarely more or less throughout; external surface of corolla lobes glabrous, bitextured; presence of basal hair tufts within the corolla tube. The first four characters are common to all the members of the group, whereas bitextured corolla lobes are absent from *A. macrocalyx.*

Group IX includes *Astroloma xerophyllum*, *A. stomarrhena,* two undescribed species also informally referred to *Astroloma* (*A.* sp. Kalbarri (D. and B. Bellairs 1368)), *A.* sp. sessile leaf (J.L. Robson 657)) and two other undescribed species tentatively assigned to *Leucopogon* (*L.* sp. Ongerup (A.S. George 16682) and *L.* sp. ciliate Eneabba (F. Obbens and C. Godden s.n. 3/7/2003). The taxa from Group IX can be distinguished from those of Group I by their terete filaments, the absence of basal hair tufts in the corolla tube (except in *A. stomarrhena*) and corolla lobes that are spreading from the base and recurved or revolute throughout. With the exception of the red flowered *A. stomarrhena,* they all have white flowers, which is never the case in *Astroloma s.s.* Compared to the taxa from other groups containing leucopogonoids from Western Australia (see section *Leucopogon and segregates*), those of Group IX differ by their larger, prominently striate sepals together with the following character combination: inner corolla tubes variously hairy below the throat, sepals at least as long as the corolla tube and corolla lobes spreading from the base. If Group IX was to be recognized as a distinct taxon, the name *Stomarrhena* is available if lectotypified on *S. xerophylla* DC.

Astroloma conostephioides, A. pinifolium, *A.* sp. Grass Patch (A.J.G. Wilson 110) and *A. baxteri* do not belong to the *Styphelia-Astroloma* clade. The first three constitute a moderately supported clade (63/0.90) – here referred to as the *A. conostephioides* group – which is robustly placed sister to *Conostephium* Benth. (100/1.0). Since support for the *A. conostephioides* group is

weak, inclusion in a more broadly defined *Conostephium* could be considered. There are however important morphological differences between *Astroloma conostephioides, A. pinifolium*, and *A.* sp. Grass Patch and the species of *Conostephium*, mainly in the corolla. In the *A. conostephioides* group the corolla tube is more or less cylindrical throughout or expands towards the lobes, whereas in *Conostephium* the upper portion of the tube tapers markedly towards the lobes. The corolla lobes in the former are always significantly longer than the latter and variously hairy or scabrous on their outer surfaces, rather than glabrous. With the exception of *A. pinifolium,* members of the *A. conostephioides* group have fleshy appendages in the lower corolla tube from which hair tufts arise. Although *Conostephium* may also exhibit hairs in the lower tube, they are not associated with fleshy appendages. Moreover, the staminal filaments in the *A. conostephioides* group are adnate to the top of the tube with anthers fully or partially exserted while in *Conostephium* the filaments are attached close to the base or at the middle of the tube and the anthers are included. Given that there is a strong morphological basis to recognize the *A. conostephioides* clade as a second genus, *Stenanthera* R.Br. - first described by Brown (1810) to accommodate *S. pinifolia* - should be reinstated.

In common with the members of the *A. conostephioides* group, *A. baxteri* differs from *Astroloma s.s.* in having an inflorescence axis that apparently terminates in a flower with no bud-rudiment and a corolla that is subtended by an undifferentiated series of floral bracts, bracteoles and sepals on an elongated floral axis. In *Astroloma s.s.* (and across the *Styphelia-Astroloma* clade, with the exception of *Croninia*) the axis extends beyond the uppermost flower and terminates in a bud rudiment. Although *A. baxteri* has always been regarded as an oddity, it does resemble *Brachyloma* section *Lissanthoides* Benth., with which it groups (88/1.0) by having lobes which are keeled distally on their adaxial surface, with very short hairs about the keel and glabrous abaxially, and inflexed tips to the lobes. Therefore, the species is accordingly transferred to *Brachyloma.*

Astroloma sp. Baal Gammon (B.P.Hyland 10341) belongs to *Leucopogon* Group VII (69/1.0) (Figure 2.1). Despite its phrase name, this species clearly does not fall within the circumscription of *Astroloma.* It has anthers exserted from the corolla tube, rather than included within, corolla lobes hairy on their external surfaces and terete rather than flattened or compressed filaments.

- ***Styphelia and segregates***

The original circumscription of the genus (Brown, 1810) characterises *Styphelia* as having anthers strongly exserted from the corolla tube, and corolla lobes typically revolute and strongly coiled abaxially. Brown's concept was based on eastern Australian taxa only and while these attributes are very distinctive, the results indicate that they have evolved independently in the eastern Australian (Group VI) and the western Australian lineages (Group II and III) (**Figure 2.1**). As Group VI includes *S. tubiflora*, the lectotype of the genus (Sleumer, 1964), it is here referred to as *Styphelia s.s.* This is a morphologically consistent and strongly supported clade (100/1.0). They are the only group of *Styphelia* whose members exhibit hairs in tufts at the base of the corolla tube.

Styphelia exarrhena, initially described as *Leucopogon exarrhenus* F.Muell. and currently assigned to *Styphelia,* does not exhibit the characters that define *Leucopogon* (see below, in the *Leucopogon* section), but it has the anthers strongly exserted and the corolla lobes revolute and strongly coiled abaxially characteristic of *Styphelia.* As with the western *Styphelia* segregates, it lacks the hair tufts at the base of the corolla present in *Styphelia s.s*. *Styphelia exarrhena* is sister to Group VI (*Styphelia s.s.*) and could be united with it, but further morphological examination is needed to determine whether it should be placed within *Styphelia s.s.* or in a separate taxon.

Unlike the eastern Australian taxa, the western Australian *Styphelia* are polyphyletic and rather heterogeneous in their morphology. Four groups can be identified on the basis of their morphology and the estimated molecular phylogeny: 1) *S. tenuifolia s.l.* and *S. melaleucoides s.l.*, 2) *S. intertexta, Leucopogon* sp. Kau Rock (M.A.Burgman 1126) WA Herbarium, *S.* sp. Bullfinch (M. Hislop 3574) and *S.* sp. Great Victoria Desert (N. Murdoch 44), 3) *S. exserta* and *S. pulchella,* and 4) *S. hainesii.*

Styphelia tenuifolia and *S. melaleucoides* constitute Group II (100/1.0). They have leaves glabrous, flat or concave, more or less smooth; flowers cream; and fruit distinctively ovoid and tapering to a more or less acute apex. A very similar fruit is also seen in *Coleanthera* and *Leucopogon s.l. p.p.* Group IV. If Group II were to be recognized at genus rank, the name *Soleniscia* DC. is available (*S. tenuifolia* being the type).

Styphelia intertexta, Leucopogon sp. Kau Rock, and *S.* sp. Bullfinch form Group III (100/1.0). They have leaves that are grooved and hairy abaxially with margins revolute; flowers white; and fruit globose-ellipsoid with an obtuse apex. *Styphelia intertexta* and *L.* sp. Kau Rock are almost indistinguishable morphologically apart from their stamens; the former has long-exserted anthers whereas in the latter they are partially included. Another undescribed species, *S.* sp. Great Victoria Desert (N. Murdoch 44) (not sampled), is also placed in this group as it shows the same morphological attributes. Group III is sister to *Coleanthera myrtoides* (100/1.0).

Coleanthera Stschegl. is endemic to south Western Australia, and comprises only three species: *C. coelophylla* (A.Cunn.) Benth., *C. virgata* Stschegl. and *C. myrtoides*. The first two are listed as Priority One and Presumed Extinct (DEC Conservation Codes for Western Australian Flora) respectively and were not sampled for this study. The main diagnostic character for the genus is the presence of anthers connate around the style and the absence of a nectary. Although *A. stomarrhena* also exhibits this feature, its anthers and filaments are densely hairy and the corolla is red, whereas in *Coleanthera* the filaments are glabrous and the corolla is white or pink. *Coleanthera myrtoides* has an ovoid fruit that tapers to a more or less acute apex, very much like the fruit of the members of Group II. Yet the results of this study suggest that it is more closely related to Group III than to Group II. DNA sequences from independent samples should be analysed in order to confirm the relationships of *C. myrtoides* with respect to *Styphelia s.l.,* Groups II and III. Moreover, the taxonomic significance of fruit characters to delimit and predict groups in the *Styphelia-Astroloma* clade also needs to be assessed.

Styphelia pulchella is sister to the clade containing *Astroloma s.s.* (Group I)*, Styphelia s.l.* groups II, III and VI and *Leucopogon s.l. p.p.* groups Groups IV, V and VII (Figure 2.1). Its closest relative is predicted to be *S. exserta* (F.Muell.) Sleumer (not sampled), as they are very similar morphologically. They have leaves glabrous, concave, striate abaxially; flowers white and a fruit cylindrical or narrow ellipsoidal, usually radially asymmetrical. DNA sequence data of *S. exserta* is needed to corroborate this relationship.

Styphelia hainesii is the only western species with the following combination of characters: leaves obtuse rather than pungent and flowers red with a well-defined zone of hairs in the basal third of the corolla tube. Although Bayesian analyses indicate it is sister to *Styphelia* Group III, the

posterior probability value for this relationship is very weak (0.55) and this grouping is not resolved in parsimony analyses. Therefore, its phylogenetic relationships with respect to Groups II, III, IV and V remain unclear.

The species of *Styphelia* from New Caledonia (Virot, 1975) (100/1.0) constitute Group XII. They are unrelated to the Australian *Styphelia* and very dissimilar in morphology. They all possess turbinate-shaped flowers with included anthers, glabrous corollas and ovaries, and leaves with acute but not pungent apices. Although placed sister to Group XI (*Leucopogon blepharolepis* + *L.* sp. Moore River), this relationship is unsupported and further investigation is needed to detail their origins and phylogenetic relationships.

- ***Leucopogon and segregates***

The current concept of this genus embraces a very large part of the diversity in the Styphelieae (Powell, 1992; Powell *et al.* 1996). It includes about 130 of the 350 species in the tribe. The most widely accepted concept of *Leucopogon* is based on the circumscription of Brown (1810), who defined the genus by the presence of a conspicuous beard of white hairs on the corolla lobes, anthers partially enclosed within the corolla tube, with or without sterile tips, ovary 2–5-locular, and flowers in axillary or terminal spikes. Even though Brown's circumscription of *Leucopogon* has been generally accepted, it fails to reflect the morphological disparities among the various species groups. Cladistic analysis using mainly floral and leaf characters provided strong evidence that *Leucopogon* is polyphyletic (Powell *et al.* 1997), and demonstrated the need for a narrower concept. Powell (1992) recognized *Leucopogon s.s.* as the largest segregate group so far. *Leucopogon s.s.* is outside the *Styphelia-Astroloma* clade (Figure 2.1) and is usually identified by the co-occurrence of at least three of the following characters: anther tips sterile, inflorescences terminal and upper axillary, style included within the corolla tube, and sepals as long as or longer than the corolla tube (Hislop and Chapman, 2007). In this study *L. melaleucoides,* included in *Leucopogon s.s.* by Powell (1992), is shown to belong to *Acrothamnus* (96/1.0).

The species of *Leucopogon* that do not belong to the *Leucopogon s.s.* clade and that are inside the *Styphelia-Astroloma* clade are here referred to collectively as leucopogonoids. Unlike *Leucopogon s.s.,* the leucopogonoids share the character of resuming vegetative growth from the apex of the inflorescence-bearing region of the stem after flowering (Powell, 1992). Powell (1992)

classified the leucopogonoids into two informally named groups: ‘Axonanthus’ and ‘Gynoconus’, ‘Axonanthus’ was characterised by a long style that is usually exserted from the corolla tube, multiporate pollen, and twisted corolla hairs. ‘Gynoconus’ was characterised by a broadening style base that merges smoothly into the ovary apex to produce a cone-like gynoecium, four-colporate pollen and narrow-conical fruit. The species previously assigned by Powell (1992) to ‘Axonanthus’ and ‘Gynoconus’ are listed in Table 2.3. The results presented here concur with Taaffe *et al.* (2001) that the characters that define these groups are homoplasic and consequently, ‘Axonanthus’ and ‘Gynoconus’ are not monophyletic. The leucopogonoids are here arranged in six groups: IV, V, VII, VIII, X, and XI). Although they are very diverse in their morphology, the pattern of morphological variation between (and sometimes within) these groups is complex. The discrepancies between them are not always discrete and the boundaries are unclear.

Leucopogon rotundifolius and *L. cuneifolius* constitute Group IV (99/1.0) (Figure 2.1**),** which emerges from a polytomy with Groups II, III and V. Members of Group IV resemble the members of Group II in having a corolla tube hairy (below the lobes), corolla lobes spreading from the base and recurved, but not revolute, and a fruit ovoid that tapers to a more or less acute apex. *Leucopogon* sp. Boorabbin (K.R. Newbey 8374), another undescribed western leucopogonoid (not sampled), also exhibits these attributes and is placed in this group by its morphology. DNA sequence data of this taxon are required to confirm its position within group IV. Additionally, more variable DNA markers are necessary to resolve the phylogenetic relationship between the species that belong to Groups II and IV. Resolution at this level of the phylogeny is critical to evaluate the taxonomic utility of the observed fruit similarities between these groups, and may lead to the discovery of new informative characters for generic delimitation in the *Styphelia-Astroloma* clade.

Group V is a large well-supported (97/1.0) clade of western species that is comprised of three strongly supported sub-clades (Figure 2.1). Group V includes all the western taxa outside of the *L. conostephioides* complex (Group VIII) with inflorescences widely spreading or pendulous, as well as many with flowers erect. Although fairly uniform in terms of its critical morphological characters, no clear potential synapomorphies have been identified so far by which the group as a whole might be recognised to the exclusion of all other leucopogonoids. The *L. racemulosus* +*L.* sp. Murdoch sub-clade is the only one that is clearly diagnosed by a potential morphological synapomorphy, namely the possession of a fruit that is zygomorphic, compressed and

asymmetrically ellipsoid at maturity, and with the style displaced from the apex to a point well down the upper margin.

Group VII (66/1.0) contains only eastern taxa with flowers pendulous, including *Astroloma* sp. Baal Gammon. Like Group V, Group VII is uniform in its morphology and not clearly diagnosable by any combination of characters. Within the group, the collections of *Leucopogon fraseri* from Tasmania and New Zealand are monophyletic (68/0.74) but do not group with *Leucopogon fraseri* from mainland Australia. Although the support values for this pattern are low, it is congruent with previous studies that have shown that these three entities are not conspecific.

Group VIII consists of taxa belonging to the *L. conostephioides* complex (96/1.0) (Figure 2.1). It is clearly a distinct group with respect to its morphology and recognisable by the following character combination: flowers pendulous (excluding *L. hispidus*), nectary of partite scales, stigma unexpanded and undifferentiated from the style, style long-exserted from the corolla tube, ovary variously hairy in most taxa, locules 2 or 3 (4 or 5 in *L.* sp. Coujinup), sepals acute or acuminate and longer than the corolla tube (except shorter in *L.* sp. Coujinup), leaves pungent, usually adaxially concave, and a dry drupe. Not sampled in this study but placed in the group by their morphology are *L. rigidus* and L. sp. Carnamah.

Group X (99/0.95) consists of eastern and western Australian taxa. It is the most morphologically heterogeneous of the leucopogonoid groups and it does not show any morphological integrity. Within it, several smaller, often well-supported sub-clades with discrete morphological differences can be recognized (Figure 2.1). Group X is currently under deeper morphological examination to assess the taxonomic implications of these differences.

Although *Croninia kingiana* belongs to Group X, it does not cluster with any of the other *Leucopogon* segregates and differs greatly in morphology from the rest of the species within the group. It exhibits a number of unusual features: inflorescence axis not terminating in a bud rudiment, paired keeled fleshy bracts at the base of the inflorescence, conspicuous flowers with large pale-coloured bracteoles and sepals, corolla-tube cylindrical with the lobes spreading horizontally immediately above the sepals, linear bifurcate anthers, hirsute style and villous ovary (Powell, 1993). These morphological attributes were the basis for the erection of a separate genus for *C. kingiana* (Powell, 1993).

Group XI (100/1.0) is comprised of two representatives (*Leucopogon blepharolepis* and *L.* sp. Moore River) of a very distinctive morphological group that is characterized by a leaf-like flattened fruit, unique in the Styphelieae. The following western taxa also exhibit this character and are therefore predicted to belong to this group: *Leucopogon flavescens* Sond, *Leucopogon* sp. Lake Magenta (K.R. Newbey 3387), and *Leucopogon* sp. Flynn (F. Hort, J. Hort and A. Lowrie 859).

Leucopogon esquamatus is robustly resolved (87/1.0) as sister to *Styphelia s.s.* Group VI and *S. exarrhena* (Figure 2.1). It is here considered separately because it is substantially different to them. Although it resembles *Styphelia* in having long filaments and solitary flowers, *L. esquamatus* differs in the following characters: leaves petiolate, stamens inserted at the throat, corolla lobes non-revolute, a much smaller corolla tube (1-1.5 mm against 12-30 mm in *Styphelia s.s.*), corolla tube glabrous (no tufts of hairs towards the base of the corolla tube), hairs on the corolla lobes being more *Leucopogon*-like (denser, less frizzy and beadlike along their length and white), nectary absent, ovary not attenuate with the style, filaments somewhat flattened but not as much as in most of the *Styphelia,* anther with a slight tapering toward the base rather than the minute bilobing typical of *Styphelia,* and fruit cylindrical. If narrow generic concepts were to be applied in the *Styphelia-Astroloma* clade, the name *Phanerandra* Stschegl. is available for *L. esquamatus* (as *Phanerandra esquamata* (R.Br.) Stschegl.).

The *Styphelia-Astroloma* clade exemplifies the main challenges of translating phylogenetic relationships into taxonomic classifications and prioritizing the principle of monophyly in taxonomy. Yet these challenges ought to be overcome in order to build a phylogenetic classification that promotes a stable and non-arbitrary nomenclature, and provides biologically meaningful units of classification that accurately describe the morphological diversity of the clade. The general approach in the Epacridoideae has been to accept only monophyletic genera and for the sake of consistency, only monophyletic genera should be considered in a taxonomic revision in the *Styphelia-Astroloma* clade. Hence, two possible approaches could be taken to accommodate these results into the existing classification scheme: 1) circumscribe the *Styphelia-Astroloma* clade as a single genus or 2) erect further segregate genera that correspond to the groups resolved here. Given the high morphological diversity within the clade, the first approach would result in a very large genus with low information content and poor predictive value. Moreover, no morphological

Table 2.3 Leucopogonoid taxa and their placement in the informal groups proposed by Powell (1992) and the present study.

Taxon	Powell (1992)	Puente-Leliévre et al. (2013)
L. alittii	Axonanthus	Group V
L. cordifolius	Axonanthus	Group V
L. corynocarpus	Axonanthus	Group X
L. crassifolius	Axonanthus	Group X
L. crassiflorus	Axonanthus	Group X
L. cuneifolius	Axonanthus	*
L. ericoides	Axonanthus	Group X
L. esquamatus	Axonanthus	*
L. fletcheri	Axonanthus	Group VII
L. fraseri	Axonanthus	Group VII
L. juniperinus	Axonanthus	Group VII
L. muticus	Axonanthus	Group X
L. neoanglicus	Axonanthus	Group VII
L. nutans	Axonanthus	Group V
L. oxycedrus	Axonanthus	Group V
L. ovalifolius	Axonanthus	Group V
L. pendulus	Axonanthus	Group V
L. propinquus	Axonanthus	Group V
L. setiger	Axonanthus	Group VII
L. strictus	Axonanthus	Group V
L. appressus	Gynoconus	Group X
L. cymbiformis	Gynoconus	Group X
L. leptospermoides	Gynoconus	Group X
L. pogonocalyx	Gynoconus	Group X
L. tamminensis	Gynoconus	Group X

*No group assigned.

characters to diagnose the *Styphelia-Astroloma* clade have yet been identified. On the other hand, the erection of segregate genera that correspond to the groups previously discussed here would result in generic circumscriptions that better account for the morphological diversity of the *Styphelia-Astroloma* clade, and the reinstatement of already available generic names would generate less nomenclatural turmoil. Nonetheless, the large number of segregate clades would entail the description of several new, small genera, which may lead to a very complex taxonomy, particularly where generic diagnostic characters have not been identified. In the light of the current knowledge both approaches should be considered, but the final decision of which one is preferable can only be made after further morphological examination.

2.5 Taxonomy

2.5.1 New combinations

Acrothamnus melaleucoides (A.Cunn. ex DC.) Puente-Lel. *comb. nov.*
Basionym: *Leucopogon melaleucoides* A.Cunn. ex DC. in Prodromus Systematis Naturalis Regni Vegetabilis 7 (2): 750 (1839). Type: "in sterilibus dumetis Novae-Hollandiae ad Hunters-river legit cl. A. Cunningham aug. fl.. (v.s. à cl. inv.)"

Leucopogon linifolius A.Cunn. ex DC. in Prodromus Systematis Naturalis Regni Vegetabilis 7 (2): 747 (1839).
Styphelia linifolia (A.Cunn. ex DC.) F.Muell. in Fragmenta Phytographiae Australiae 6 (42): 36 (1867).

Brachyloma baxteri (A.Cunn. ex DC) Puente-Lel. *comb. nov.*
Basionym: *Astroloma baxteri* in *Prodromus Systematis Naturalis Regni Vegetabilis* 7 (2): 739 (1839).
Type: "ad Novae-Hollandiae oram merid. legit cl. Baxter."

Styphelia baxteri (A.Cunn. ex DC.) F.Muell. in Fragmenta Phytographiae Australiae 6 (42): 35 (1867).

Cyathodes squamuligera B.D.Jacks. [nom. illeg.] in Jackson, B.D., Index Kewensis 1 (1): 677 (1893).
Stenanthera squamuligera F.Muell. in Fragmenta Phytographiae Australiae 4 (27): 97 (1864).

2.5.2 Reinstated names

Stenanthera conostephioides Sond.
Sonder, O.W. in Lehmann, J.G.C. (Ed) (1845), Plantae Preissianae 1(2): 296.
Type: "Ad Port Adelaide, leg. Th. Siemssen, 1839"

Astroloma conostephioides (Sond.) F.Muell. ex Benth. Flora Australiensis 4: 158 (1868).
Styphelia behrii (Schltdl.) Sleumer. Florae Malesianae Precursores XXXVII. Materials towards the knowledge of the Epacridaceae mainly in Asia, Malaysia and the Pacific. Blumea 12 (1): 152 (1964).

Stenanthera pinifolia R.Br.
Brown, R. (1810), Prodromus Florae Novae Hollandiae: 538.
Type: "(J.) v.v."
Astroloma pinifolium (R.Br.) Benth. in Flora Australiensis 4: 15 (1868).
Styphelia pinifolia (R.Br.) Spreng. in Systema Vegetabilium 1: 659 (1824).

2.6 Conclusions

This study presents an extensively sampled phylogenetic framework of the *Styphelia-Astroloma* clade. Although this clade is well supported by the molecular data, no diagnostic morphological characters have yet been identified. The majority of taxa that belong to the *Styphelia-Astroloma* clade were arranged in twelve groups. Of these Groups I, II, III, IV, VI, VIII, XI and XII are morphologically distinct and can be diagnosed by different character combinations. Conversely, taxa from Groups V, VII and X are morphologically heterogeneous and inconsistent, and cannot be diagnosed by any morphological character**.** *Styphelia pulchella, S. hainesii, S. exarrhena, Leucopogon esquamatus,* and *Coleanthera myrtoides* remain ungrouped either because

their phylogenetic relationships are not clear or because they do not show strong morphological affinities with any of the groups.

The *Styphelia-Astroloma* clade typifies the big challenges of reconciling phylogenetics and taxonomy. Further morphological examination of the more problematic groups is necessary to provide a strong basis for a classification that embraces informative, stable and predictable generic concepts that accurately describe the morphological diversity and the phylogenetic relationships within the clade.

Chapter 3 Extinction and recolonization in the New Zealand flora: the case of the fleshy-fruited epacrids (Styphelieae, Epacridoideae, Ericaceae)

ABSTRACT

The origins and evolutionary history of the New Zealand flora has been the subject of much debate. The recent description of *Cyathodophyllum novaezelandieae* from early Miocene sediments in New Zealand provides possible evidence for the antiquity of the fleshy fruited epacrids (tribe Styphelieae, Ericaceae) in New Zealand. Yet the extant species in this tribe are thought to be very closely related to or conspecific with Australian taxa, suggesting recent trans-Tasman origins. In order to investigate the origins and evolution of the extant New Zealand Styphelieae molecular phylogenetic trees based on sequences of three plastid regions that include representatives of all the genera of the tribe and eight of the ten New Zealand species were generated. The range of minimum ages of the New Zealand lineages was estimated using Bayesian relaxed-clock analyses with different calibration methods and relative dating. Each of the eight extant species of New Zealand Styphelieae is a distinct lineage that is nested within an Australian clade. In all except one case the sister is from Tasmania and/or the east coast of mainland Australia; for *Acrothamnus colensoi* the sister is in New Guinea. Estimated dates indicate that all of the New Zealand lineages diverged from their non-New Zealand sisters within the last 7 Ma. Time discontinuity between the fossil *Cyathodophyllum novae-zelandiae* (20-23 Ma) and the origins of the extant New Zealand lineages (none older than 5 Ma) indicates that the fossil and extant Styphelieae in New Zealand are not related. The relative dating analysis showed that to accept this relationship, it would be necessary to accept that the Styphelieae arose in the early-mid Mesozoic (210-120 Ma), which is starkly at odds with multiple lines of evidence on the age of Ericales and indeed the angiosperms. The results presented here do not support the hypothesis that Styphelieae have been continuously present in New Zealand since the early Miocene. Instead they suggest a historical biogeographical scenario in which the lineage to which *C. novae-zelandiae* belongs went extinct in New Zealand, and the extant New Zealand Styphelieae are derived from Australian lineages that recolonised (presumably by long distance dispersal) no earlier than the late Miocene to Pliocene.

3.1 Introduction

Historical biogeography seeks to explain patterns of species distribution in terms of biogeographic processes such as dispersal and vicariance. Given the dual nature of New Zealand as a Gondwanan continental fragment with features of a geologically active oceanic island, the origins and diversification of the New Zealand biota have been considered important to biogeographic theory in general. The landmass that became New Zealand broke away from Gondwana around 80 million years ago (Ma). Subsequently, during the Oligocene (ca. 38-26 Ma), this landmass became significantly reduced in size as a consequence of erosion and marine transgression, which has been identified as the cause of a generalised bottleneck effect observed in several New Zealand plant and animal groups. However, the extent of this reduction remains controversial. The fossil record indicates a very high rate of biotic turnover since the Cretaceous (ca. 145-65 Ma). Moreover, the importance of Tertiary trans-oceanic long distance dispersal in the assembly of the modern New Zealand biota has been widely documented in invertebrates, birds and plants. However findings on wrens, mammals, velvet worms and Araucariaceae do not support the hypothesis that New Zealand was completely submerged during the Oligocene.

In a review of the origins and evolution of the mountain flora of New Zealand Winkworth *et al.* (2002) illustrated how well-resolved molecular phylogenies can help gain a better understanding of the evolutionary history of plant groups as well as testing explicit historical biogeographical hypotheses. In the present study we investigate the phylogenetic relationships and tempo of evolution of the New Zealand flora using the southern heaths (tribe Styphelieae, Ericaceae) as a case study.

The centre of taxonomic diversity of the Styphelieae lies in Australia but significant radiations have also occurred in New Zealand, New Caledonia and montane New Guinea, with outliers in South East Asia, Hawaii and other Pacific islands (Kron *et al.* 2002). In New Zealand five genera and 10 species occur: *Acrothamnus* Quinn (1 sp.), *Leptecophylla* C.M.Weiller (2 spp.), *Leucopogon* R.Br. (5 spp.), *Montitega* C.M.Weiller (1 sp.) and *Pentachondra* R.Br. (1 sp.) (Figure 3.1).

Extant Styphelieae are diverse and distinctive, yet their fossil record is poor. Fossils from southeastern mainland Australia and Tasmania suggest that the tribe had diversified in the Oligocene-Early Miocene and that they had radiated substantially by the beginning of the Pleistocene (ca. 2.6-0.01 Ma) (Jordan and Hill, 1995, 1996; Jordan *et al.* 2007). No fossil Styphelieae from New Zealand had been reported until very recently, when *Cyathodophyllum novae-zelandiae* G.J. Jord. and Bannister was erected for leaves from Late Oligocene-Early Miocene deposits from the south of the South Island (Jordan *et al.* 2010). *Cyathodophyllum novae-zelandiae* dates to 20-23 Ma (D.E. Lee, personal communication), and implies the presence of members of the tribe during the Early Miocene in New Zealand. This fossil presents potential evidence for the antiquity of Styphelieae in New Zealand. However, the lack of strong morphological affinities between this fossil and any of the extant taxa suggest that *C. novae-zelandiae* may represent a different, possibly extinct lineage of Styphelieae in New Zealand (Jordan *et al.* 2010).

The aim of this study was test the hypothesis that Styphelieae have been continuously present in New Zealand since the Late Oligocene-Miocene by estimating the phylogenetic relationships and the age of the extant Styphelieae lineages in New Zealand. This hypothesis predicts that the age of at least one extant clade of New Zealand Styphelieae will overlap with that of *C. novae-zelandiae*.

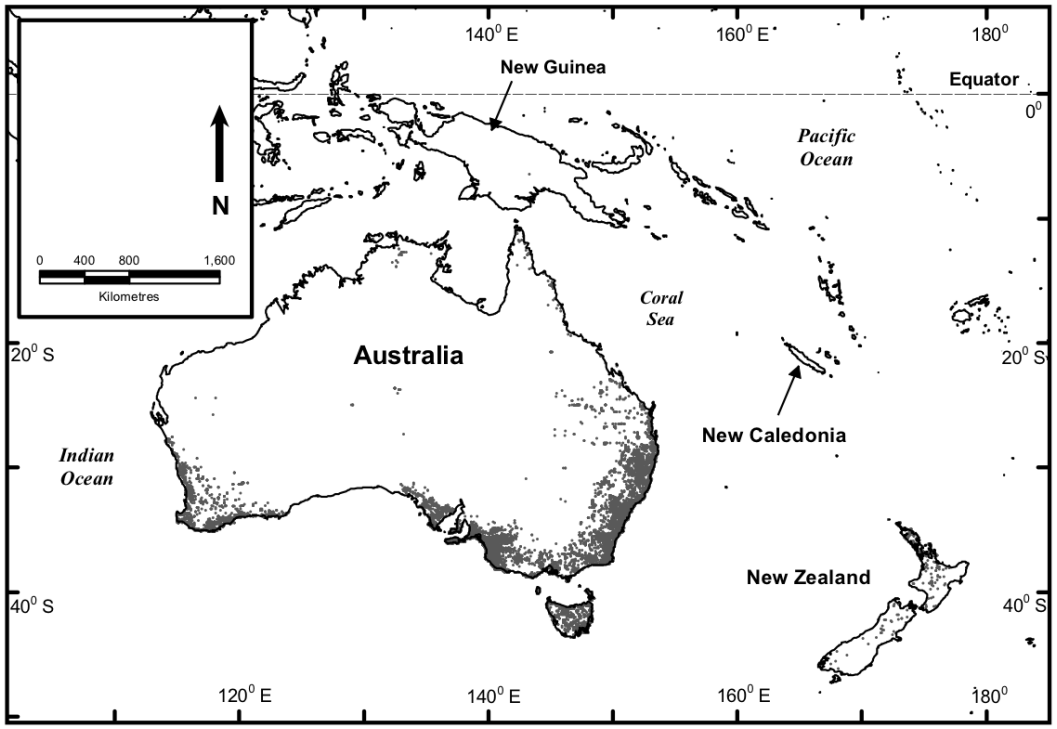
3.2 Materials and methods

3.2.1 Sampling

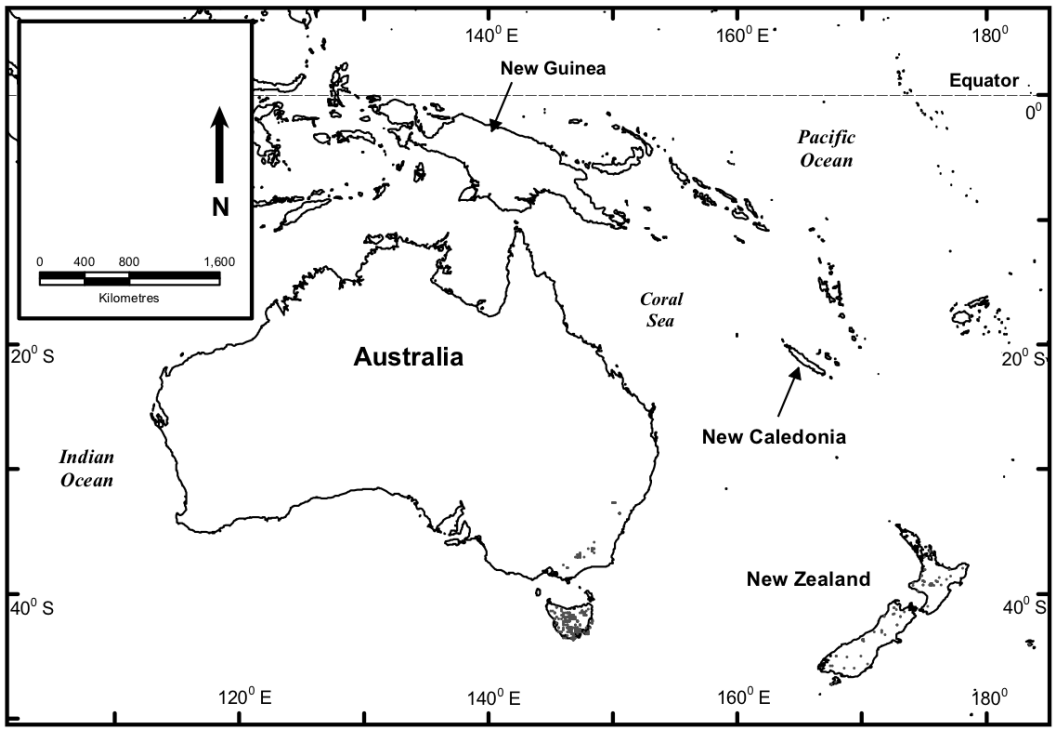
Fifty-five taxa with representatives from all Styphelieae genera, including eight of the ten New Zealand taxa as currently recognised, were selected for the analysis. Samples of *Leucopogon nanum* and *Leucopogon parviflorus* from the Chatham Islands, New Zealand, were not available. We included eight taxa from the tribes Epacrideae, Richeae, and Cosmelieae as outgroup (Kron *et al.* 2002; Johnson *et al.* 2011). The plastid loci *rbc*L*, matK* and the *atp*β-*rbc*L intergenic spacer were selected to provide informative data at different taxonomic levels.

Figure 3.1 Current distribution of the genera of Styphelieae (Epacridoideae, Ericaceae) that occur in New Zealand based on herbarium collections. Information taken from the Australia Virtual Herbarium (http://chah.gov.au/avh/) and Atlas of Living Australia (http://www.ala.org.au/). (a) *Leucopogon* (not monophyletic) (b) *Acrothamnus* (c) *Pentachondra* (d) *Leptecophylla* (e) *Montitega*.

a)



b)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New Caledonia
Indian Ocean
New Zealand
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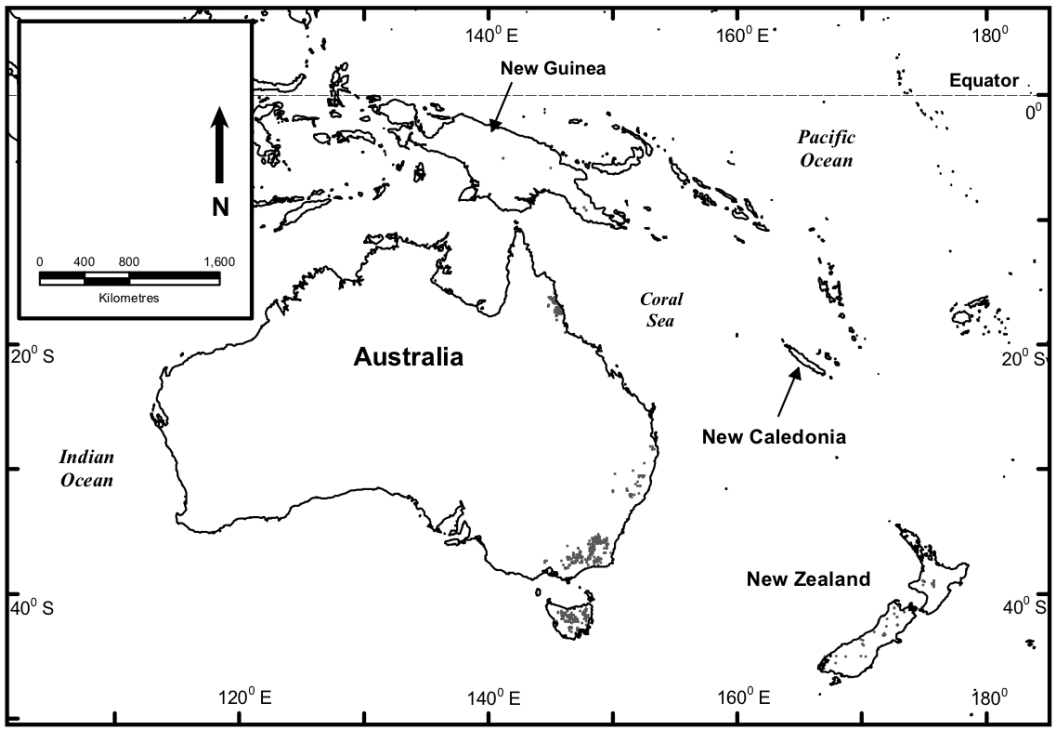
120⁰ E
140⁰ E
160⁰ E
180⁰

c)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Indian Ocean
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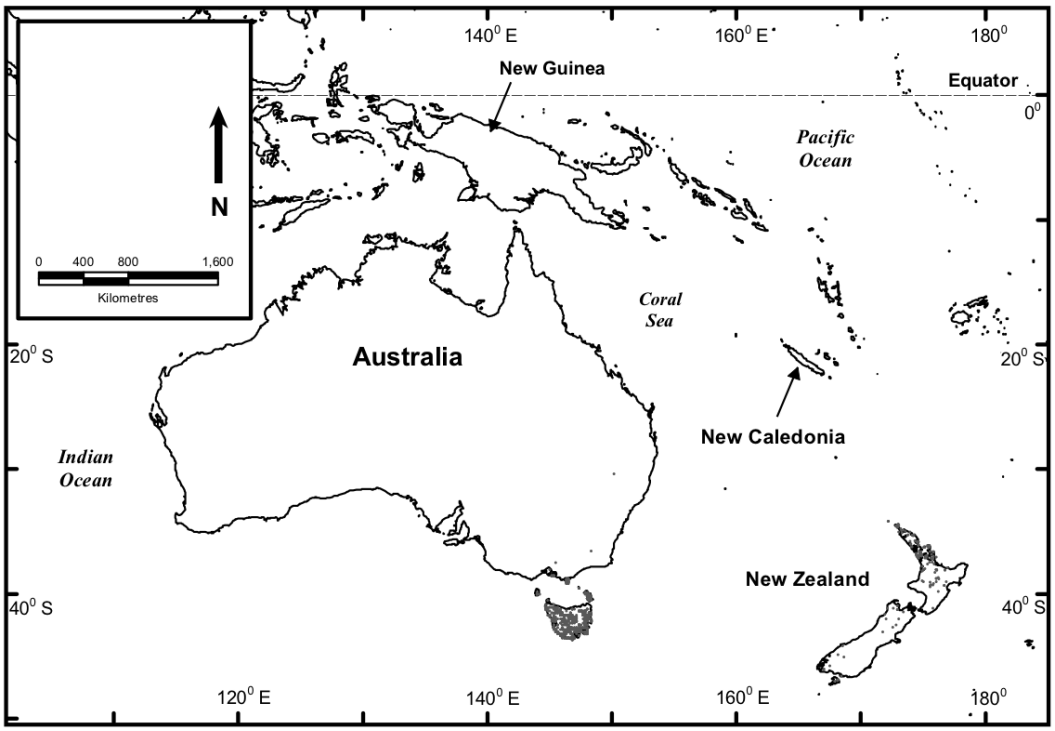
160⁰ E
180⁰

d)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New Caledonia
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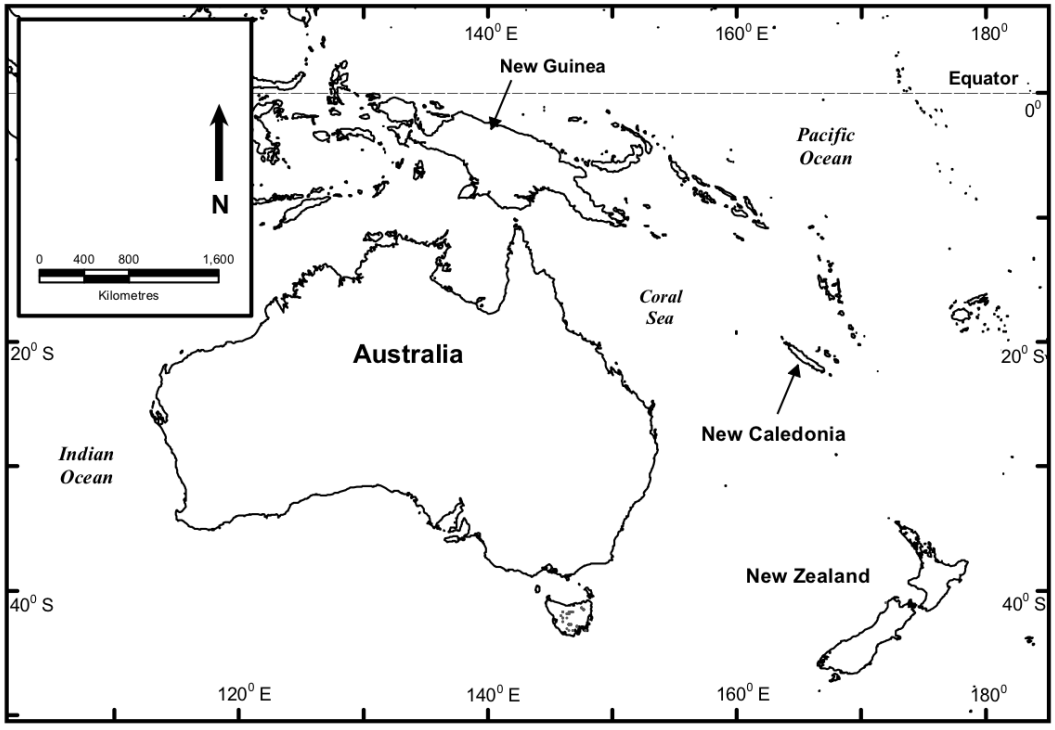
160⁰ E
180⁰

e)


140⁰ E
160⁰ E
180⁰
New Guinea
Equator
0⁰
Pacific Ocean
N
0 400 800 1,600
Kilometres
Coral Sea
20⁰ S
Australia
20⁰ S
New Caledonia
Indian Ocean
New Zealand
40⁰ S
40⁰ S
120⁰ E
140⁰ E
160⁰ E
180⁰

3.2.2 DNA extraction, amplification and sequencing

For newly collected samples, total genomic DNA was extracted from silica dried leaf material at the Australian Genome Research Facility (AGRF). Tissue samples (25–50 mg) were ground to a fine powder by bead milling with 3 mm tungsten carbide beads in a TissueLyser II (30 Hz, 2 x 60 s pulses; Qiagen Pty Ltd, Doncaster, Australia). DNA extraction was performed using the Nucleospin Plant II system (Machery-Nagel GmbH and Co, Düren, Germany) according to the manufacturer's instructions using the SDS buffer set option (PL2/3). Regions were amplified using standard PCR primers and protocols (Sang *et al.* 1997; Crayn and Quinn, 2000; Levin *et al.* 2003; Tate and Simpson, 2003; Kress and Erickson, 2007; Ki-Joong Kim, unpublished). Primer sequences are reported in Table 2.1. PCR products were cleaned using Exo-SAP-IT (USB Corporation, Cleveland, Ohio, USA). DNA was bidirectionally sequenced on an AB3730xl 96-capillary sequencer at the AGRF. Several *rbc*L and *mat*K sequences were generated at the Biodiversity Institute of Ontario, University of Guelph, Canada (www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification-Plants.pdf,www.dnabarcoding.ca/CCDB_DOCS/CCDB_PrimerSets-Plants.pdf). Voucher details and GenBank accession numbers for all sequences are listed in Appendix 2.1. Sequences were automatically aligned and manually adjusted using Geneious Pro 5.4 software (Drummond *et al.* 2010).

Table 3.1 Gene region, aligned length, number of potentially parsimony-informative characters (and %), and number of missing taxa (and %).

DNA region	Aligned length (bp)	Informative characters (%)	# Missing taxa (%)
*rbc*L	552	49 (8.9)	18 (28.6)
*mat*K	1477	217 (14.7)	9(14.3)
*atp*B-*rbc*L	1180	269 (22.8)	9(14.3)
Combined	3209	535	36

3.2.3 Phylogenetic analyses

Each of the three plastid loci was analysed independently as well as combined using Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI). Parsimony analyses were performed with PAUP* Version 4.0b10 (Swofford, 2002). Parsimony-informative characters were unordered and equally weighted, gaps were treated as missing data. Heuristic searches were performed with TBR branch swapping and 1000 random stepwise addition replicates. Relative clade support was estimated using jackknife (10,000 replicates, 33% character deletion, 'Jac' resampling emulated).

Maximum Likelihood analyses were carried out in Garli 2.0. (Genetic Algorithm for Rapid Likelihood Inference). Sequences data were run under a GTR model, as determined by the AICc. AICc was selected as the criterion for model selection because it is not hierarchical in nature and also corrects for small sample sizes (approximately 40 and below) (Akaike, 1974). Bootstrap analysis (100 replicates) was conducted to determine node support.

Bayesian inference analyses were executed in MrBayes v3.2. (Ronquist *et al.* 2012). The most appropriate nucleotide substitution model parameters for each partition was chosen using the corrected Akaike Information Criterion (AICc) in jModeltest (Posada 2008). A separate GTR+I+gamma model was applied to each partition. The Markov Chain Monte Carlo (MCMC) search was run for ten million generations with a tree sampled every 1000 generations. Two simultaneous analyses started from different random trees (Nruns=2), each with four Markov chains (Nchains=4). To ensure that the two runs converged on a stationary distribution, analyses were run until the average standard deviation of split frequencies was <0.01. The first 25% of the trees were discarded from each run as the burn-in. A Bayesian majority-rule consensus tree was calculated in MrBayes with posterior probability values plotted. Trees were viewed and exported using Figtree v1.3.1. (http://tree.bio.ed.ac.uk/software/figtree/). The trees from all analyses were rooted on Prionoteae as previous analyses indicate this tribe is sister to all other taxa included in this study (Kron *et al.* 2002).

3.2.4 Divergence time estimation

Relaxed clock molecular dating MCMC analyses were executed in BEAST v.1.6.2. (Drummond and Rambaut, 2007). Evolutionary models applied to each partition are shown in Table 2.1. Estimation of all model parameters was unlinked across the partitions. Substitution rates were estimated under an assumption of a relaxed clock with the rates in each branch independently drawn from an assumed log-normal distribution (uncorrelated log-normal model – UCLN). The degree of autocorrelation of substitution rate variation was estimated directly from the data (covariance statistic) and was not assumed a priori. The tree branching prior was Yule speciation process birth rate (constant speciation rate per lineage) (Yule, 1924). The monophyly of the ingroup was assumed *a priori* (Kron *et al.* 2002; Quinn *et al.* 2003). Five independent MCMC runs for the combined dataset were executed for ten million generations, sampling the topology every 1,000 generations. The degree of autocorrelation and whether the data satisfy the assumption of a molecular clock were determined by assessing if the credibility interval (CI) of the coefficient of variation was significantly removed from zero. Analyses with empty alignments were ran to ensure that the data and not the priors generated the results. The output was examined using Tracer v.1.5 to optimize priors and to assess effective sample sizes. LogCombiner v.1.6.2. and TreeAnnotator v.1.6.2. (Drummond and Rambaut, 2007) were used to combine and summarize the information in the tree output files (excluding the burn-in) to generate a maximum clade credibility chronogram scaled to mean node heights with 95% highest posterior density (HPD) intervals on the branch divergence estimates. Trees were drawn using FigTree v1.3.1.
(http://tree.bio.ed.ac.uk/software/figtree/).

- ***Calibration dates***

– *Fossil calibration*

Oldest fossils of putative Styphelieae occur in Oligocene-Early Miocene sediments and suggest that the tribe was highly diverse in this period. Therefore, we constrained the divergence time of the Styphelieae (stem age) setting log-normal priors of mean 21.5 Ma (standard deviation (stdv) = 19.8-23.3) to provide minimum and maximum bound of 19.8 Ma and 23.3 Ma for the most recent common ancestor (MRCA). Additionally, *Trochocarpa* fossils from the Pleistocene were used to constrain the *Trochocarpa* crown node to a lognormal mean 2.2 (1.8 – 2.6) Ma (stdv=0.11)

based on the age range from.

– *Secondary calibration point*

Alternatively, secondary calibration was tested as an alternative method to estimate time divergence in the absence of geological events and reliable fossils in order to compare the results with the direct fossil calibration analyses. Divergence times were estimated by scaling the relative node heights into time by setting the divergence of the Styphelieae (stem) to 22.66-32.61 Ma as per Wagstaff *et al.* (2010). The compound error associated with secondary calibrations was incorporated into the analysis using age calibration constraints (rather than point estimates) in the form of statistical distributions (95% HPD) from the original study (Wagstaff *et al.* 2010). Accordingly, a comparable gene sampling (*mat*K and *rbc*L) and the same Bayesian methods were also used. A normal prior distribution was considered the most appropriate as uncertainty of the age used (the mean of the distribution) is equally distributed on either side of the calibration node (Forest, 2009).

– *Relative dating*

The use of epacrid fossils to calibrate dated phylogenetic trees of Styphelieae is problematic as most of them cannot be placed with confidence on any particular branch within the crown group given the lack of clear morphological affinities with any extant taxon (Jordan *et al.* 2010). Moreover, previous cladistic analyses shown that many of the important taxonomic characters in Styphelieae are highly homoplasic and that some genera are not monophyletic e.g. *Leucopogon, Styphelia* and *Astroloma* (Taaffe, 2001;Quinn, 2003). Even in cases where they could be morphologically related to extant taxa, as in the case of the Pleistocene *Astroloma*-type fossils, the fact that the genus as currently circumscribed is not monophyletic reduces the possibility of calibrating the phylogenetic tree at the right node. *Monotoca*-type fossil pollen known from the mid-late Miocene (Martin, 1993) could not be placed with confidence either since a comprehensive pollen survey (C. Puente-Lelievre, unpublished) suggests that the monad pollen type (of which *Monotoca*-type is a special case) is widespread throughout the Styphelieae. Although these fossils represent important evidence for the antiquity and evolution of the tribe, their use as calibration points is not reliable as they are not morphologically comparable with extant taxa (Jordan *et al.* 2007).

In order to test and compare the results of the fossil calibrated phylogenies, relative rather than absolute time scales from the molecular data were inferred. This approach allows testing the null hypothesis that parallel distributions are a result of contemporaneous divergences caused by a single biogeographic event or as a result of independent arrivals. Relative dating allows for the evaluation of temporal congruence by fixing the root node to an arbitrary value (in this case 1.0) and inferring the relative timing of lineage divergences. Different case scenarios can be explored by giving the root node different ages and examining the ages of the internal nodes relative to the root. With the aim of investigating the plausibility of a continuous presence of Styphelieae in New Zealand, the divergence time of Styphelieae was inferred when the oldest extant New Zealand lineages (*Leptecophylla* and *Acrothamnus colensoi*) were scaled to 20 Ma (i.e. the minimum age contemporaneous with *Cyathodophyllum novae-zelandiae*).

3.3 Results

3.3.1 Phylogenetic analyses

This study includes 34 sequences obtained from Genbank and 57 newly generated sequences: 39 rbcL and 18 matK. Aligned length, number of parsimony-informative characters (and percent), and percent missing data (not including alignment gaps) for each DNA region are given in Table 1. The combined matrix contained a total of 3209 characters; rbcL= 552 bp, matK= 1477 and atpβ-rbcL= 1180 (including indels) of which 535 are parsimony-informative. No conflicts among the topologies from the analyses of the single data partitions were identified (i.e. no conflicting branches received a posterior probability (PP) $\geq$ 0.95, trees not shown), thus we report the results of analyses of the combined dataset only. Results from MP, ML and BI analyses shown general congruence for topology, node support and branch lenght. Heuristic searches with 1000 replicates of random taxon addition found one island of 9402 trees of 1636 steps, consistency index (CI)= 0.74, retention index (RI)= 0.80, rescaled consistency index (RC)= 0.59. One of the MP phylograms is shown in Figure 3.2. Support values from MP, ML and BI analyses are mapped onto it.

The tribe Styphelieae is well supported as monophyletic (100/100/1.0). Within the tribe there is no substantial topological disagreement with previously published phylogenies. In this

study, as in previous ones, the monophyly of the genera as currently recognised is well supported with the exception of *Leucopogon* R.Br.*, Styphelia* Sm. and *Astroloma* R.Br. (Taaffe *et al.* 2001; Quinn *et al.* 2003).

All New Zealand Styphelieae are nested within larger Australian clades. It is noteworthy that none of the New Zealand taxa sampled is closely related to each other. In general, the closest relatives are from the east coast of mainland Australia and Tasmania, except for *Acrothamnus colensoi* for which the sister taxon is *Acrothamnus suaveolens* from New Guinea. The collections of *Leucopogon fraseri* from Tasmania and New Zealand included in this study cluster together (66/63/0.99) but are separate from what is currently recognised as *Leucopogon fraseri* in New South Wales, Australia. This is congruent with previous studies that shown that these three entities are not conspecific (Taaffe *et al.* 2001; Dawson and Heenan, 2004). Similarly, the three sampled subspecies of *Leptecophylla juniperina* (subsp. *juniperina, oxycedrus* and *parvifolia*) do not form a clade.

3.3.2 Divergence time estimations

Analyses run without the data and sampling only from the prior distribution compared to the posterior distribution confirm that the priors did not dominate the phylogenetic signal in the data. Reconstructions from separate runs of the combined analyses produced identical topologies and overlapping ranges of likelihood scores, which indicated that all runs had reached stationarity. The estimated coefficient of variation of the branch rates was 0.49 (95% HPD upper 0.63, 95% HPD lower 0.35). This value indicates significant rate heterogeneity among branches - the DNA regions sequenced are not evolving in a clock-like manner. The combined BEAST runs produced sufficient effective sample sizes (>200) for all measured parameters indicating appropriate sampling of the posterior distribution.

Divergence times of the New Zealand Styphelieae from their sister lineages using different estimation methods (fossil calibration and secondary calibration) are shown inTable 3.2.As the resolution inside the *Leptecophylla* clade is poor (PP< 0.95), we report the estimated age of the next deeper well supported node (where *L. juniperina* subsp. *parviflora* diverge from the remaining *Leptecophylla* species). Along with *Acrothamnus colensoi*, these two lineages are the oldest from

New Zealand. The resulting chronograms from the combined Bayesian analyses using fossil and secondary calibration are shown in Figure 3.3 and Figure 3.4. Both analyses indicate that the 95%HPD of the eight New Zealand lineages falls between 5 Ma and the Recent. When the age of *Leptecophylla* and *Acrothamnus colensoi* was scaled to 20 Ma in the relative dating analysis to assume contemporaneity with *C. novae-zelandiae*, the divergence time for the stem of Styphelieae was 120-210 Ma (Figure 3.5).

3.4 Discussion

The molecular data provide evidence for the importance of trans-oceanic dispersal in establishing the distribution of the Styphelieae. The results show that the eight extant Styphelieae in New Zealand sampled in this study are all recent independent arrivals most likely from mainland Australia and Tasmania. *Acrothamnus colensoi* is the oldest divergence, and the intraspecific divergences within *Leucopogon fraseri* and *Pentachondra pumila* are the youngest (Table 3.2). For the taxa that were not sampled in this study, *Leucopogon nanum* M.I.Dawson et Heenan and *Leucopogon parviflorus,* there is evidence to suggest that they also have very recent origins in New Zealand. *Leucopogon nanum* is part of the *Leucopogon fraseri* complex (Dawson and Heenan, 2004), which was shown to have diverged recently from its conspecific most recent common ancestor in Tasmania. *Leucopogon parviflorus* is one of the most widespread epacrid species. It occurs along the western, southern and eastern Australian coastlines where it is common in dune communities. This species occurs in New Zealand only in the Chatham Islands (De Lange *et al.* 2003), and this metapopulation has been previously shown to diverge from its Australian conspecifics no earlier than the late Cenozoic (Heenan *et al.* 2010). In all cases, macrofossils associated with extant Styphelieae genera (e.g. *Astroloma, Leucopogon* and *Monotoca*) are younger than the mean inferred ages of those genera as determined by the present dating analysis.

Figure 3.2. One of 9402 equally parsimonious trees obtained from the combined analyses. Branch lengths are proportional to amount of change. Branch support values are to the left of nodes in the following order: MP Jacknife/ML Bootstrap/BI posterior probability. Tree length=1636, CI=0.74, RI=0.80, RC=0.59.

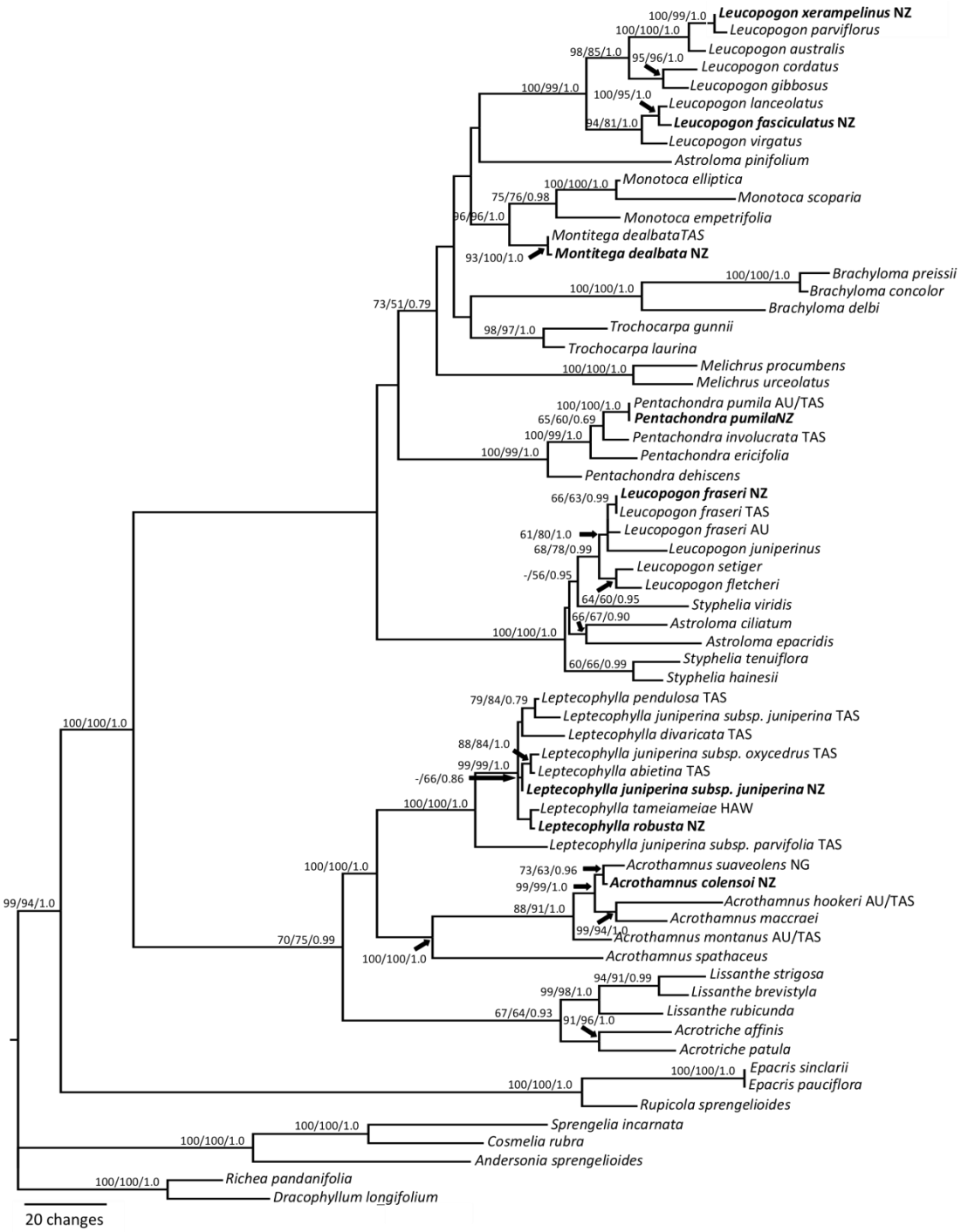


Figure 3.3 Bayesian maximum credibility chronogram based on three plastid DNA regions, direct fossil calibration and uncorrelated lognormal model. Black and grey bars represent the 95% highest posterior density interval for the branching times. Black bars are only used for the nodes from which New Zealand lineages diverge. Bars appear only on nodes that receive more than 95% posterior probability. AU: Mainland Australia; TAS: Tasmania; NZ: New Zealand; HAW: Hawaii; NG: New Guinea. Taxa with no label occur in mainland Australia. Arrows indicate the constrained nodes.

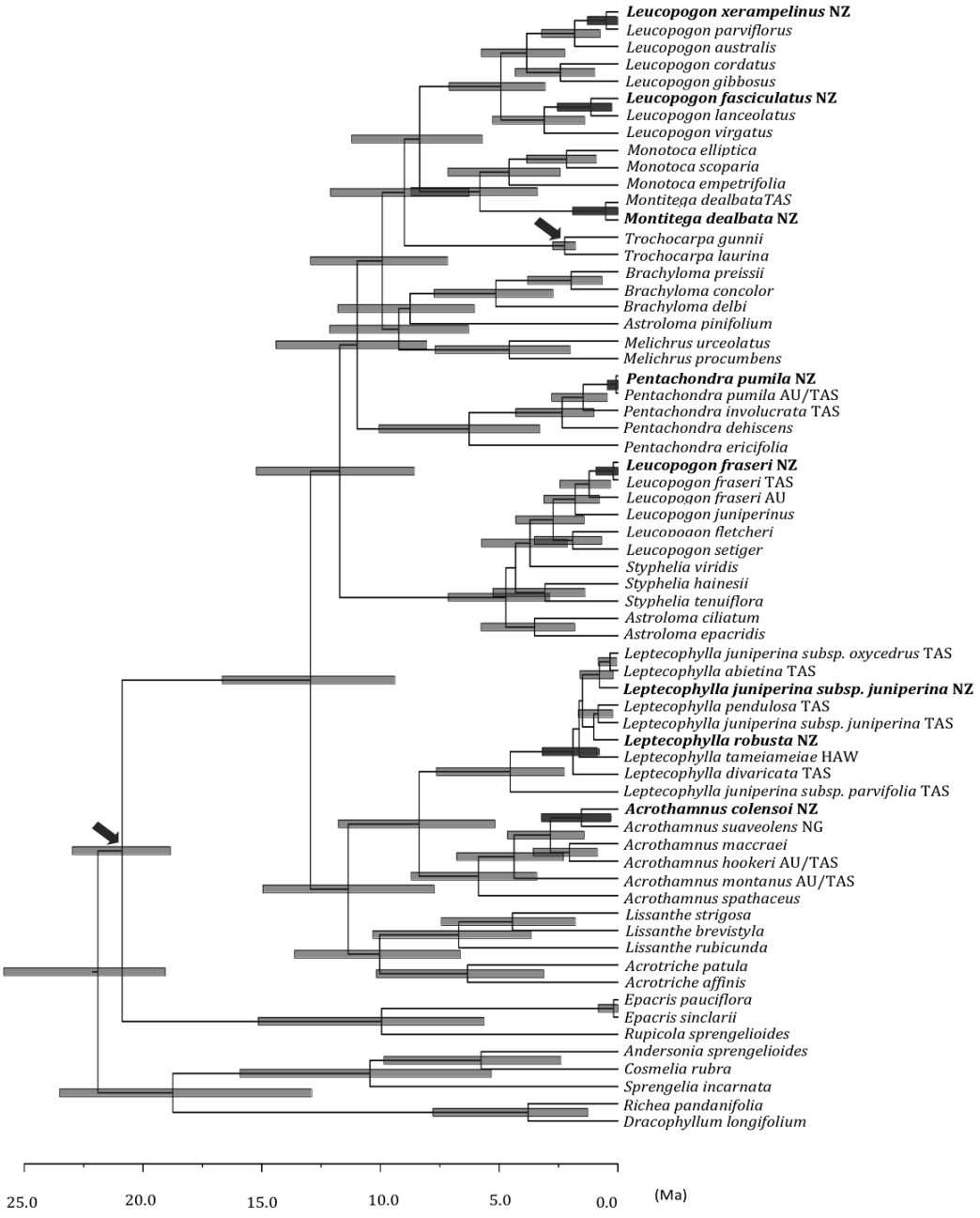


Figure 3.4 Bayesian maximum credibility chronogram based on three plastid DNA regions, uncorrelated lognormal model and normal distribution secondary calibration. Black and grey bars represent the 95% highest posterior density interval for the branching times. Black bars are only used for the nodes from which New Zealand lineages diverge. Bars appear only on nodes that receive more than 95% posterior probability. AU: Mainland Australia; TAS: Tasmania; NZ: New Zealand; HAW: Hawaii; NG: New Guinea. Taxa with no label occur in mainland Australia.

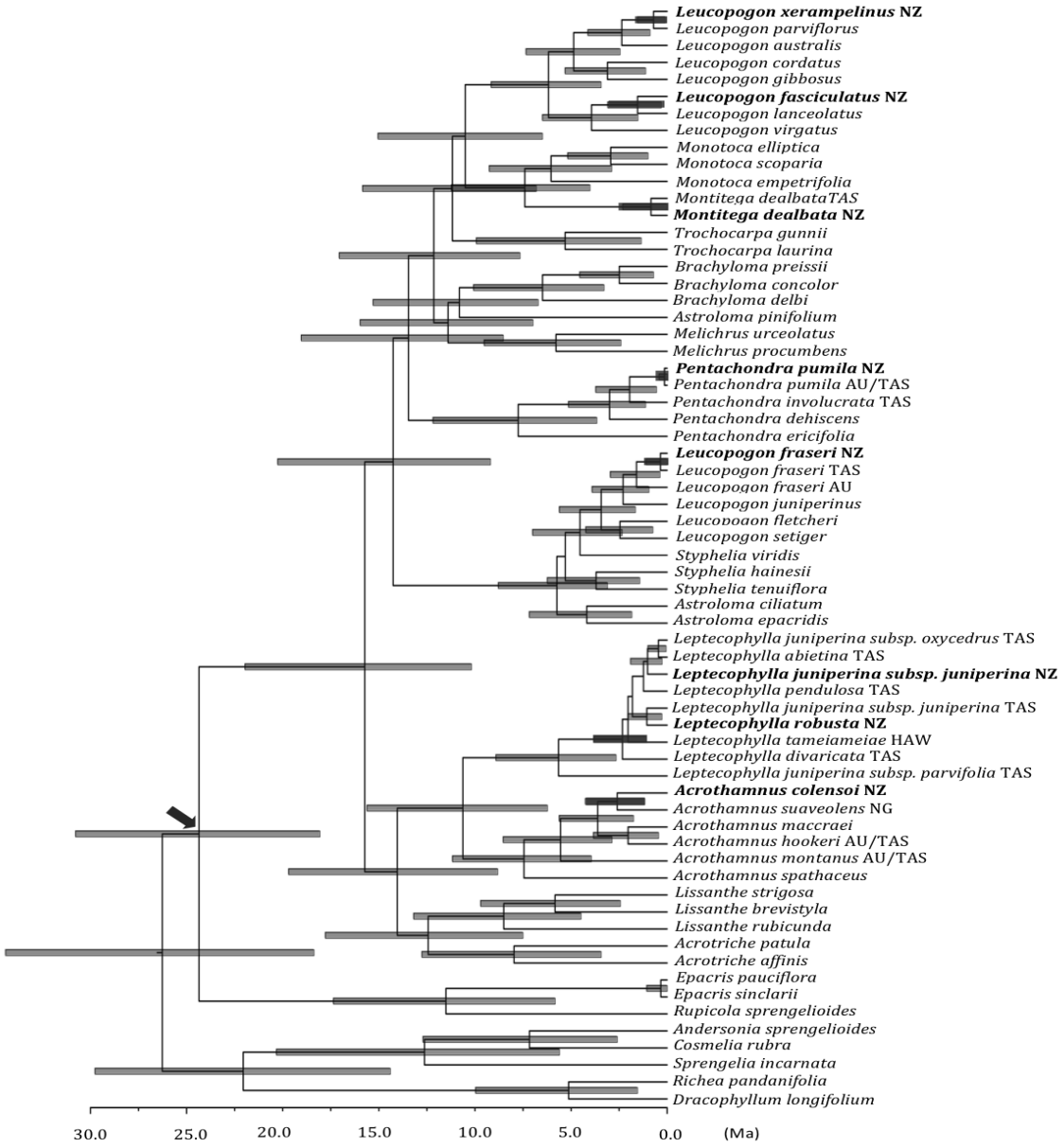


Figure 3.5 Bayesian maximum credibility chronogram showing posterior estimates of relative branching times from the partitioned analyses of three plastid DNA regions, uncorrelated lognormal model. Root was scaled to 165 in order to make the diverge times of Leptecophylla and Acrothamnus colensoi 20 Ma. Black and grey bars represent the 95% highest posterior density interval for the branching times. Black bars are only used for the nodes from which New Zealand lineages diverge. Bars appear only on nodes that receive more than 95% posterior probability. AU: Mainland Australia; TAS: Tasmania; NZ: New Zealand; HAW: Hawaii; NG: New Guinea. Taxa with no label occur in mainland Australia. Vertical light grey area highlights the age of *Cyathodophyllum novaezelandieae*.

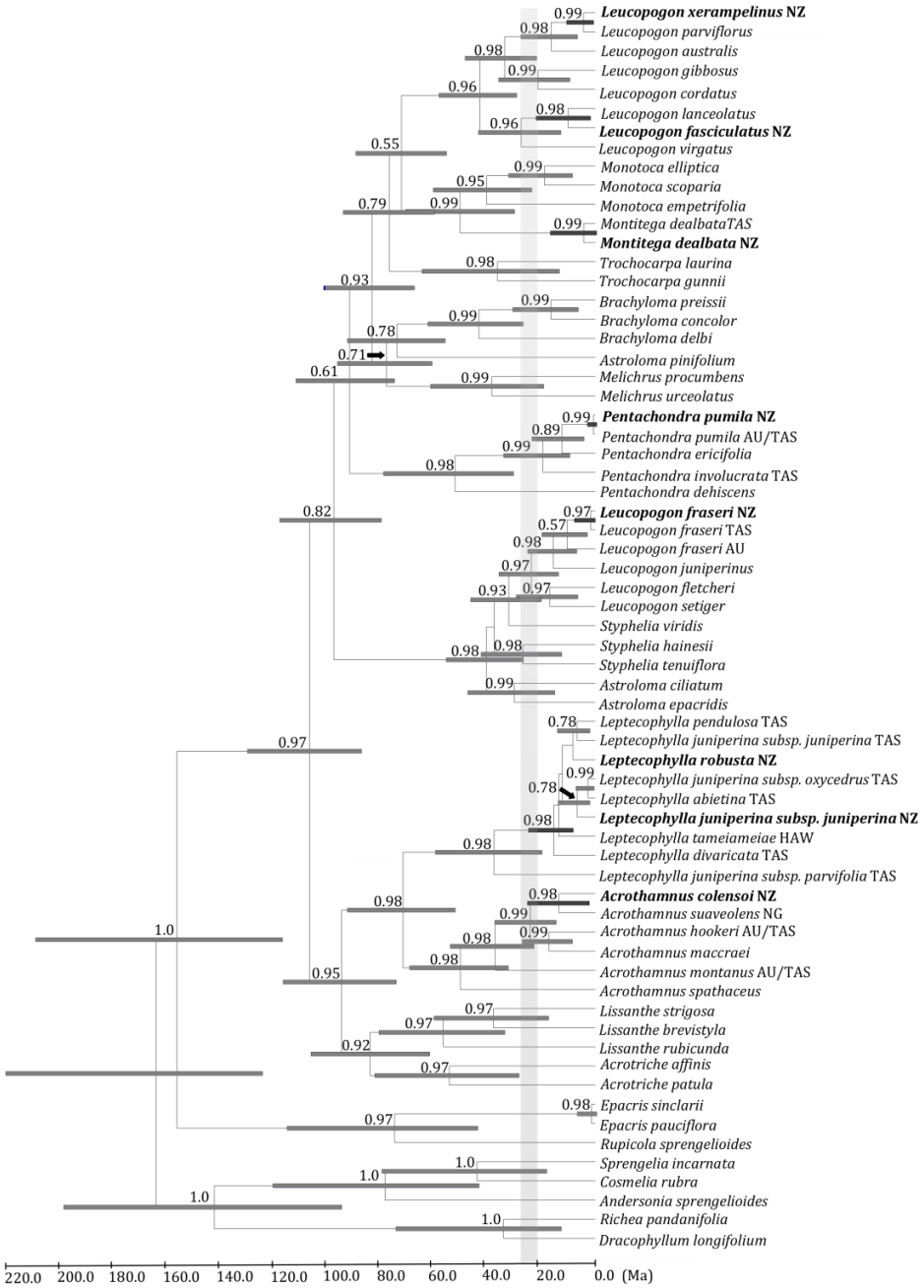


Table 3.2 Divergence estimates for the New Zealand Styphelieae (Epacridoideae, Ericaceae) lineages given as Ma. Bayesian estimates are presented as means with 95% confidence intervals of the highest posterior density (HPD). *Dates given are for the nearest supported node (PP>0.95). It is the same node for L. robusta and L. juniperina subsp. juniperina.

	Mean estimated age (95%HPD) Ma	
	Fossil calibration	**Secondary calibration**
Acrothamnus colensoi	1.53 (0.31-3.21)	2.03 (0.45-3.84)
Leucopogon xerampelinus	0.49 (0.07-1.26)	0.71 (0.09-1.54)
Leucopogon fraseri	0.19 (0.0-0.92)	0.35 (0.0-1.10)
Leucopogon fasciculatus	1.13 (0.25-2.55)	1.54 (0.30-3.09)
Pentachondra pumila	0.08 (0.0-0.42)	0.14 (0.0-0.42)
Montitega dealbata	0.51 (0.01-1.90)	0.84 (0.01-2.31)

Considering that single and secondary calibration points tend to underestimate node ages , it is likely that these results are biased towards younger ages. Nonetheless. the oldest age in the 95% HPD of the oldest lineage (*Acrothamnus colensoi*) is much younger than *Cyathodophyllum novae-zelandiae* fossils. To accept the hypothesis of lineage continuity (i.e. forcing the age of *Leptecophylla* and *Acrothamnus colensoi* to 20 Ma), it would be necessary to accept that the origins of Styphelieae date to 160 (120-210 Ma) (Figure 3.5). This scenario is highly unlikely as the estimated age of angiosperm origins is 180-140 Ma. Moreover, the Ericalean clade is not older than 100-92 Ma and the maximum estimated age for Ericaceae is 58-50 Ma (Wikström, 2001; Bell, 2010).

The fact that none of the extant New Zealand lineages overlaps in time with *Cyathodophyllum novae-zelandiae* and that clear morphological affinities are lacking indicate that the extant Styphelieae in New Zealand are not related to *C. novae-zelandiae*. The lineage to which it belongs became extinct in New Zealand, and the extant New Zealand Styphelieae are derived from Australian lineages that recolonised no earlier than the late Miocene to Pliocene.

As there is not evidence for land connections between New Zealand and Australia since the Late Eocene, it is here hypothesised that this biogeographical pattern is the result of long distance dispersal. Two possible mechanisms of dispersal are considered: anemochory and zoochory. The west-wind drift and ocean currents may be a plausible explanation for some Southern Hemisphere plant distributions (Winkworth *et al.* 2002). Consistent with this expectation, Australian and New Zealand plant fossil records indicate that most shared taxa occurred first in Australia and later in New Zealand. The young ages of the extant New Zealand Styphelieae and the fact that they are nested within older Australian clades indicate that their arrival in New Zealand postdated the establishment of the westerly winds during the Miocene (23–5 Ma) and the intensification of the eastward flow throughout the late Tertiary.

Yet wind-mediated dispersal is strongly distance dependent and requires suitable fruits or seeds. Given that the Styphelieae possess small fleshy fruit, biotic vectors such as birds appear to be more probable dispersal agents (Kubitzki, 2004). Even though birds might be also affected by the same weather systems that influence wind dispersal, they can transport propagules over very long distances and in various directions in the Southern Hemisphere (Winkworth *et al.* 2002). The New Zealand falcon has been documented as a potential long distance dispersal agent for fleshy-fruited plants that inhabit open alpine ecosystems, such as *Leucopogon fraseri* (Young and Bell, 2010). Moreover, adaptations for zoochory such as long flowering and fruiting seasons and sweet, resinous fruits have been reported for species of *Leucopogon* (McIntyre *et al.* 1995; Metcalf, 1996).

In New Zealand, Styphelieae often occur in coastal lowland areas and mountain forests as their closest relatives do in Australia and New Guinea, which is consistent with the model of long-distance dispersal proposed by Jordan *et al.* (2010). The incidence of disjunct and closely related species between Tasmania and New Zealand hints at recent multiple dispersal events between the two landmasses (Jordan, 2001) e.g. *Pentachondra pumila* and *Montitega dealbata*. Further studies at population level could reveal patterns of contemporary gene flow (if any) between the landmasses and elucidate its direction and strength. Also, more detailed work on non-monophyletic species - *Leucopogon fraseri* and *Leptecophylla juniperina* – is needed in order to determine appropriate species circumscriptions. In addition, a deeper knowledge of the pattern of variation in pollen morphology, in particular its phylogenetic distribution, would improve our ability to identify fossil pollen and lead to a better understanding of the relationships between fossil and extant lineages.

The causes of past extinction are uncertain. The age of the fossil *C. novae-zelandiae* (20-23 Ma) on the stem of Styphelieae does not rule out the possibility of its demise being associated with the Oligocene 'drowning' of New Zealand (Landis *et al.* 2008; Biffin *et al.* 2010). On the other hand, the conditions that facilitated the Styphelieae recolonization are likely to relate to the emergence of alpine and subalpine environments and the development of subarid areas during the Pliocene (5-2 Ma) (Raven, 1973; Winkworth *et al.* 2005). Geological changes during this epoch created opportunities for the colonization of novel and rapidly changing environments as has been documented for many elements of the New Zealand flora (Raven, 1973).

3.5 Conclusions

The results presented here do not support the continuous presence of Styphelieae in New Zealand since the Early Miocene (*C. novae-zelandiae*). The closest relatives of the extant New Zealand Styphelieae are from mainland Australia and Tasmania, except for *Acrothamnus colensoi*, which is sister to *A. suaveolens* from New Guinea. The Styphelieae recolonised New Zealand independently during the Pliocene-Pleistocene (5-0.5 Ma). The mechanism of dispersal was not investigated but is likely to be zoochory. The recolonization of New Zealand seems to be associated with the emergence of alpine environments and subarid areas during the Pliocene.

Chapter 4 Evolution and systematic utility of pollen characters in the *Styphelia-Astroloma* clade (Styphelieae, Epacridoideae, Ericaceae).

ABSTRACT

The Styphelieae are unusual with respect to their pollen. Unlike the other Ericaceae, three different pollen types occur within the tribe: pseudomonads, tetrads with variable sterility (A-Type) and regular tetrads (T-Type). Although pseudomonads are rare in flowering plants, they are very common in Styphelieae. In order to assess the diversity of pollen types and pollen morphological characters in the *Styphelia-Astroloma* clade a representative pollen survey was conducted. The evolution of these charcaters in the clade was investigated by optimization onto the Bayesian consensus tree of the combined chloroplast and nuclear dataset presented in Chapter 2 using the software Mesquite. Pseudomonads are universally distributed in the *Styphelia-Astroloma* clade and pollen type proved to be of no taxonomic use within the clade. Conversely, the examined pollen morphological characters (exine ornamentation, number of apertures, presence/absence of a thickened annulus around the apertures, and size of the pollen tetrads at maturity) are variable, consistent and useful to diagnose Groups I – XI. With the exception of pollen type, for which pseudomonads have a single origin, the different character states have derived multiple times independently in the *Styphelia-Astroloma* clade.

4.1 Introduction

Styphelieae are atypical in Ericaceae Juss. with regards to pollen morphology. Even though the pollen grains are shed in tetrads as in the majority of the family (with the exception of *Andersonia macranthera* which exhibits regular monads; , they present various levels of sterility. These levels were described by Smith-White (1955) as follows: 1) T-type, tetrads comprised of four fully developed microspores; 2) A-type, permanent tetrads comprised of four or fewer functional microspores resulting in triads, dyads, monads or more rarely nullads with, three, two, one or no functional grains, respectively, within the wall of the original microspore-mother cell; 3) S-type, permanent tetrads with postmeiotic nuclear migration, unequal division of cytoplasm and subsequent nuclear abortion of three microspores (reported in certain species of *Styphelia* Sm. and *Astroloma* R.Br.)*;* or 4) S'-type, permanent tetrads with initially equally-sized microscopores of which only one fully develops while the remaining three become flattened against the functional microspore. S'-type pollen has only been recorded in species belonging to *Leucopogon sensu stricto (s.s.)*: namely, *L. assimilis, L. distans, L. gibbosus, L. glabellus, L. parviflorus, L. revolutus* (Furness, 2009; Smith-White, 1955; Taaffe *et al.* 2001). Despite the differences in their development, S and S'-type do not show any external distinction and in both cases the non-functional grains remain as cryptic elements of the tetrad. Thus, they are generally called pseudomonads. Pseudomonads often resemble regular monads, but they differ in consisting initially of four microspores while monads consist of only one pollen grain developed from a single microspore. Moreover, pseudomonads have a continuous exine layer laid down around all four microspores in the tetrad, whereas true monads have an exine deposited around the cellulose wall (intine) of a single microspore (McGlone, 1978a). While true monads have not yet been recorded in Styphelieae, pseudomonads appear to be particularly common in the tribe.

Although the ontogeny of the different pollen types has been well studied , their evolution in the tribe remains unclear. Different interpretations of the evolution of pollen type in Ericaceae have been proposed. The most recent interpretation was based on a phylogenetic framework (Furness, 2009) and indicates that (1) regular monad pollen is plesiomorphic in Ericaceae and pollen shed in permanent tetrahedral tetrads has evolved from this condition, and (2) tetrads with variable sterility (A-type) have arisen from regular tetrads (T-type) several times in the

Epacridoideae and this condition is ancestral to pseudomonads. These conclusions appear appropriate to describe the general evolutionary pattern of pollen type in Ericaceae. Nevertheless, a deeper examination is needed to elucidate the evolution of pseudomonads in Styphelieae, the only Ericaceae lineage where they occur. The detailed phylogenetic framework presented in Chapter 2 provides a comprehensive basis to re-evaluate the evolution of pollen type in this tribe.

Previous investigations have focused on pollen development while external morphological characters such as exine ornamentation (only visible in detail with Scanning Electron Microscopy SEM), size, shape and variation in the number of apertures have not been described systematically. This chapter details a representative pollen survey within the Styphelieae focusing on the *Styphelia-Astroloma* clade, with the aims of documenting the diversity of pollen morphology, and testing the homology of the various states against the molecular phylogeny (Chapter 2, Figure 2.1). The purpose of this survey was to (1) reconstruct the evolution of pollen morphology in Styphelieae and (2) identify new morphological synapomorphies to underpin a genus-level taxonomic revision of the *Styphelia-Astroloma* clade.

4.2 Methods

4.2.1 Sampling

Taxa were chosen to represent the majority of the lineages identified in the molecular-based hypotheses of phylogenetic relationships within the *Styphelia-Astroloma* clade presented in Chapter 2 (Figure 2.1 - Groups I to XI, pollen samples for Group XII (New Caledonian *Styphelia*) were not available). With the aim of comparing the variation in other Styphelieae genera and to infer the ancestral state of the observed characters (see below – *4.2.3 Definition of Characters*) in the *Styphelia-Astroloma* clade, representatives of the following genera were included on the basis of their position in the molecular phylogeny: *Acrothamnus, Acrotriche, Brachyloma*, *Conostephium*, *Leptecophylla, Leucopogon s.s., Lissanthe, Monotoca, Pentachondra,* and *Stenanthera*. Pollen type for eleven species previously included as the outgroup in Chapter 2 were scored from the literature: *Epacris impressa*, *Rupicola sprengelioides*, *Lysinema ciliatum* (Epacrideae), *Dracophyllum kirkii, D. patens*, *Richea scoparia* (Richeeae), *Cosmelia rubra*, *Andersonia sprengelioides* (Cosmelieae), *Oligarrhena micrantha*, *Needhamiella pumilio* (Oligarrheneae), and *Prionotes cerinthoides* (Prionoteae). The full list of species examined is

provided in Appendix 4.1.

4.2.2 SEM observations

Pollen grains were extracted from dried herbarium specimens (NSW, PERTH). Streiber (1999) demonstrated that the exine features are identical in acetolysed and the untreated pollen grains. Therefore, pollen samples were directly mounted on the stubs using double-sided sticky tape, sputter-coated with gold (Gold Sputter Coater: Emitech K550) and examined using a Zeiss EVOLS15 Scanning Electron Microscope (SEM) fitted with a Robinson Backscatter Detector at the Australian Museum (Sydney, Australia).

4.2.3 Definitions of characters

The terminology employed here to describe the pollen morphology is that used by Hesse *et al.* (2009). A glossary can also be found at http://www.pollen.mtu.edu/glos-gtx/glos-int.htm. Characters coded were pollen type, exine ornamentation, number of apertures, presence/absence of a thickened annulus around the apertures, and size (longitudinal diameter) of the pollen tetrads at maturity. Since it is impossible to distinguish S type and S' type pollen using SEM, no assumption about the ontogenesis of the pollen grains was made. Hence, pollen grains comprised by permanent tetrads with only one fully developed microspore are generally called pseudomonads. Observations of shape were made but not scored as separate states because the observed variation was continuous and no discrete character states could be discerned.

1. Pollen type: permanent tetrads with only a single fully developed microspore (pseudomonad) (0); permanent tetrads comprised of four and fewer functional microspores: triads, dyads, pseudomonads or nullads (variable sterility, A-type) (1); complete tetrads comprising four, more or less equal-sized, functional pollen grains (T-type) (2)
2. .Exine ornamentation: Nine discrete types of ornamentation of the exine layer can be recognised within the Styphelieae: psilate, a smooth surface (0) (Figure 4.1); perforate, surface of exine with holes less than 1μm in diameter (1); (Figure 4.1e.; 4.2.c, e); granulate, sculptural elements of different sizes and shapes, all smaller than 1 μm in diameter (2) (Figure 4.8b, 4.8c); gemmate, globular exine elements >1 μm in diameter (3)

(Figure 4.5.b–f); areolate, small, mostly convex exine islands (4) (Figure 4.10.a–c); rugulate, elongated exine elements longer than 1 μm, irregularly arranged (5) (Figures. 4.7, 4.8.d), verrucate, wart-like elements >1 μm in width, broader than high (6) (Figure 4.3.a,b); striate, elongated exine elements separated by predominantly parallel grooves. Here, the term refers to the elevated elements and not the grooves (7) (Figure 4.12a).

3. Number of apertures: 0 (0), 3 (1), 4 (2), 5 (3), 6 (4), >6 (5).
4. Annulus: absent (0), present (1), depressed (2).
5. Size categories were defined to represent the differences in dimension ranges between the Groups: small (<30 μm) (0), medium (30-60 μm) (1) and large (>60 μm) (2).

4.2.4 Character optimization

Analyses including the pollen characters into the DNA sequences matrix were ran independently and produced a tree of identical topology with no changes in the support values. Therefore, pollen characters were optimized onto the Bayesian consensus tree of the combined chloroplast (cDNA) and nuclear encoded ribosomal DNA (nrDNA) sequences under the assumption of parsimony in mesquite v.2.5 (Maddison and Maddison, 2008). All characters were treated as unordered and unweighted. The analysis only includes taxa for which SEM images were obtained. Character states scored for each species are presented in Appendix 4.1.

4.3 Results

Scanning electron micrographs were obtained for 83 species including eight undescribed species (Figures 4.1 – 4.15). The character reconstruction analysis comprises a total of 92 species. Five phylogenetic trees are presented, one for each pollen character scored (Figures 4.16 – 4.20). Pseudomonads are usually distinguishable by the scar left from the aborted microspores. However, it was not possible to make reliable observations on pollen type in highly ornamented pollen grains as the remains of the aborted microspores may be obscured. For these cases, the interpretation of pollen type was based on previous light microscopy reports from the literature (Smith-White, 1955; Venkata-Rao, 1961; Furness, 2009).

Figure 4.1. Scanning electron micrographs of pseudomonads in Group I (*Astroloma s.s.*): a) *Astroloma ciliatum*, b) *A. epacridis*, c) *A. humifusum*, d) *A. pallidum* (A.J.G. Wilson, unpubl.), e) *A. prostratum*, f) *A*. sp. Dumbleyung (A.J.G. Wilson 146). Pollen grains in this group have psilate or perforate ornamentation, 6 apertures, 45 – 110 µm, annulus absent or present. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

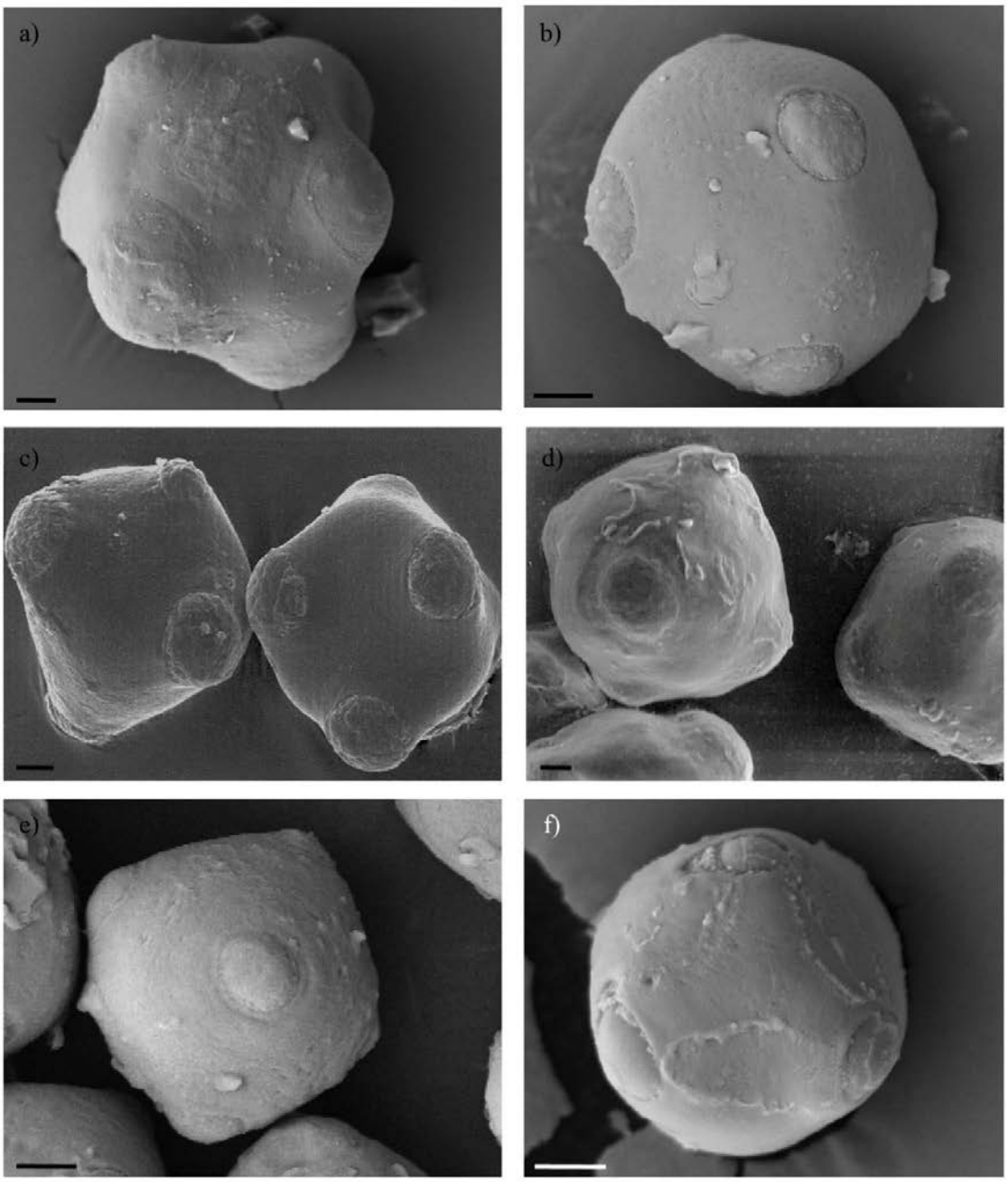


Figure 4.2. Scanning electron micrographs of pseudomonads in Group I (*Astroloma s.s*): a) *Astroloma serratifolium*, b) *A.* sp. Cataby, c) *A.* sp. Nannup, d) *A. macrocalyx,* e) *A. tectum.* d and e from A.J.G. Wilson (unpubl.). Pollen grains in this group have psilate or perforate ornamentation, 6 apertures, 45 – 110 µm, and annulus absent or present. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

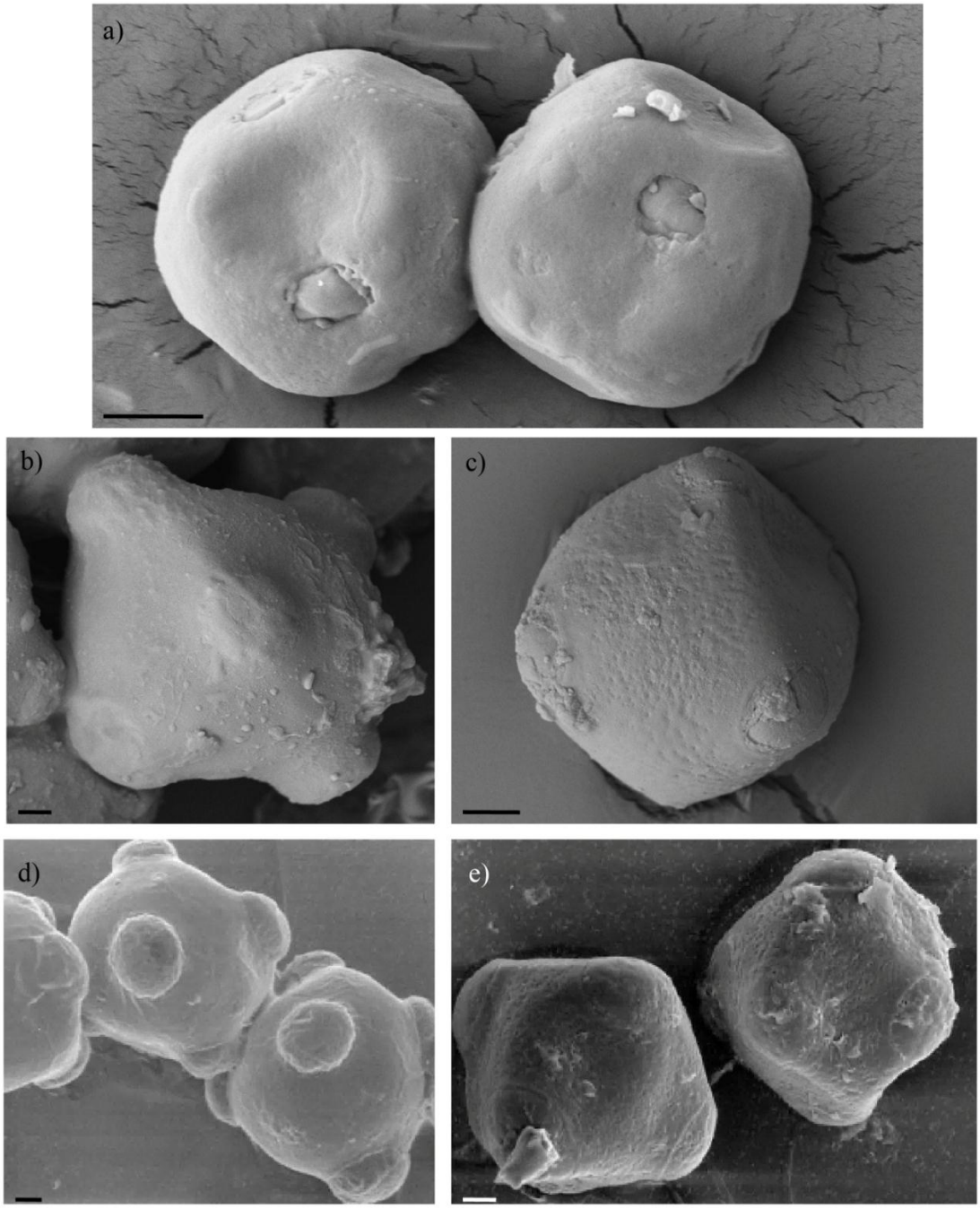


Figure 4.3 Scanning electron micrographs of pseudomonads in Group II (*Styphelia s.l.*): a) *Styphelia melaleucoides*, b) *S. tenuifolia*. Pollen grains in this group have verrucate ornamentation, >6 apertures, 35 – 48 μm, and annulus absent. Group III (*Styphelia s.l.*): c) *Styphelia intertexta*. Pollen grains in this group have perforate ornamentation, >6 or 6 apertures, 20 – 28 μm, and annulus absent. d) *Coleanthera myrtoides*. Voucher information can be found in Appendix 4.1. Scale bars = 10 μm.

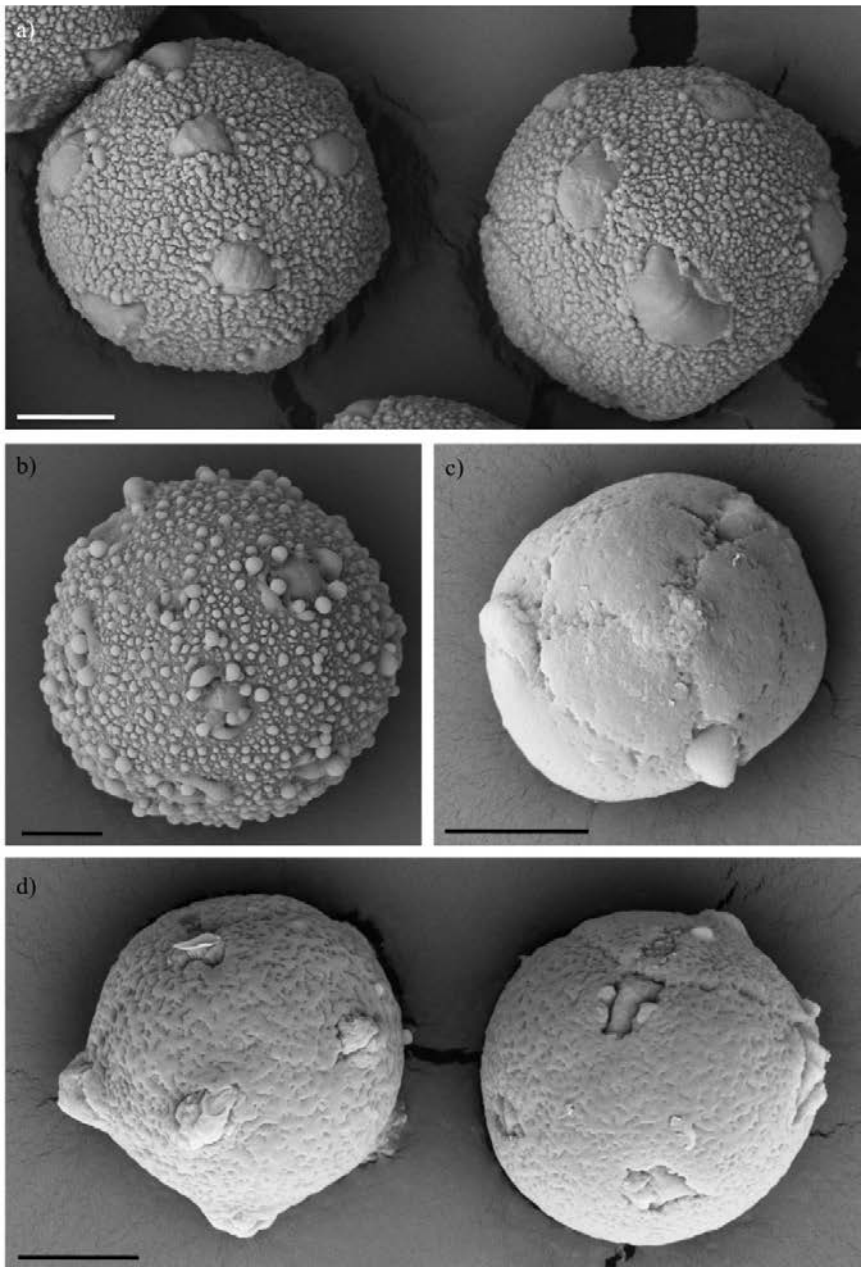


Figure 4.4*:* Scanning electron micrographs of pseudomonads in Group V (*Leucopogon s.l. p.p.*): a) *Leucopogon cuneifolius*, b) *L. ovalifolius*, c) *L.cordifolius*, d) *L. oxycedrus*, e) *L. allittii*, e) *L. propinquus*, f) *L. pendulus*. a, b and f from C. Quinn (unpubl.). Pollen grains in this group have psilate, perforate ornamentation, >6, 6 apertures, 25 – 45 µm, and annulus absent. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm

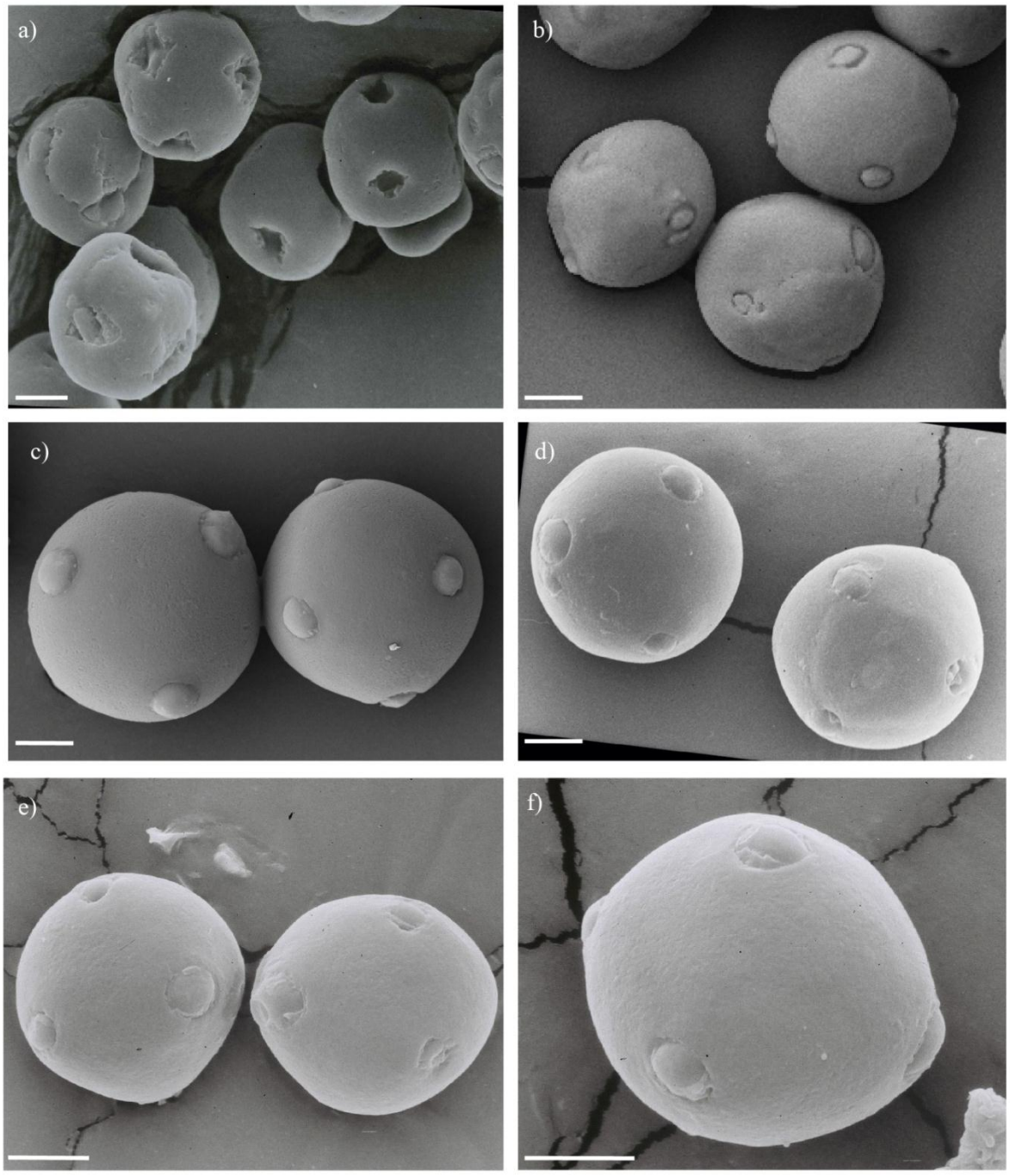


Figure 4.5*:* Scanning electron micrographs of pseudomonads in Group V (*Leucopogon s.l. p.p.*): a) *Leucopogon strictus*. Group VII (*Styphelia s.s.*): b) *Styphelia longifolia*, c) *S. triflora*, d) *S. laeta*, e) *S. adscendens*, f) *S. viridis*. Pollen grains in this group have gemmate and granulate ornamentation, >6 apertures, 45 – 80 µm, and annulus absent. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

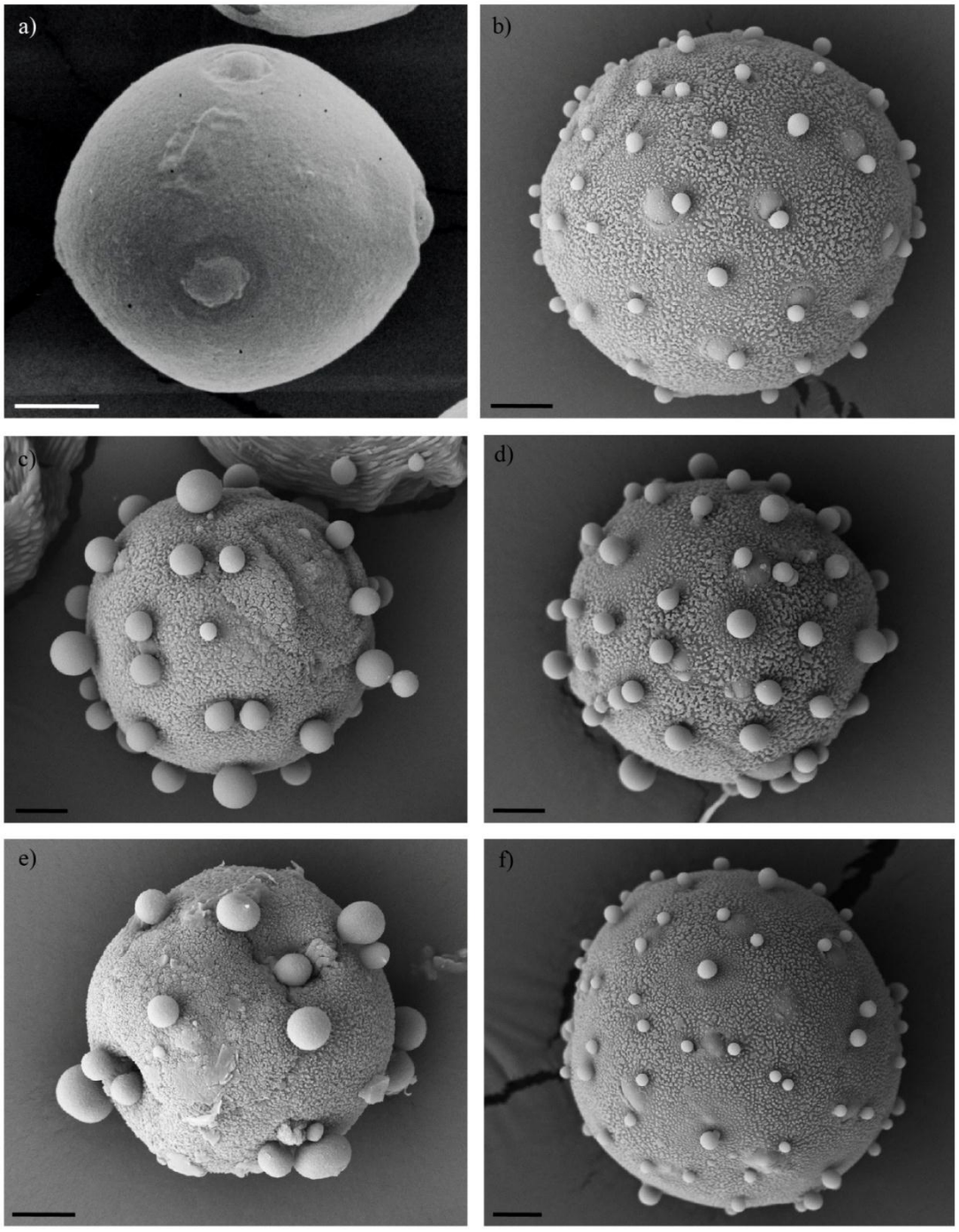


Figure 4.6*:* Scanning electron micrographs of pseudomonads in Group VII (*Leucopogon s.l. p.p.*): a) *Astroloma* sp. Baal Gammon, b) *Leucopogon fletcheri*, c) *L. juniperinus*, d) *L. neoanglicus*, e) *L. setiger*, f) *L. sonderensis.* c and e from C. Quinn (unpubl.). Pollen grains in this group have perforate or granulate ornamentation, 6 apertures, 30 – 70 µm, and annulus present. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

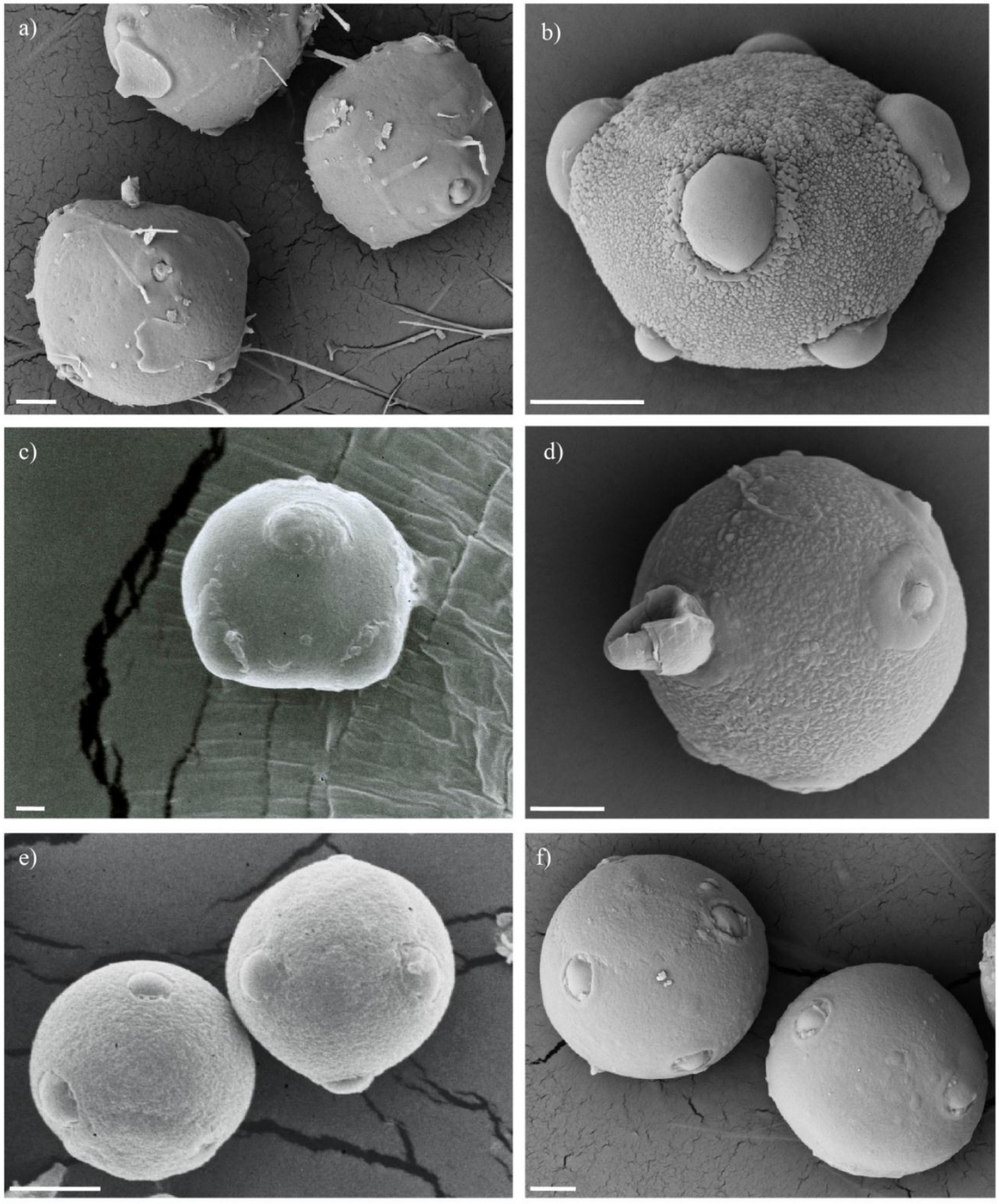


Figure 4.7*:* Scanning electron micrographs of pseudomonads in Group VIII (*Leucopogon conostephioides* complex): a) *Leucopogon conostephioides*, b) *L. pubescens*, c) *L.* sp. Newdegate, d) *L.* sp. short style. Pollen grains in this group have rugulate ornamentation, 6 apertures, 20 – 32 μm, and annulus absent. Voucher information can be found in Appendix 4.1. Scale bars = 3 μm.

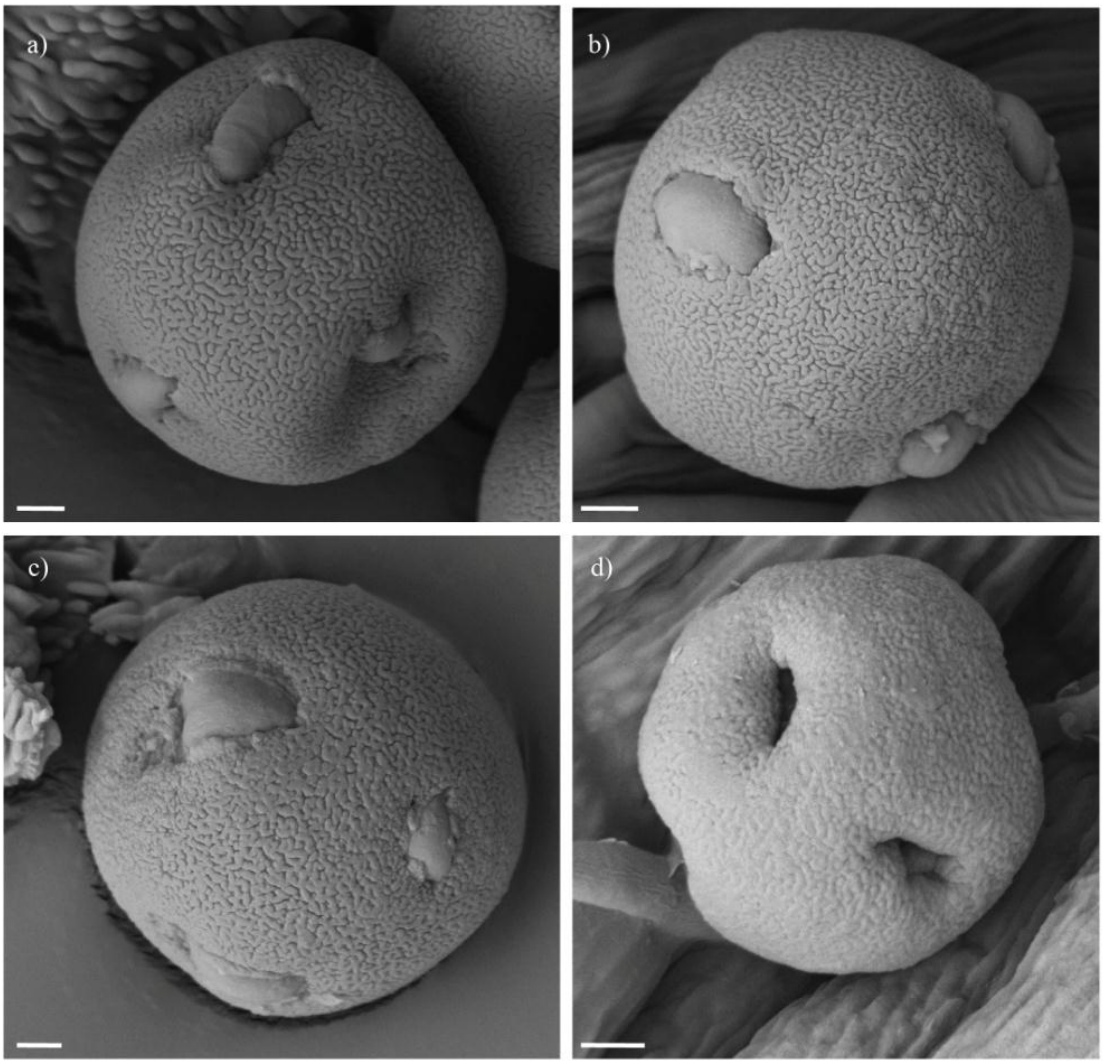


Figure 4.8*:* Scanning electron micrographs of pseudomonads in Group IX (*Stomarrhena*): a) *Astroloma stomarrhena*, b) *A. xerophyllum*, c) *Leucopogon* sp. ciliate Eneabba. Pollen grains in this group have psilate, granulate ornamentation, >6, 6 apertures, 45 – 60 µm, and annulus absent. Group XI (*Leucopogon blepharolepis* + *L.* sp. Moore River): d) *L. blepharolepis*. Pollen grains in this group exhibit rugulate ornamentation, 4 apertures, 30 – 40 µm, and annulus present. a, b from A. Wilson (unpubl.). Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

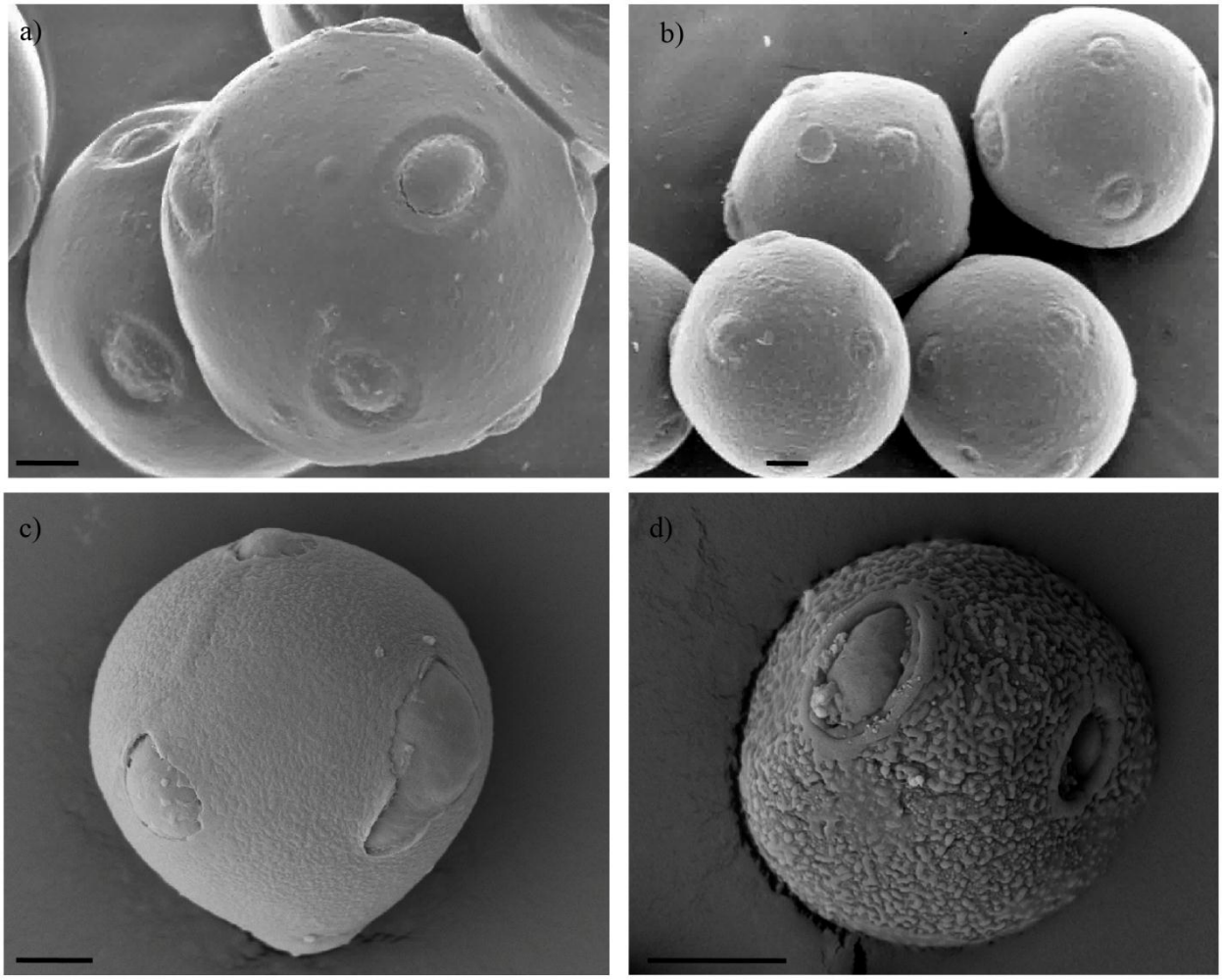


Figure 4.9 Scanning electron micrographs of pseudomonads in Group X (*Leucopogon s.l. p.p*): a) *Leucopogon appressus*, b) *L. crassiflorus*, c) *L. crassifolius*, d) *L. cordifolius*, e) *L. cymbiformis*, f) *L. ericoides*. b – f from C. Quinn (unpubl.). This is the most heterogeneous of the groups with ornamentation that varies from psilate, perforate, or granulate, usually 3-4 apertures, 15 – 45 µm and annulus absent or present. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

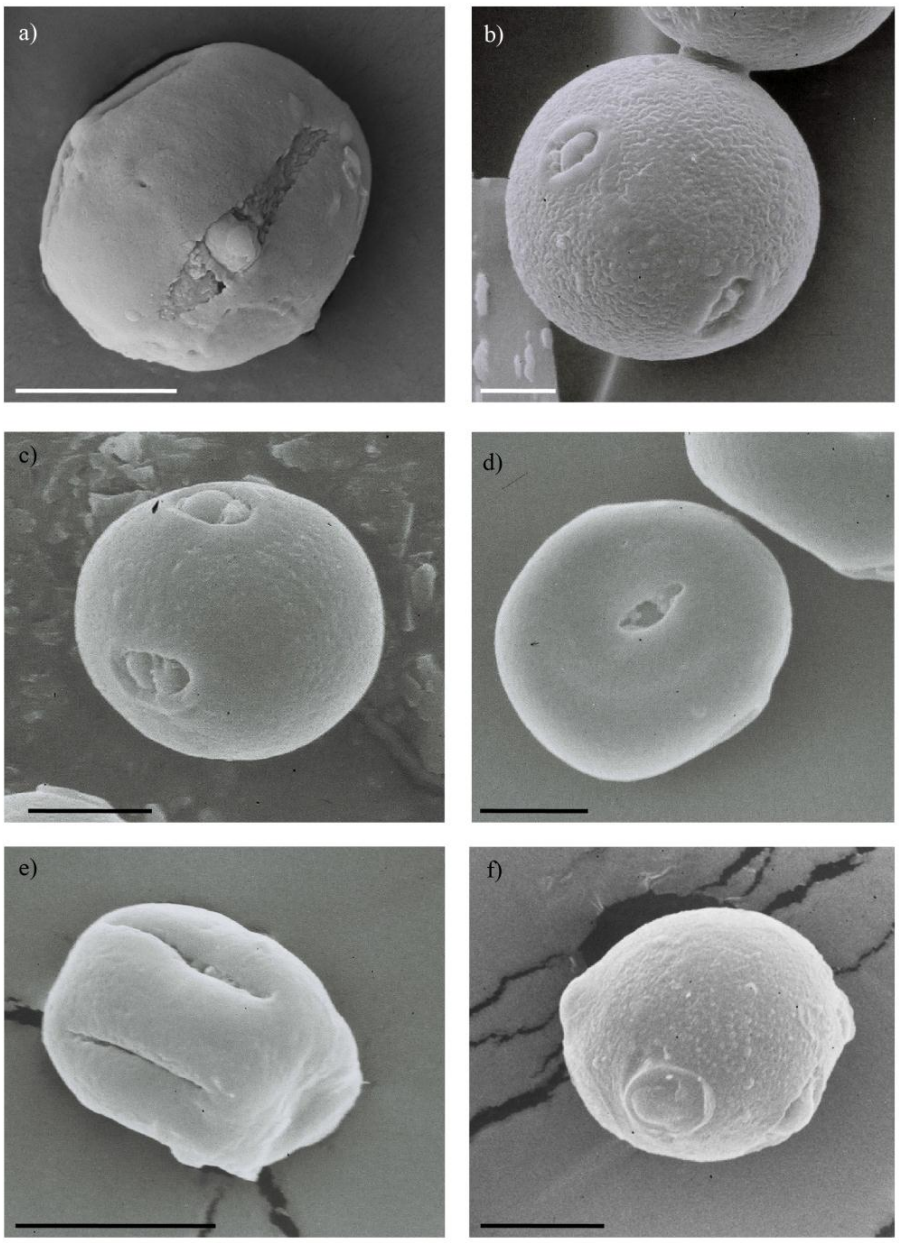


Figure 4.10 Scanning electron micrographs of pseudomonads in Group X (*Leucopogon s.l. p.p.*): a) *Leucopogon leptospermoides*, b) *L. muticus*, d) *Croninia kingiana*, d) *L. ruscifolius*. b and c from C. Quinn (unpubl.) This is the most heterogeneous of the groups with ornamentation that varies from psilate, perforate, granulate, or verrucate in *Croninia kingiana*, usually 3-4 apertures, (except for *C. kingiana* with 6), 15 – 45 μm and annulus absent or present. Voucher information can be found in Appendix 4.1. Scale bars = 10 μm.

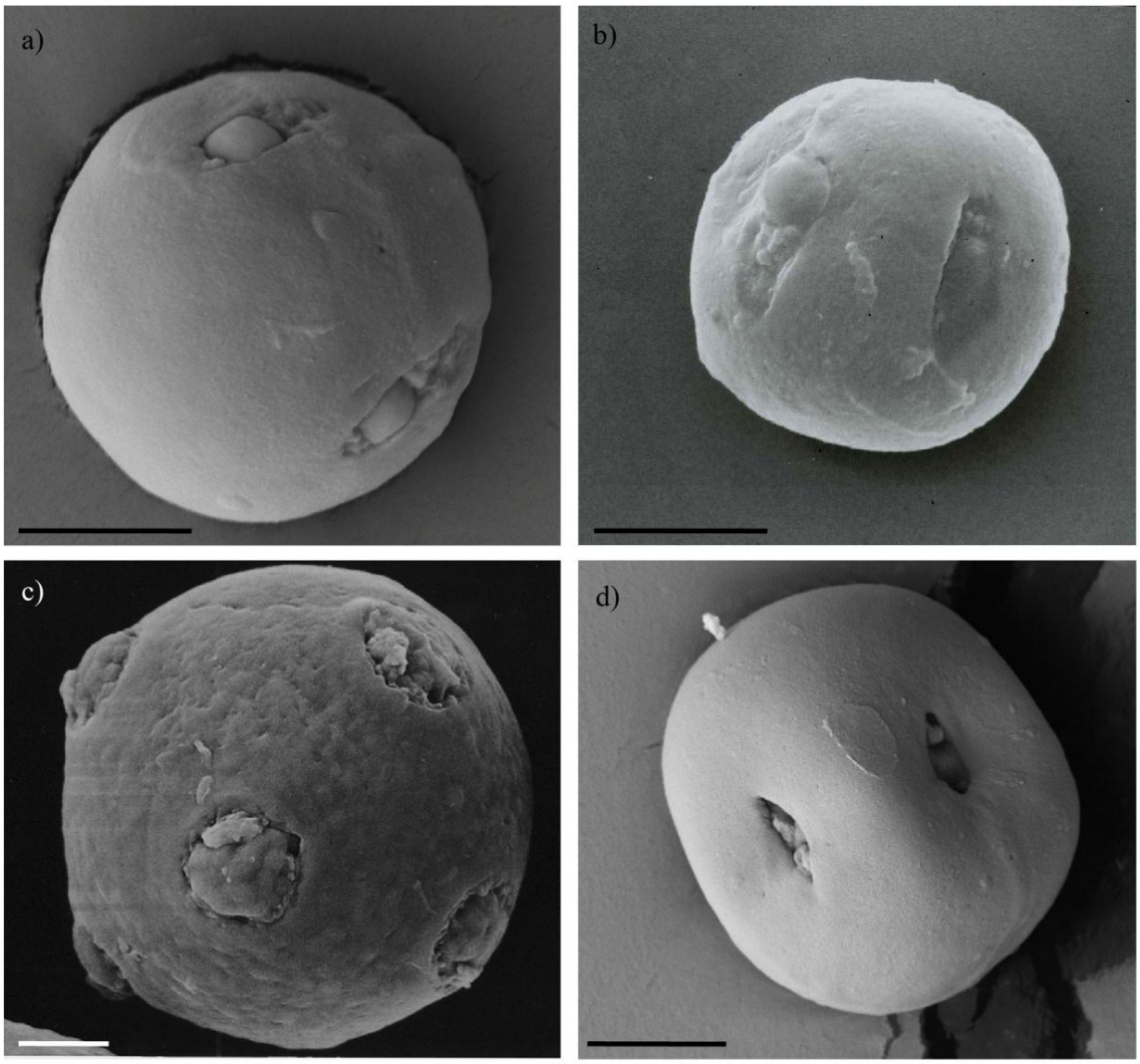


Figure 4.11 Scanning electron micrographs of pseudomonads of the ungrouped taxa: a) *Leucopogon esquamatus*: areolate ornamentation, 4,5 apertures, 35 – 40 µm, annulus absent (C. Quinn, unpubl.); b) *Styphelia exarrhena*: areolate ornamentation, 5,6 apertures, ~28 µm, annulus absent; c) *Styphelia hainesii*: areolate, 4,5 apertures, 40 – 50 µm, annulus absent; d) *Styphelia pulchella*: gemmate, verrucate, >6 apertures, ~35 µm, annulus absent. b and d from Streiber, 1999. Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

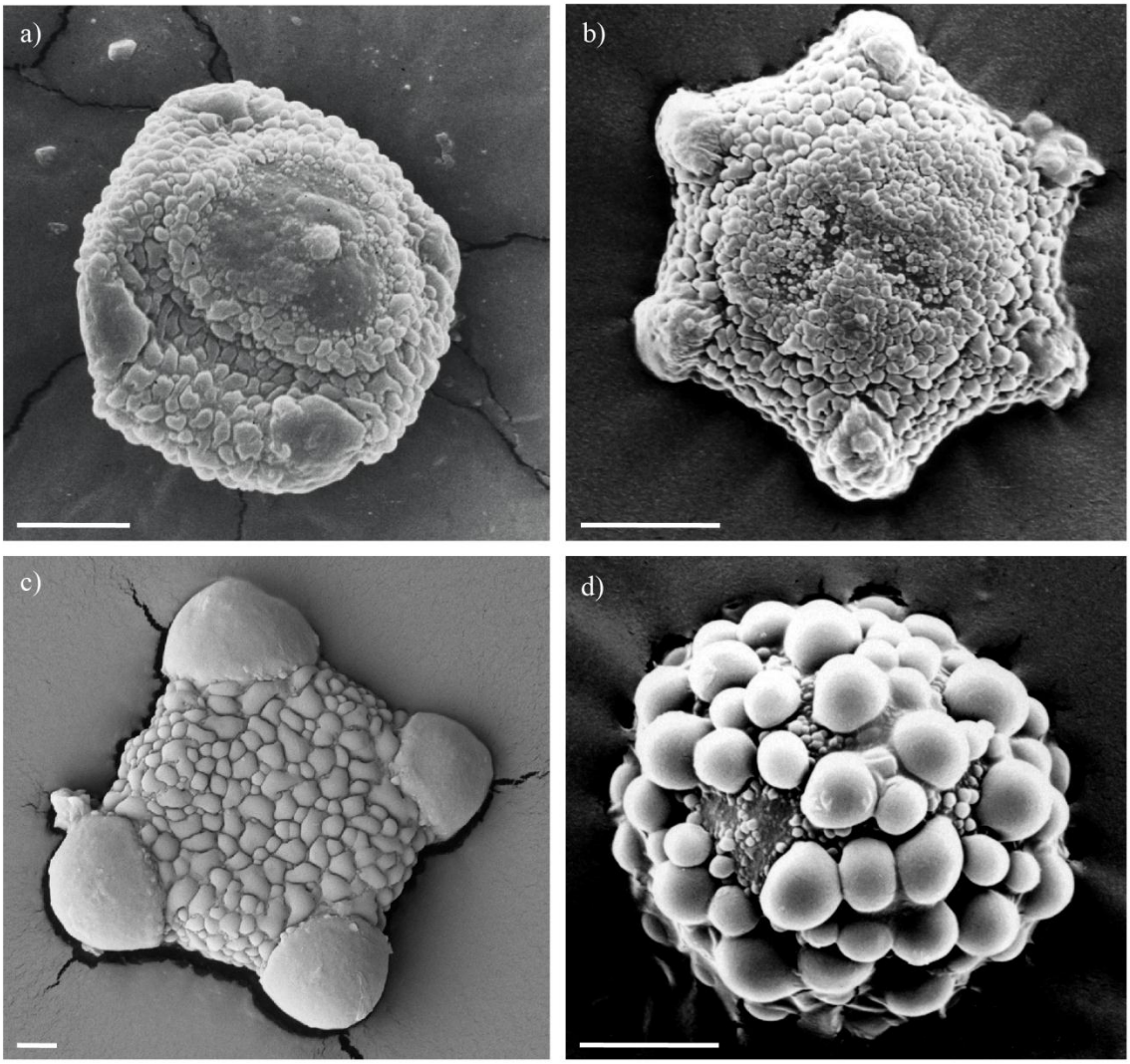


Figure 4.12*:* Scanning electron micrographs of pollen grains in *Stenanthera* (A-Type): a) *Astroloma conostephioides,* b) *A. pinifolium.* c) *A.* sp. Grass Patch. *Brachyloma:* d) *Astroloma baxteri* (pseudomonad)*,* e) *Brachyloma scortechinii,* f) *B.daphnoides*. a and f from C. Quinn (unpubl.); d and e from Streiber, 1999; b from A.J.G. Wilson, unpubl. Voucher information can be found in Appendix 4.1. Scale bars = 20 µm.

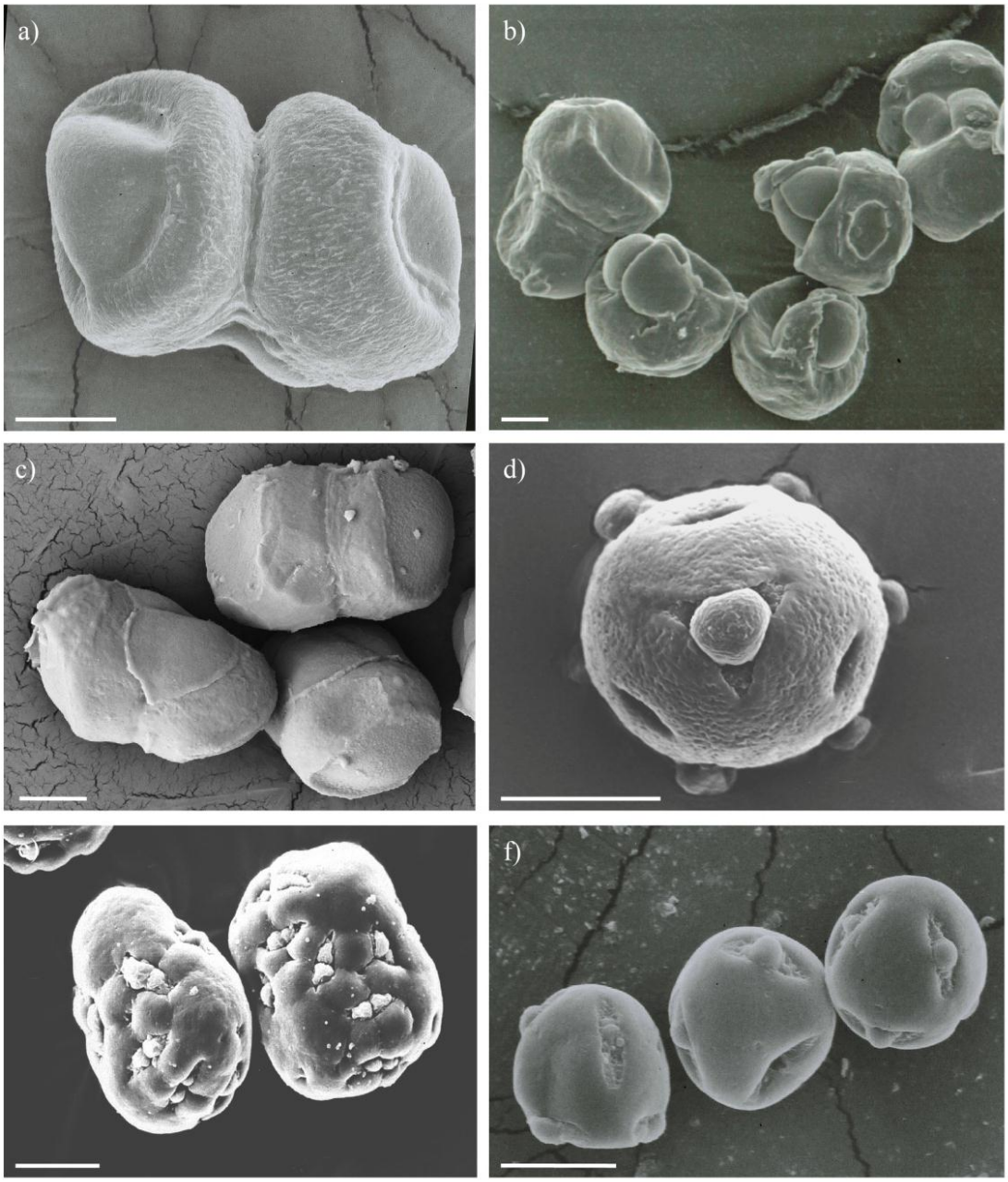


Figure 4.13 Scanning electron micrographs of pollen grains in *Leucopogon s.s.* (pseudomonads): a) *Leucopogon amplexicaulis*, b) *L. australis*, c*) L. bossiaea*, d) *L. virgatus*. *Lissanthe*: e) *Lissanthe pluriloculata* (A-Type) f) *L. strigosa* subsp. *subulata* (T-Type). All images except d from C. Quinn (unpubl.). Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

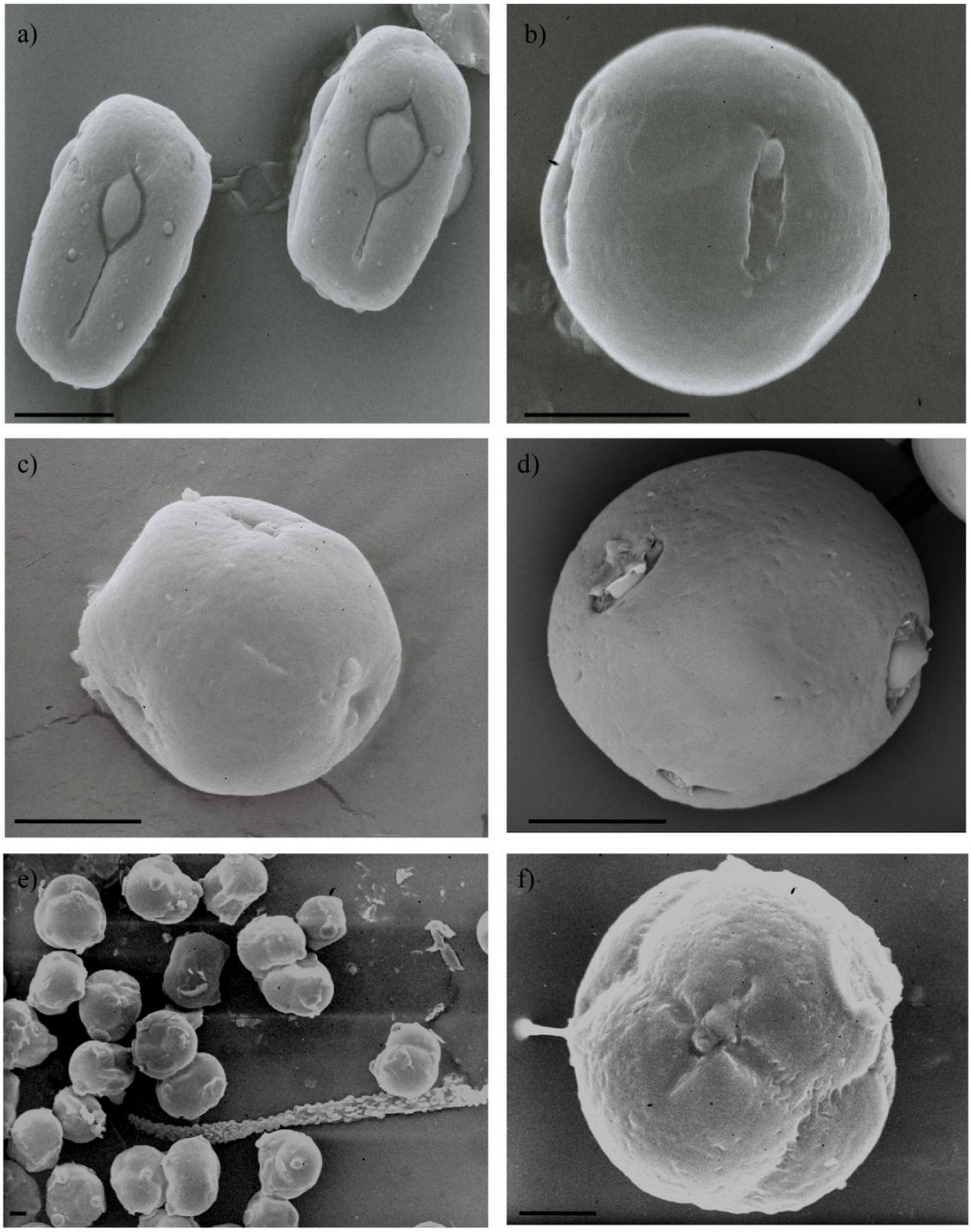


Figure 4.14 Scanning electron micrographs of pollen grains in *Acrothamnus* (A-Type): a)

Acrothamnus colensoi, b) *A. hookeri*, c) *A. maccraei*, d) *A. suaveolens*. *Monotoca* (pseudomonads): e) *Monotoca elliptica*, f*) M. rotundifolia*. a, c – f from C. Quinn (unpubl.). Voucher information can be found in Appendix 4.1. Scale bars = 10 µm.

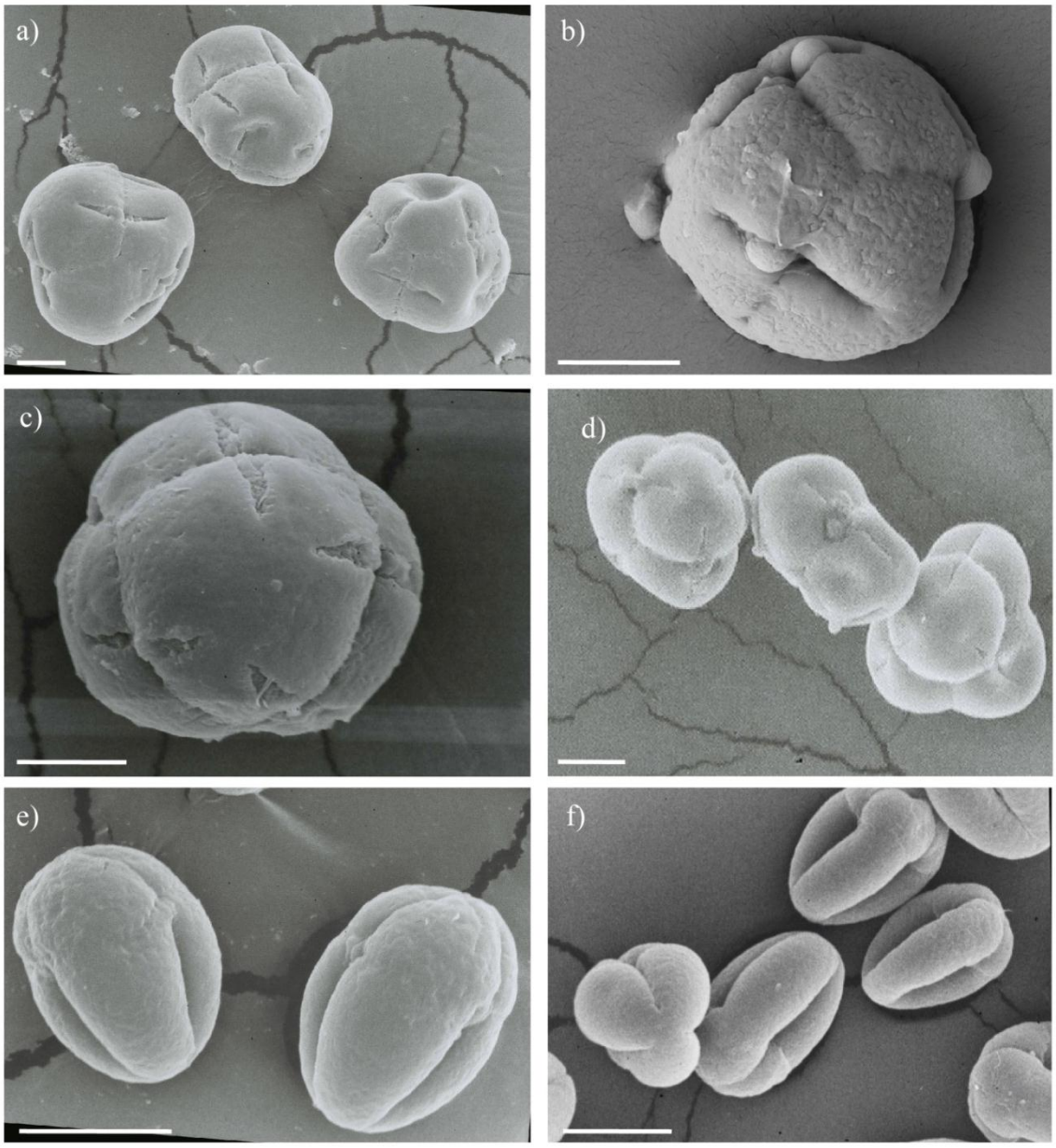


Figure 4.15: Scanning electron micrographs of pollen grains in *Leptecophylla* (A-Type): a) *Leptecophylla abietina,* b) *L. juniperina. Pentachondra:* c) *Pentachondra involucrata* (A-Type), d) *P. pumila* (T-Type). *Oligarrheneae:* e) *Needhamiella pumilio* (A-Type), f) *Oligarrhena micrantha* (pseudomonad). Images provided by C. Quinn, unpubl. Voucher information can be found in Appendix 4.1. Scale bars = 10 μm

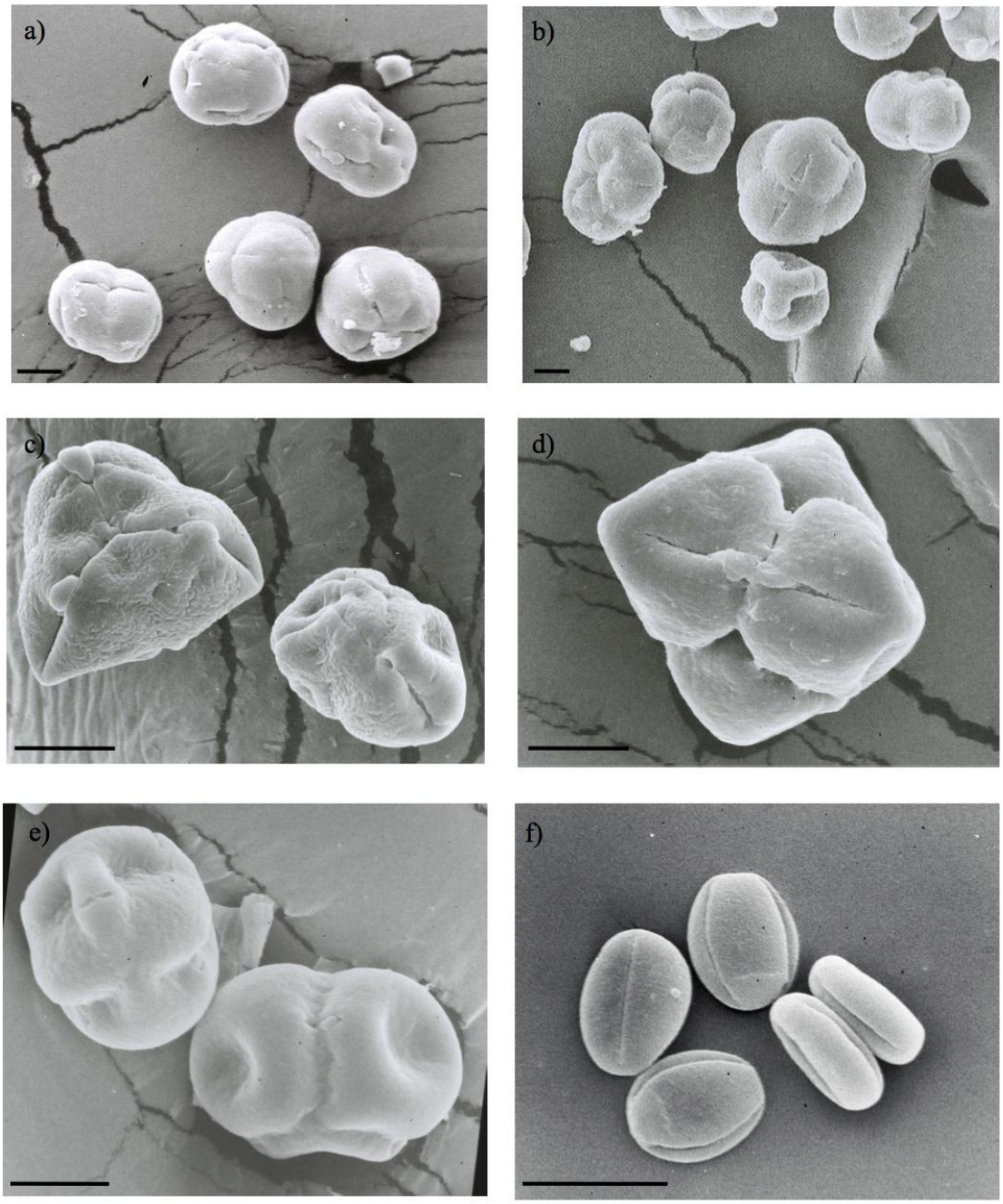
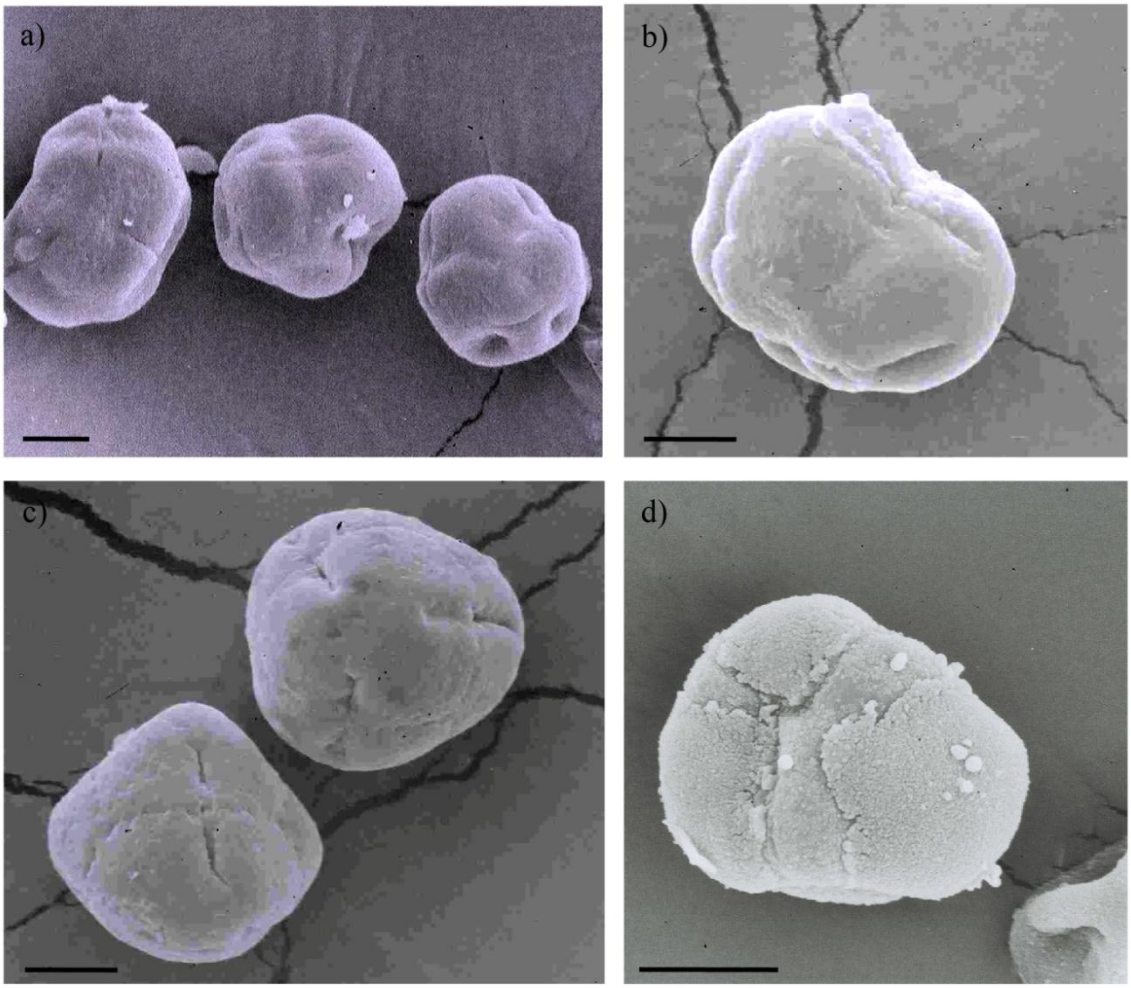


Figure 4.16 Scanning electron micrographs of pollen grains *Acrotriche*: a) *Acrotriche affinis*, b) *A. cordata*, c) *A. patula*. *Conostephium*: d) *C. pendulum*. Images provided by C. Quinn (unpubl.). Voucher information can be found in Appendix 4.1. Scale bars = 10 μm.



4.3.1 Pollen type

Pseudomonads were present in all species sampled within the *Styphelia-Astroloma* clade (Table 4.1, Figure 4.1– 4.11) as well as in *Monotoca* and *Leucopogon s.s.* (which are placed outside the *Styphelia-Astroloma* clade) (Figure 4.13.a–d; 4.14.e, f). *Acrothamnus, Acrotriche, Conostephium, Leptecophylla, Pentachondra involucrata, Stenanthera* and *Needhamiella pumilo* all exhibit permanent tetrads with different levels of abortion (A-type) (Figures. 4.12, 4.14 – 4.16). Permanent tetrads (T-type) were observed in *Brachyloma* (Figure. 4.12.f), *Lissanthe* (Figure. 4.13.e, f) and *Pentacondra pumila,* (Figure. 4.15.d). None of the species sampled from the

Styphelia-Astroloma clade displays T or A-type pollen. The species included from Epacrideae, Cosmelieae, Prionoteae and Richeeae are reported in the literature to exhibit regular tetrads (T-type). True monads were not found in any of the taxa included in this study.

4.3.2 Pollen morphology

Eight different exine ornamentation types were observed in the *Styphelia-Astroloma* clade, from smooth (psilate) to highly ornamented pollen grains (e.g. gemmate, verrucate, rugulate). Perforate and psilate ornamentations are the most common. They are present in Group I (*Astroloma s.s.*), Group III (*Styphelia*), and Groups IV, VII and X (*Leucopogon s.l. p.p*). Species of Group VI (*Styphelia s.s.*) are the only ones with both globular exine elements larger than 1 μm (gemmate) and elements of variable size and shape, all smaller than 1 μm (granulate) (Figure 4.5.b–f). Although *S. pulchella* has gemmate ornamentation as well, it also shows wart-like exine elements (verrucate) (Figure 4.11.d). In *S. pulchella,* the globular ornamentation elements are generally larger in diameter and more densely distributed than in *Styphelia s.s.*; *L. esquamatus, S. exharrena,* and *S. hainesii* are the only ones with areolate ornamentation (Figure 4.10.a–c). Rugulate ornamentation was observed in all the taxa sampled from Group VIII (*L. conostephioides* complex) (Figure 4.7) and Group XI (*L. blepharolepis*) (Figure 4.8.d). Group X includes taxa with five different ornamentation types: psilate, perforate, granulate and verrucate (Figures 4.9; 4.10).

The number of apertures in the *Styphelia-Astroloma* clade varies between six and three (Figures 4.1 – 4.11), with the exception of *Styphelia s.s.* and the Western Australian *Styphelia* Group II, which have numerous apertures (>6). Generally, there is no variation in the number of apertures within the Groups, except for *Leucopogon s.l. p.p.* Group X with 3, 4 (*L. crassiflorus*) and >6 (*Croninia kingiana*) apertures. Infraspecific variation was observed in *L. fletcheri* (Group VII) with 6-7 apertures, *L. crassifolius* and *L. ericoides* (Group X) with 4 – 5 apertures.

A slightly thickened annulus was observed in some members of Group I (*Astroloma s.s.*), e.g. *A. prostratum* (Figure 4.1.e), *A.* sp. Dumbleyung (Figure 4.1.f), *A.* sp. Nannup (Figure 4.2.c), V (*L. cordifolius, L. oxycedrus, L. propinquus, L. strictus*) (Figure 4.4.b–f), X (*L. crassifolius, L. crassiflorus, L. ericoides*, and IX (*L.* sp. ciliate Eneabba) (Figure 4.8). All of the *Leucopogon s.l.*

Table 4.1 Summary of the pollen character states present in Groups I-XI in the *Styphelia-Astroloma* clade (Figure 2.1). The character states for each taxon sampled can be found in Appendix 4.1.

Group	Pollen type	Ornamentation	No. apertures	Size (µm)	Annulus
I: *Astroloma s.s.*	Pseudomonad	Psilate, perforate	6	45 – 110	Absent or Present
II: *Styphelia s.l.*	Pseudomonad	Verrucate	>6	35 – 48	Absent
III: *Styphelia*	Pseudomonad	Perforate	>6, 6	20 – 28	Absent
IV: *L. rotundifolius* + *L. cuneifolius*	Pseudomonad	Psilate to perforate	6	20 – 28	Absent
V: *Leucopogon s.l. p.p.*	Pseudomonad	Psilate, perforate	>6, 6	25 – 45	Absent
VI: *Styphelia s.s.*	Pseudomonad	Gemmate, granulate	>6	45 – 80	Absent
VII: *Leucopogon s.l. p.p.*	Pseudomonad	perforate, granulate	>6, 6	30 – 70	Present
VIII: *Leucopogon conostephioides* complex	Pseudomonad	Rugulate	6	20 – 32	Absent
IX: *Stomarrhena*	Pseudomonad	Psilate, granulate	>6, 6	45 – 60	Absent
X: *Leucopogon s.l. p.p.*	Pseudomonad	Psilate, perforate, granulate, verrucate	3-6*	15 – 45	Absent or Present
XI: *L. blepharolepis* + *L.* sp. Moore River	Pseudomonad	Rugulate	4	30 – 40	Present
Leucopogon esquamatus	Pseudomonad	Areolate	4,5	35 – 40	Absent
Styphelia exarrhena	Pseudomonad	Areolate	5,6	~28	Absent
Styphelia hainesii	Pseudomonad	Areolate	4,5	40 – 50	Absent
Styphelia pulchella	Pseudomonad	Gemmate, verrucate	>6	~35	Absent

* Six apertures only in *Croninia kingiana*.

p.p. in Group VII (Figure 4.6) and *C. kingiana* (Figure 4.10.c) exhibit a raised annulus around the pores. *Astroloma xerophyllum* and *A. stomarrhena* (Group IX) have a rather depressed, instead of a thickened, annulus (Figure 4.8.a, b).

Group I (*Astroloma s.s.*) exhibit the largest pollen grains (45 – 110 μm) and Group VIII (*L. conostephioides* complex) the smallest (20 – 32μm). All taxa sampled in the *Styphelia-Astroloma* clade have more or less spherical or ovoid mature pseudomonads, except for some species of *Astroloma s.s.* (e.g. *Astroloma* sp. Nannup (Figure 4.2.c), *A. prostratum* (Figure 4.1.e), *A. pallidum* (Figure 4.1.d), *A. tectum* (Figure 4.2.e) which are hexagonal, and *S. exharrena* (Figure 4.11.b) with star-shape pollen grains.

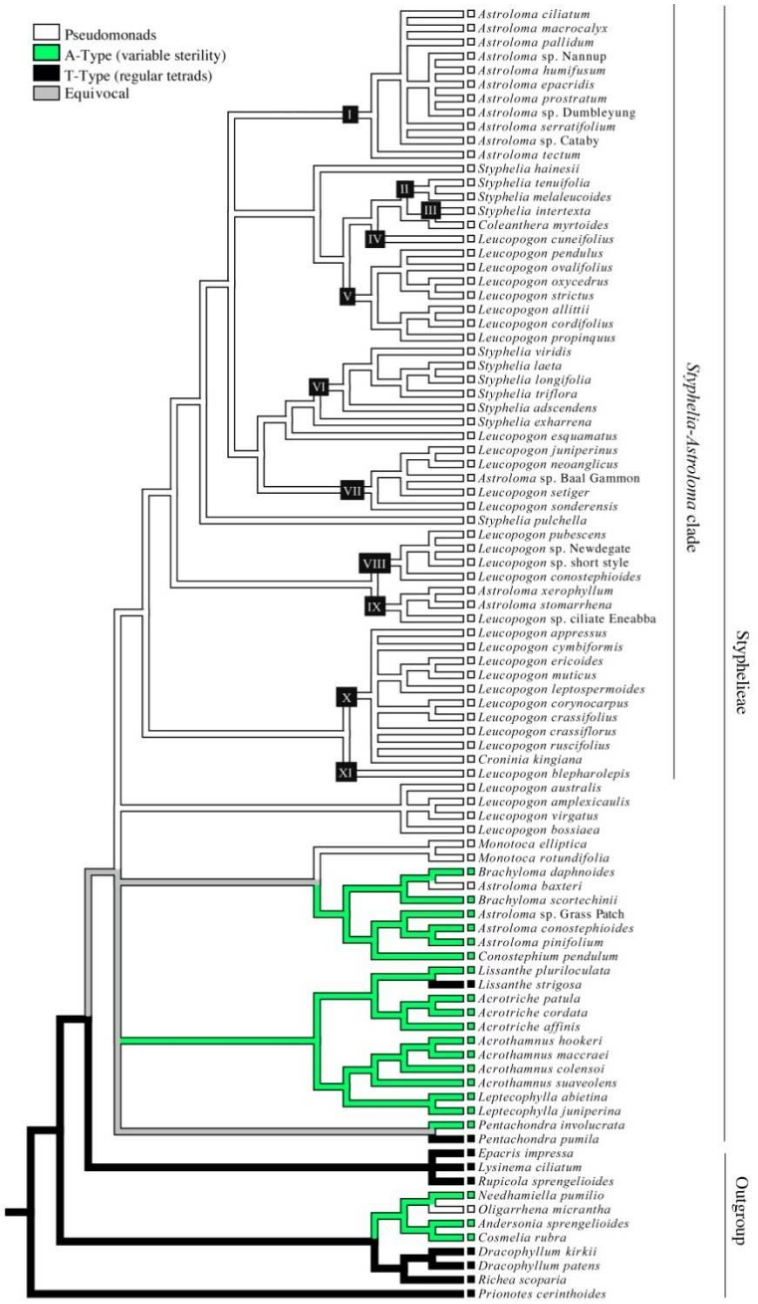
4.4 Discussion

4.4.1 Pollen type

The results of the extensive pollen type survey presented here confirm the conclusions reached by previous authors based on much less comprehensive sampling that normal tetrads are rare within the Styphelieae and true monads do not occur in the tribe (Smith-White 1955, McGlone 1978a, Kron *et al.* 2002, Furness 2009). Regular tetrads (T-type) are plesiomorphic, and A-type pollen has arisen at least twice in the Styphelieae. Pseudomonads are derived in the tribe with a single origin inferred within the *Styphelia-Astroloma* clade (Figure 4.17). This analysis however does not present strong evidence for pseudomonads being derived from A-type pollen as suggested by Furness (2009). A broader pollen sampling including all the genera of the tribe would help clarify the evolution of pseudomonads in the Styphelieae.

If the occurrence of pseudomonads was related to a particular pollination vector, it would be expected that the majority of the species within the Styphelieae would have similar pollinators. Instead, a broad range of pollinators has been reported within the tribe. *Conostephium* (A-type) is insect pollinated, *Brachyloma* (A and T-type depending on the species) and *Leptecophylla* (A-Type) have been reported to be bird pollinated. *Acrotriche* (T-type) , is pollinated by mammals and ants. Both *Astroloma* and *Styphelia* (S-type) are bird and insect pollinated. Hence, it seems unlikely that the occurrence of pseudomonads in the Styphelieae relates to their pollination syndrome, as the variation in their pollination vectors is not consistent with the variation in pollen type. Given the

Figure 4.17 Pollen type optimised in the Bayesian phylogenetic tree of Styphelieae using maximum parsimony. Branch colour corresponds to pollen types, as indicated in the box. Numbers I to XI correspond to the groups as per Chapter 2.



high diversity of floral morphology within the tribe, it seems more likely that the different pollination syndromes might be associated with floral features. Additional investigation of the pollination biology of the species within the *Styphelia-Astroloma* clade is needed to better estimate the diversity of pollination syndromes, their pattern of evolution and their relationships with respect to floral morphology.

Besides Styphelieae, pseudomonads have only been reported in Cyperaceae with remarkable developmental similarities. The fact that Cyperaceae are predominantly wind pollinated suggests once again that pollen type is not greatly influenced by pollination mechanisms. Furthermore, Cyperaceae is the only family in Poales that exhibits ovaries with one ovule per locule. Styphelieae is one of only two tribes in Ericaceae (the other is Oligarrheneae) with ovaries that contain a single ovule per locule. As suggested, the occurrence of pseudomonads could potentially relate to the reduction in number of seeds per fruit given that fully fertile tetrads may become superfluous and only one fertile grain may be necessary to fertilize each ovule. Yet the presence of A-Type pollen in members of the multiseeded tribes Oligarrheneae, Cosmelieae and Richeae (Furness, 2009) argues against this theory.

Schneemilch and Kokkinn (2011) found a correlation between the proportion of tetrad types and the variation in floral colouration among six species of *Acrotriche* (A-Type), and suggested it as a possible indicator of phylogenetic relationships within the genus. It seems unlikely that this correlation would be applicable on a larger scale (i.e. across the *Styphelia-Astroloma* clade) as variable sterility (A-Type pollen) is absent in all the taxa sampled within the clade and still floral colouration varies (white, cream, green, pink and red).

4.4.2 Exine ornamentation

The *Styphelia-Astroloma* clade displays the highest diversity in ornamentation in Epacridoideae, ranging from smooth (psilate) to areolate, gemmate, granulate, verrucate, and rugulate. Perforate ornamentation is present in Groups I, III, IV, VII and X. Species of *Styphelia s.s.* (Group VI) share gemmate ornamentation with *S. pulchella,* but differ by having granulate instead of verrucate ornamentation under the globular exine elements (Figures. 4.3.b–f; 4.11.d) as well as in the diameter and distribution of their exine elements. Unrelated taxa exhibit the same

ornamenation type, e.g. *L. esquamatus, S. exharrena,* and *S. hainesii* with areolate ornamentation (Figure. 4.11.a–c); *L. conostephioides* complex (Group VIII) (Figure 4.7.) and *L. blepharolepis* (Figure. 4.8.d) with rugulate ornamentation. Group X is the most diverse with respect to ornamentation. It includes five different types: psilate, perforate, granulate and verrucate (Figure. 4.9; 4.10). Each ornamentation type has arisen independently in multiple lineages in the *Styphelia-Astroloma* clade (Figure 4.18).

Pollen ornamentation types have been associated with pollination syndromes in some groups of plants. Smooth (psilate) pollen grains are often present in plants that are wind/water pollinated whereas sculptured pollen grains are characteristic of plants pollinated by biotic vectors. Several studies (Hesse, 1981; Ferguson and Pearce, 1986; Whitehead, 1969; Sannier *et al.* 2009) have shown however that the relationships between the ornamentation type and the pollination system depend on the family and vary among taxonomic groups. Further research on the possible links between exine ornamentation, pollination syndromes and floral morphology in the *Styphelia-Astroloma* clade would improve our understanding of their biology and allow more accurate interpretations on the patterns of exine ornamentation diversity. Nevertheless, such analyses are currently unfeasible because the pollination syndrome has not been determined for the majority of the Styphelieae.

4.4.3 Pollen apertures

The Styphelieae are the most morphologically diverse tribe in the Epacridoideae as well as the most geographically widespread and species-rich (over 320 species are currently recognized). Crayn and Quinn (2000) suggested that rapid cladogenesis in Styphelieae might have occurred as a result of the development of the indehiscent fleshy fruit in this lineage, which likely increased dispersal potential and thus exposure to novel environments. The largest diversity of the tribe occurs in the *Styphelia-Astroloma* clade (ca. 200 species). Interestingly, the number of apertures in this clade rises from three to four or more apertures. With the exception of some species within Group X, which diverged relatively early within the clade, all the species sampled have more than three apertures. Generally, there is no variation in the number of apertures within the groups, except for Group X with three, four (*L. crassiflorus*) and more than six (*Croninia kingiana*) apertures.

Figure 4.18 Exine ornamentation optimised in the Bayesian phylogenetic tree of Stypehlieae using maximum parsimony. Branch colour corresponds to ornamentation type, as indicated in the box. Numbers I to XI correspond to the groups as per Chapter 2.

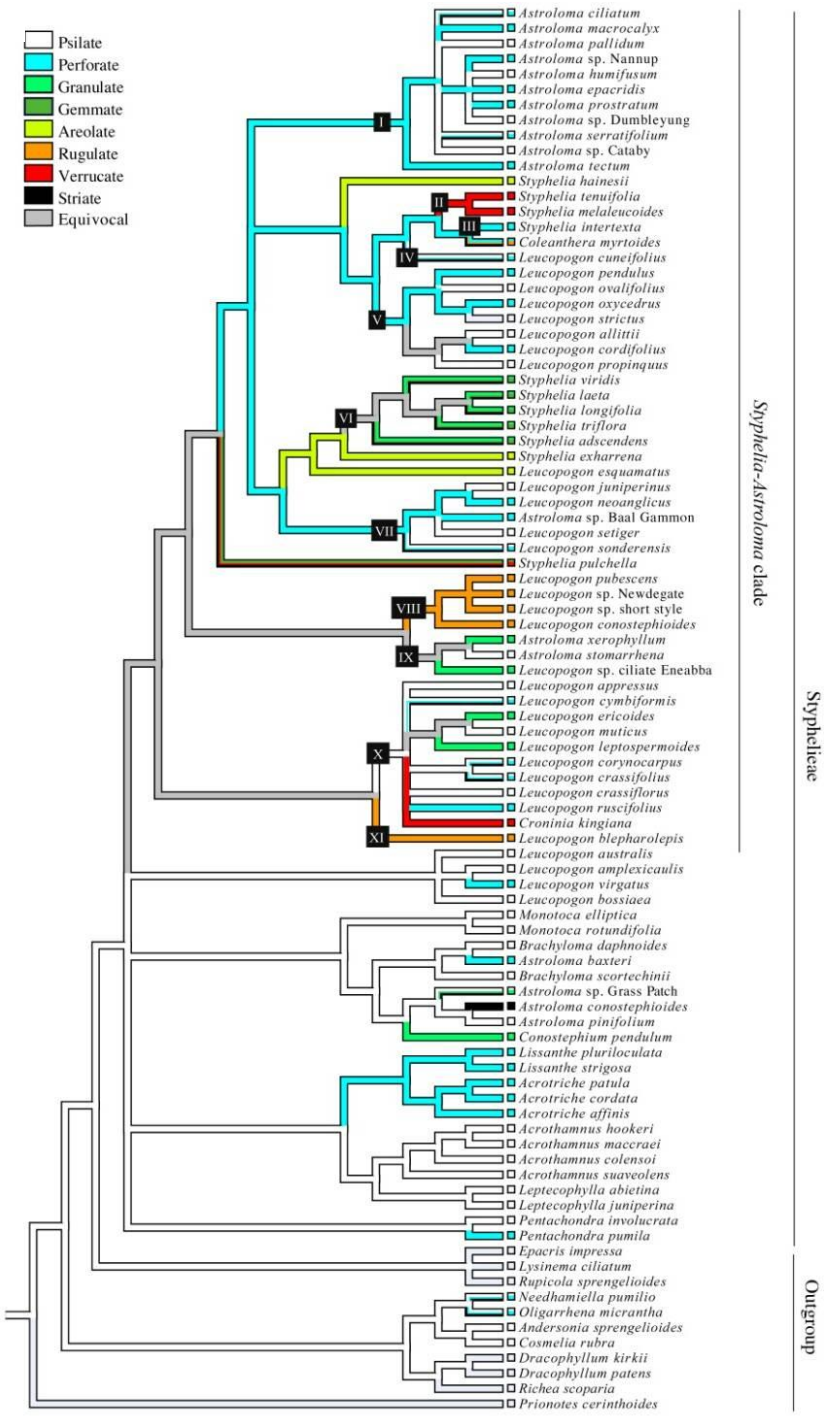


Figure 4.19 Number of apertures optimised in the Bayesian phylogenetic tree of Styphelieae using maximum parsimony. Branch colour corresponds to the number of apertures, as indicated in the box. Numbers I to XI correspond to the groups as per Chapter 2.

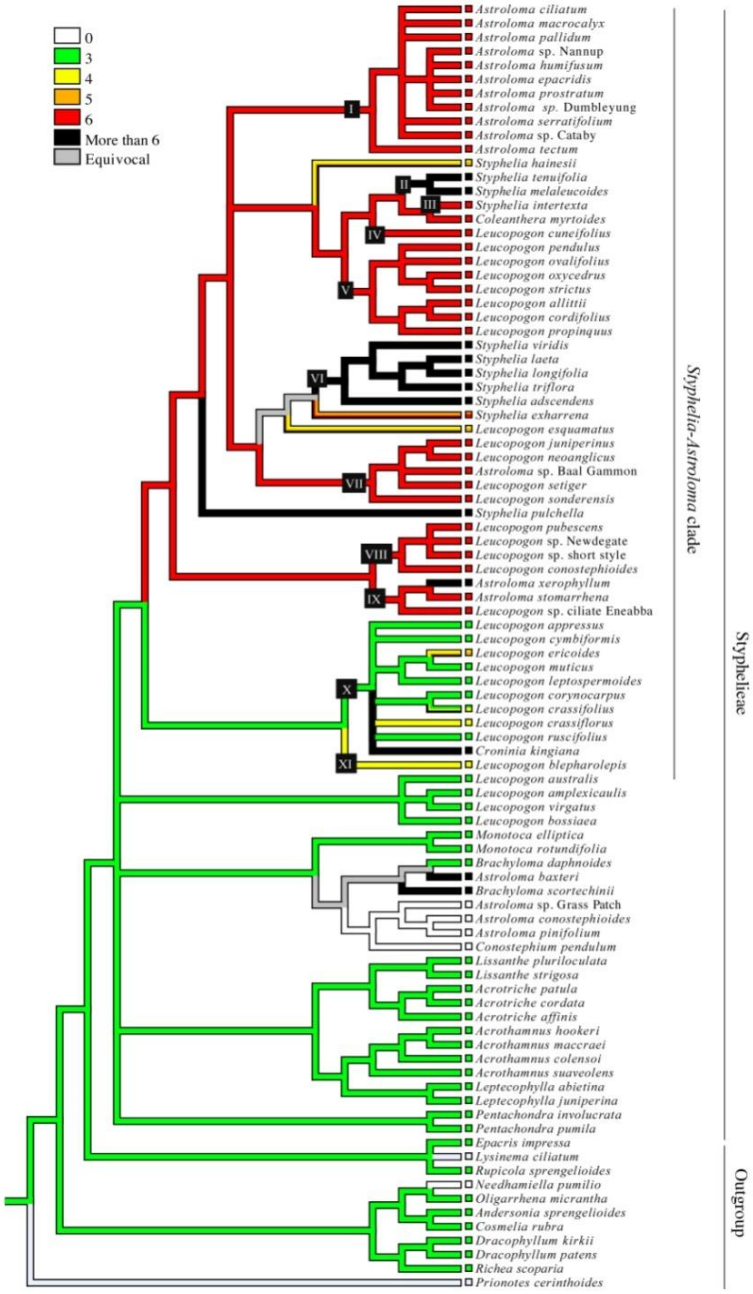


Figure 4.20 Presence/absence of a thickened annulus optimised in the Bayesian phylogenetic tree of Stypehlieae using maximum parsimony. Branch colour corresponds to presence/absence of an annulus, as indicated in the box. Numbers I to XI correspond to the groups as per Chapter 2

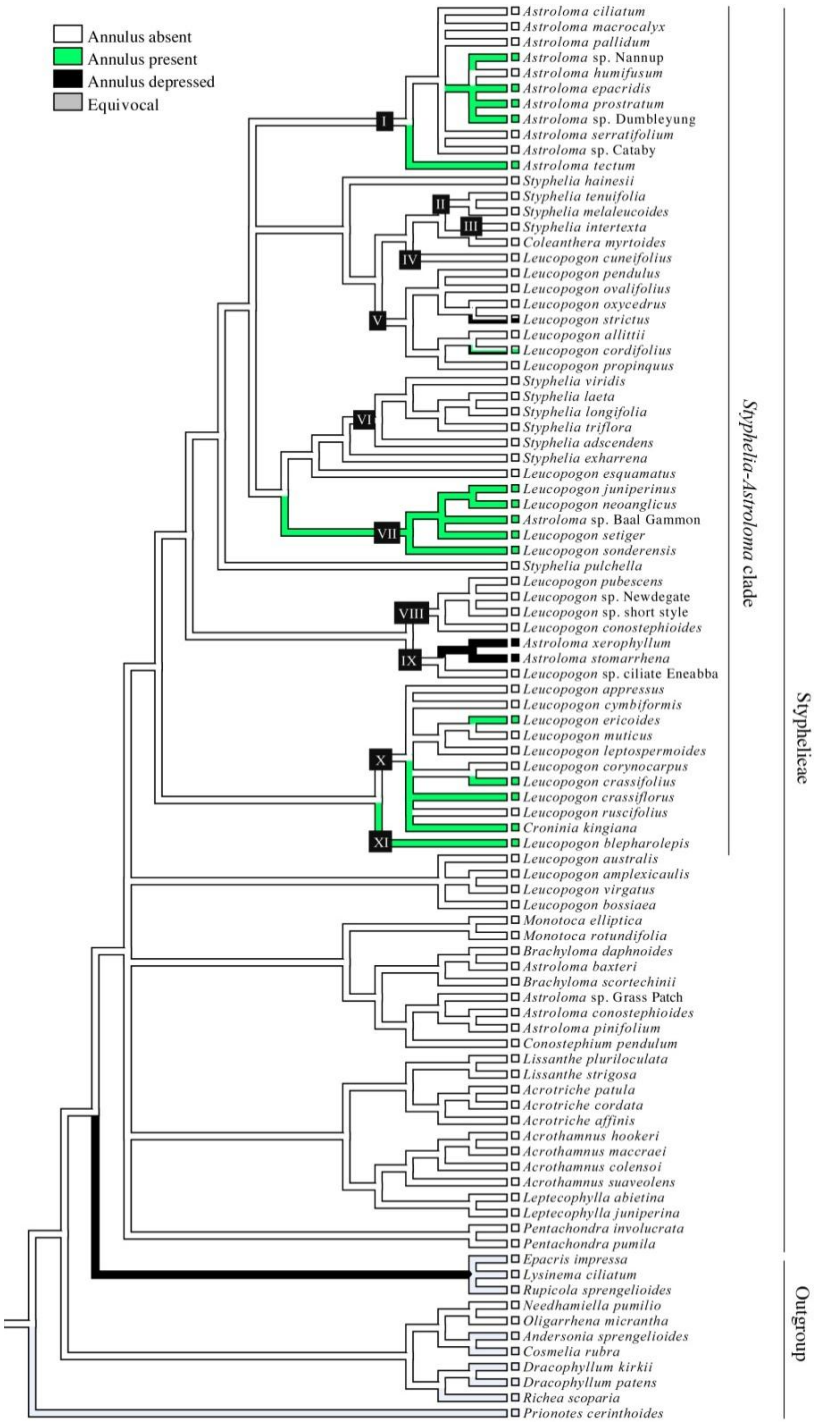
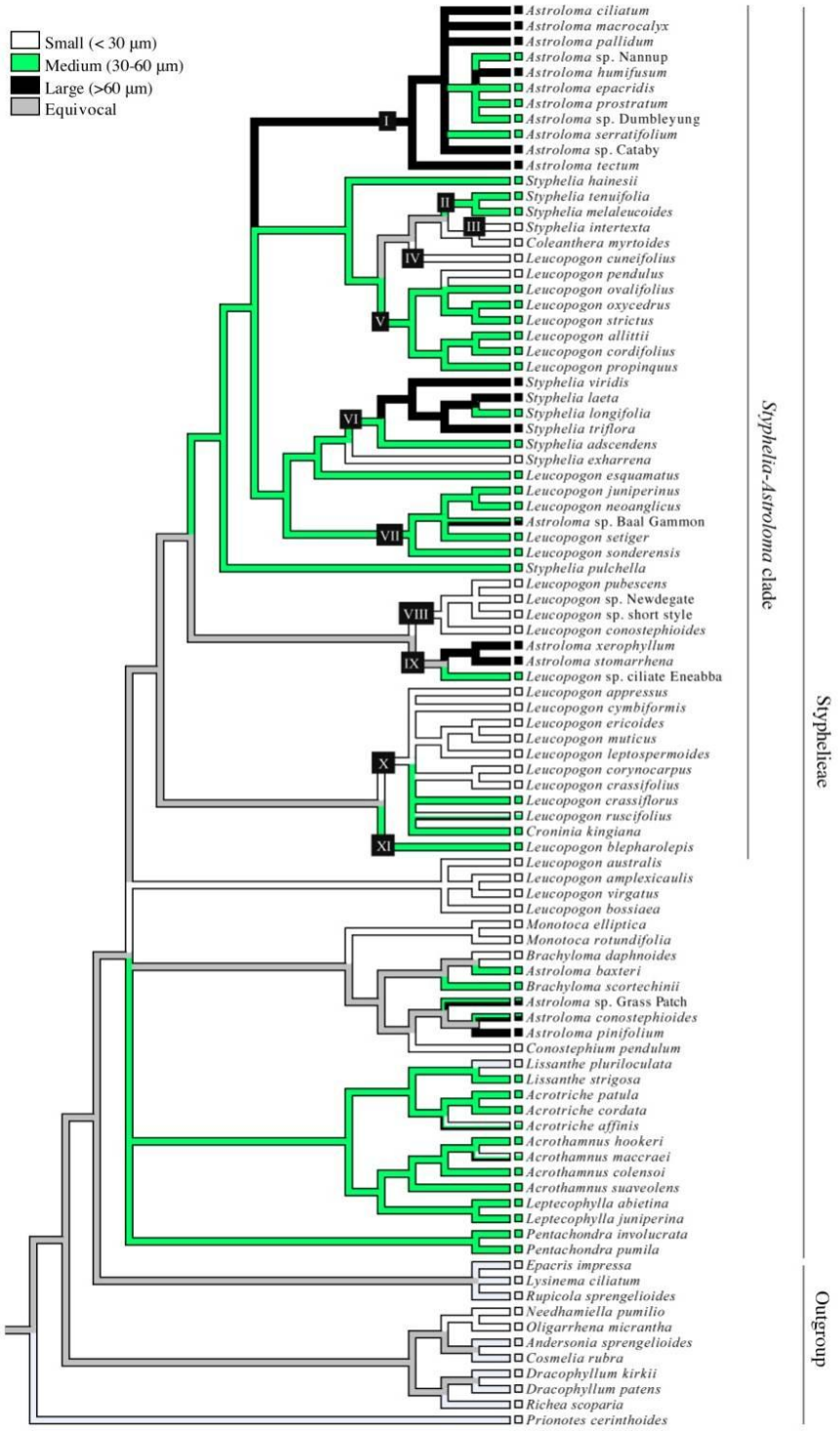


Figure 4.21 Size of the pollen grain optimised in the Bayesian phylogenetic tree of Styphelieae using maximum parsimony. Branch colour corresponds to pollen size, as indicated in the box. Numbers I to XI correspond to the groups as per Chapter 2



Besides *Astroloma baxteri* (Figure 4.12.d) and *Brachyloma scortechinii* (Figure 4.12.e), none of the taxa sampled outside the *Styphelia-Astroloma* clade possess more than three apertures. Three-aperturate pollen is plesiomorphic in the Styphelieae. Pollen grains with more than three apertures have emerged independently in the tribe and become more common in the *Styphelia-Astroloma* clade, where only some species appear to have retained 3-aperturate pseudomonads (Figure 4.19).

Furness and Rudall (2004) argued that a high number of pollen apertures, which increases the prospective germination sites and facilitates contact between at least one aperture and the stigmatic surface, is generally associated with high radiation in eudicots as it may provide a selective advantage by optimizing fertilization rates. The association between an increase in the number of pores and the fleshy fruit are potentially important factors driving the high diversification of the *Styphelia-Astroloma* clade. The potential influence of these traits in the diversification rates in the *Styphelia-Astroloma* clade could be investigated using analytical tools to estimate diversification parameters in a Bayesian framework, which accounts for uncertainties in the divergence times and incomplete taxon sampling.

None of the taxa sampled outside the *Styphelia-Astroloma* clade shows a thickened annulus around the aperture (Figures 4.12 – 4.16). Absence of an annulus is plesiomorphic in the Styphelieae and in the *Styphelia-Astroloma* clade (Figure 4.20). Within the clade, the presence of an annulus occurs in some species within groups I, IX and X and it consistently occurs to some extent in all the taxa sampled from Group VII.

4.4.4 Shape and size

Pollen shape appears to be rather homogeneous in the *Styphelia-Astroloma* clade. With the exception of *Astroloma s.s.,* in which the pollen grains can be more or less hexagonal, shape is generally ovoid to spheroid in the taxa sampled. On the other hand, size is more diverse. *Astroloma s.s.* (Group I) exhibit the largest pollen grains (45 – 110μm; Figures 4.1; 4.2; Appendix 4.1). *Leucopogon s.l. p.p.* groups IV and VI usually have medium sized pseudomonads (25 – 50 μm). *Leucopogon conostephioides* complex (Group VIII) and *Leucopogon s.l. p.p.* Group X have the smallest (25 – 35 μm). Small (<30 μm), medium (30 – 60 μm) and large (>60) pollen grains appear to have emerged multiple times in the *Styphelia-Astroloma* clade. The plesiomorphic character

state for the clade was unclear. .

Williams and Rouse (1990) showed a significant correlation between pollen and pistil size in *Rhododendron* and that extreme disparity in pollen and pistil size acts as a reproductive barrier. Unlike other Ericaceae (e.g. *Gaultheria*, Lu *et al.* 2010; *Rhododendron*, Milne *et al.* 1999), hybrids have not yet been reported in the *Styphelia-Astroloma* clade. The lack of field evidence for morphological intermediates, chromosome counts (Smith-White, 1955) and the congruence between the chloroplast and the ribosomal nuclear DNA phylogenies (Figures 2.1, 2.2) supports the contention that hybridization has not been an important evolutionary process in the group. Given the observed heterogeneity in pollen size within the *Styphelia-Astroloma* clade, it is worth investigating the potential implications for their reproductive biology. Evaluation of pistil size and pollen/pistil size ratios across the clade would elucidate the potential association between pollen size disparities with the very low rate of hybridization in the clade.

4.4.5 Taxonomic utility of pollen morphological characters

The molecular phylogenetic analyses of the combined chloroplast markers (*rbc*L, *mat*K, *atp*B-*rbc*L, *trn*H-*psb*A) and the nuclear one (ITS) presented in Chapter 2 resolve 12 main lineages within the *Styphelia-Astroloma* clade. Although these groups are relatively congruent with the distribution of some of the traditional morphological characters (Chapter 2, discussion), the challenge still remains to find new morphological attributes to diagnose groups in a phylogenetic classification of the *Styphelia-Astroloma* clade.

Because pseudomonads occur universally in the *Styphelia-Astroloma* clade, pollen type character is of no utility in delimiting groups within the clade. Ornamentation, on the other hand, is highly variable and informative. Species of *Styphelia s.s.* (Group VI) can be identified solely by their exine ornamentation. They are the only group with both gemmate/granulate pollen grains. Similarly, *S. puchella* can be recognized by its gemmate/verrucate pollen grains. Nevertheless, ornamentation by itself is not always diagnostic since the same ornamentation type is present in multiple groups (Table 1, Figure 4.18). Number of apertures, size and presence of an annulus are also variable and become informative when combined. *Astroloma s.s.* pollen grains are psilate, 6-porate pseudomonads (S-type), 45 μm or larger in diameter, and sometimes with a prominent annulus. Although they are usually spheroidal, they can also be hexagonal in shape. Species of

Stenanthera (Figure 14a, b) (ungrouped but previously included in *Astroloma*) can be distinguished from *Astroloma s.s.* by their A-type pollen, with striate, or granulate ornamentation, and no visible pores. Similarly, *Stomarrhena*, Group IX (Figure 4.8) differs from *Astroloma s.s.* in having only spherical pseudomonads with 6 or more pores, psilate to granulate ornamentation, and occasionally a depressed annulus.

Leucopogon s.l. p.p. Group X (Figures 4.9; 4.10) can be discriminated from *Leucopogon s.l. p.p.* Groups V (Figures 4.4; 4.5.a) and VII (Figure 4.6) by having smaller pseudomonads (15 – 45 μm), with 3 – 4 sulci, equatorially distributed and an annulus always absent. Although very similar to *Leucopogon s.s.* (Figure 4.13.a–d), the ornamentation in Group X is perforate rather that psilate. Pollen grains from *Leucopogon s.l. p.p.* Species from Group VII are usually larger (30 – 70 μm) than Group V (25 – 45 μm) and posses a distinctly raised annulus. Pseudomonads in both *L. conostephioides* (Group VIII) and *L. blepharolepis* (Group XI) are rugulate. Yet species from the *L. conostephioides* complex can be discriminated from Group XI by the possession of smaller pseudomonads (20 – 32 against 30 – 40 μm) with six instead of four pores, and the lack of an annulus.

The finding of pollen features that are consistent with the phylogenetic groups identified within the *Styphelia-Astroloma* clade is important for the identification of fossil pollen. Fossil data play an important role in determining the antiquity of a group and its past distribution. In Styphelieae, fossils are uncommon. Macrofossils may show pleisomorphic characters that are absent in extant taxa, which prevent their accurate identification to genus/species levels. Prior to this study, the morphological patterns in pollen diversity in the tribe were undetermined. Consequently, the pollen fossil record for Styphelieae has probably been underestimated. A better knowledge of the pollen morphological characters and variation on the extant taxa may allow a more accurate identification of unassigned fossils and perhaps improve the identification of the existing records, which should lead to an improved reconstruction of the evolutionary history of the tribe.

4.5 Conclusions

Pseudomonads are universally distributed in the *Styphelia-Astroloma* clade. Hence, there is little prospect of pollen type proving to be of taxonomic use within that clade. Conversely, different

character combinations of exine ornamentation, number of pores, size and presence of a thickened annulus on the mature tetrads show promising taxonomic utility. With the exception of pollen type, which has a single origin in the clade, pollen morphological characters have derived independently among Groups I – XI.

The groups currently recognized as *Astroloma* - Group I (*Astroloma s.s.*), Group IX (*Stomarrhena*) and *Stenanthera* – are consistent with the differences observed in their pollen attributes. *Styphelia s.s.* (Group VI) and the *Styphelia* segregates (Groups II and III) differ primarily in ornamentation and size. *Leucopogon s.l. p.p.* Groups IV, V, VIII, and X differ in the number of apertures, size and presence or absence of annulus. Thus, pollen morphological characters are informative and promising to support a phylogenetic classification of the *Styphelia-Astroloma* clade and for a more accurate identification of pollen fossils.

Chapter 5 Genetic divergence within the *Leucopogon conostephioides* complex (Styphelieae, Epacridoideae, Ericaceae): taxonomic implications and potential ecological correlates

ABSTRACT

Based on the topology of the estimated molecular phylogeny presented in Chapter 2, the taxa that belong to the *Styphelia-Astroloma* clade were arranged in twelve groups (I – XII). Group VIII consists of taxa that have been informally included in the *Leucopogon conostephioides* complex. This is a widely distributed group in south-western Western Australia and the pattern of variation within it is more consistent with the presence of several currently unrecognised, segregate taxa rather than with a single, highly variable species. Four putative taxa within the complex were sampled: *L. conostephioides, L.* sp. Bifid Eneabba, *L.* sp. Cockleshell Gully, and *L.* sp. short style. Amplified fragment length polymorphisms (AFLP) profiles were obtained for 52 individuals and yielded 1311 characters. Four genetic groups that correspond to the four putative taxa sampled were identified using four different types of analyses: NeighborNet, Bayesian clustering analysis, Neighbor joining and parsimony phylogenetic analysis. While the morphological differences between these taxa are discrete, genetic differentiation is not complete and some individuals present genetic admixture. Retention of ancestral genetic elements as a consequence of recent divergence and genetic isolation appears to be the most suitable hypothesis to explain this result. According to preliminary field observations and morphological examinations, possible factors involved in the genetic divergence within the *L. conostephioides* complex are differences in flowering time, structural changes in floral morphology, and soil type preferences. Both morphology and genetic structure within the *L. conostephioides* complex indicate that these groups are evolutionarily distinct and they merit recognition at species level.

5.1 Introduction

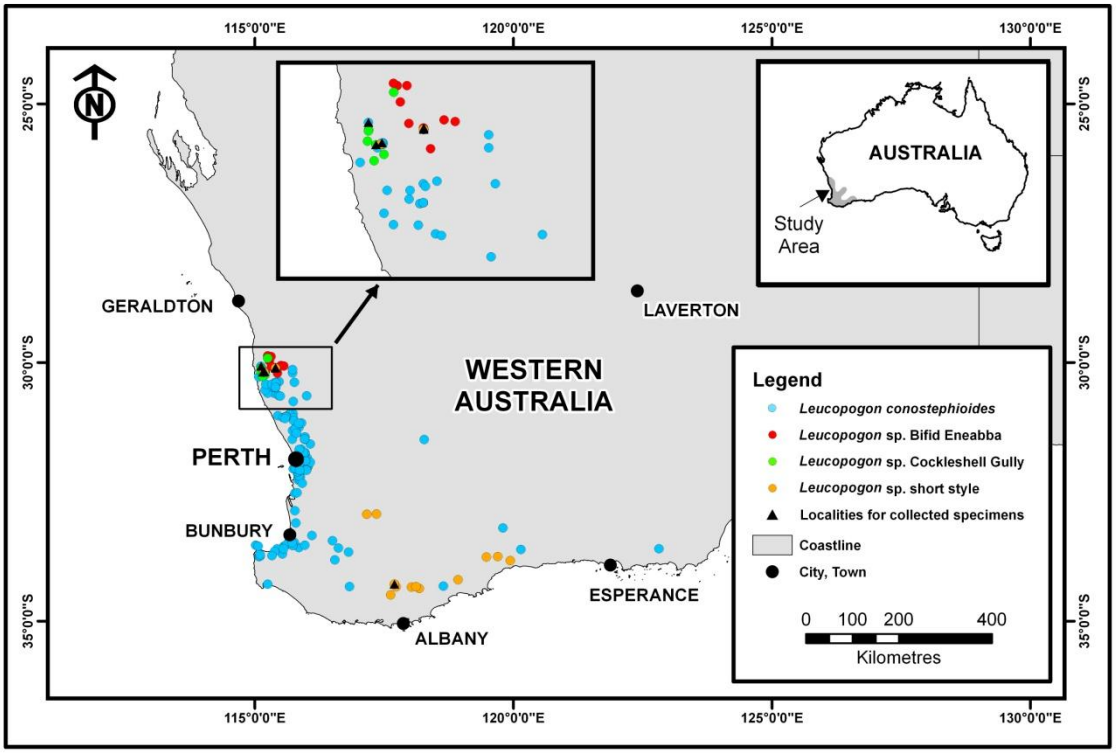
It is a matter of constant debate among systematists and the users of taxonomy how the results of phylogenetic studies should be translated into classification in order to establish monophyletic taxa. The *Styphelia-Astroloma* clade is a clear example of the compromises inherent in reconciling phylogenetics and taxonomy. Based on the topology of the estimated molecular phylogeny presented in Chapter 2 (Figure 2.1), the taxa that belong to the *Styphelia-Astroloma* clade were arranged in twelve groups (I – XII). Since the level of morphological variation differs within the groups, a taxonomic classification that better melds the morphological diversity and the phylogenetic relationships of the species within these groups require further consideration using close morphological examination and more sensitive molecular techniques. One of the groups that need further investigation in order to accurately ascertain the taxonomic rank of the taxa within it is Group VIII, the *Leucopogon conostephioides* species complex.

The *Leucopogon conostephioides* species complex is widely distributed in south-western Western Australia (Figure 5.1). It has historically been very broadly circumscribed, but the observed morphological variation in the complex is consistent with the occurrence of a number of segregate taxa rather than with a single, highly variable species (M. Hislop, personal communication (pers. comm.)).

Preliminary taxonomic investigation suggests that the *L. conostephioides* complex (Group VIII) comprises at least ten taxa including three described species (*L. pubescens* S.Moore*, L. hispidus* E.Pritz**.**, *L. conostephioides* DC.) and seven phrase-named taxa (*Leucopogon* sp. Newdegate (M. Hislop 3585), *Leucopogon* sp. short style (S.Barrett 1578), *Leucopogon* sp. Coujinup (M.A.Burgman 1085), *Leucopogon* sp. Northern ciliate (R. Davis 3393), *L*. sp. Cockleshell Gully (J.M. Powell 1749), *L.* sp. Bifid Eneabba (M.Hislop 1927) and *L.* sp. Carnamah (M.Hislop 2898) (M. Hislop, pers. comm.). The group as a whole can be identified by the following characters: flowers pendulous (excluding *L. hispidus*); nectary of partite scales; stigma unexpanded and continuous with the style, and long-exserted from the corolla tube (excluding *L.* sp. short style); ovary hairy (excluding *L*. sp. Cockleshell Gully); sepals acute or acuminate, longer than the corolla tube (excluding *L*. sp. Coujinup); leaves pungent, usually adaxially concave; fruit a ‘non-fleshy’ or nearly dry drupe (M. Hislop, pers. comm.).

Within the complex, two morphological groups are identified: 1) taxa with ovary 5-4 locular and fruit short, angular (*Leucopogon* sp. Coujinup, and *Leucopogon* sp. Northern ciliate); and 2) taxa with ovary 2-3 locular, and fruit elongated, more or less terete, or slightly ribbed (*L. pubescens, L. hispidus, L. conostephioides, L.* sp. Newdegate, *L.* sp. short style, *L.* sp. Cockleshell Gully, *L.* sp. Carnamah and *L.* sp. Bifid Eneabba) (M. Hislop, pers. comm.). In the phylogenetic analyses presented in Chapter 2 the taxa sampled from the first morphological group were monophyletic, while those from the second group formed two lineages: one containing *L. pubescens, L. hispidus, L. conostephioides, L.* sp. Newdegate, *L.* sp. short style and placed sister to the first morphological group, and the other one comprising only *L.* sp. Bifid Eneabba, which is sister to all of the other taxa in the complex (Figure 2.1).

Figure 5.1 Map showing the distribution of the taxa included in this study: *L. conostephioides s.s.*, *L.* sp. short style, *L.* sp. Bifid Eneabba and *L.* sp. Cockleshell Gully.



The *Leucopogon conostephioides* complex was chosen as the study group because the morphological differences between the taxa/populations are relatively well known, but the taxonomic implications of these differences remain uncertain (M. Hislop pers. comm.). While DNA sequence data suggested some genetic differences between the putative taxa in this group, the markers used were not sufficiently variable to resolve relationships (Chapter 2, Figure 2.1). Consequently, more sensitive genetic markers are needed to better estimate the level of genetic differentiation within the complex, resolve groups as a basis for a robust taxonomy and investigate their evolutionary diversification.

Amplified fragment length polymorphisms (AFLPs) were chosen as the appropriate technique to infer genetic population structure within the *L. conostephioides* complex because it can potentially generate a reproducible and unique fingerprint for each individual and is time and cost efficient. Contrary to the sequencing approach (based on a small number of loci) previously utilized to infer phylogenetic relationships in the *Styphelia-Astroloma* clade, the AFLP technique amplifies fragments from across the entire genome rather than from small regions within the genome. AFLP data are usually highly variable and provide an independent source of evidence to assess relationships at shallow phylogenetic levels. Among the limitations of AFLPs are the risk of homoplasy between fragments of the same size and the lack of sequence knowledge in fragment data, which can lead to erroneous estimations of phylogenetic relationships. Sufficient character sampling across the genome can overcome these limitations. Despite the dominant nature of AFLPs and the consequent difficulties in estimating allele frequencies, AFLP data can be used in a wider range of analyses including population genetics by implementing models to estimate allele frequencies in dominant data assuming Hardy Weinberg equilibrium.

The aim of this study was to assess the genetic structure among some of the representatives of Group VIII (the *L. conostephioides* complex) using AFLPs and to explore possible correlations with the morphological variation and selected environmental variables to underpin a reliable species taxonomy and infer the evolutionary processes in the group.

5.2 Methods

5.2.1 Sampling

Given the limited time frame available for this study, the sampling scheme targeted localities of geographical overlap between the putative taxa as these are where individuals potentially showing evidence of incomplete genetic segregation are expected.

Field surveys identified six populations from the Geraldton Sandplain area (Western Australia) suitable for preliminary study where a number of the segregates co-occur, often in very close proximity: two populations each of *L. conostephioides s.s.,* and *L.* sp. short style, and one population each of *L.* sp. Bifid Eneabba and *L.* sp. Cockleshell Gully (not included in the molecular phylogenetic study) (Table 5.1) Plant collections consisted of 2 – 20 individuals per population, one voucher per population, and fresh leaf/flower material for the molecular analyses. Specimens were identified by M. Hislop (Western Australia Herbarium, Perth) and vouchers deposited in PERTH and NSW (Table 5.1).

5.2.2 DNA extraction

DNA was extracted from silica-dried material (leaves and flowers). Tissue was ground to a fine powder by bead milling with 3 mm steal beads in an automatic tissue grinder, TissueLyser II (Qiagen). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Doncaster, VIC, Australia) following the manufacturer's protocol. DNA was quantified by micro-volume dilution-free UV-visual spectrophotometry using a NanoDrop (Thermo Fisher Scientific, Scoresby, VIC, Australia) and diluted with Qiagen TE buffer to 20ng/mL.

5.2.3 AFLP assays

Total genomic DNA was digested with the two restriction endonucleases *Mse*I and *Hind*3. Adaptors were ligated to restriction fragments in the same reaction. The restriction / ligation reaction mix contained 2.5 µL 10x T4-ligase-buffer with ATP, 2.5 µL NaCl (0.5M), 1.25 µL BSA (1µg/µL), 1 µL each of Msel and Hind III adaptors (2.5 pmol/µL), 0.25 uL T4-DNA ligase (5

U/μL), 0.5 μL Hind III and Msel I (10 U/μL) endonucleases and 15.5 μL DNA extract (20 ng/μL). The reaction mix was incubated in an Eppendorf Mastercycler epGradient S thermal cycler (Eppendorf South Pacific, North Ryde, NSW, Australia) for 12h at 37º C followed by a 20 min denaturation step at 80º C. All reagents were obtained from Fermentas (Thermofisher Scientific, Waltham, MA, USA).

Pre-selective and selective amplification reactions were performed using *Mse*I and *Hind*3 primers with one (+1) and three (+3) selective bases, respectively. Pre-selective amplification of restriction fragments were conducted using *Mse*1-C (GAT GAG TCC TGA GTA A_**C**) and *Hind*3-A (GAC TGC GTA CCA GCT T_**A**) primers (Gene works, Hindmarsh, South Australia, and Applied Biosystems, Carlsbad, CA, USA). The pre-selective amplification reaction mix (10 μL) contained 2.5 μL 10x PCR buffer, 0.8 μL $MgCl_2$ (25 mM), 0.5 μL *Mse*1-C and *Hind*3-A primers, 0.2 μL dNTPs (10 μM), 0.05 μL Kapa Taq polymerase (KAPA Biosystems, Boston, MA, USA), 2 μL of 1:10 dilution of restriction ligation product and 4.95 μL of distilled H_2O. The reaction mix was incubated at 94º C for 2 min, then cycled 30 times with 20 s at 94º C, 30 s at 56º C and 2 min at 72º C, followed by final elongation of 2 min at 72º C then 30 min at 60º C.

For selective amplification six suitable primer pair combinations of *Mse*1 and *Hind*3 primers +3 selective bases were chosen from 63 screened primer combinations (i.e. peaks were well separated and signal to noise ratio was high) (Table 5.2). The selective amplification reaction mix contained the same proportion of reagents used in pre-selective reactions with the following exceptions: 1:20 dilution of pre-selective amplification product and 4.45 μL of H_2O replaced the 2 μL restriction ligation product and 4.95 μL of H_2O used in the pre- selective amplification reaction mix. Selective amplification reactions were incubated at 94º C for 2 min and then subjected to 15 cycles of 94º C for 20 s, 66º C for 30 s decreasing by 0.7º C each cycle, followed by 72º C for 2 min, followed by 20 cycles of 94º C for 20 s, 56º C for 30 s and 72º C for 2 min and a final incubation at 60º C for 30 min. PCR products were multiplexed combining three primer combinations each labelled with one of 3 distinct fluorescent dyes, FAM 6, VIC and NED. Multiplexed samples, each with an internal size standard (LIZ 500), were run on an automated capillary sequencer (DNA Analyser AB3730 Applied Biosystems) at the Australian Genome Research Facility (Melbourne, VIC, Australia). Fifteen percent of the samples were amplified twice independently to determine the reproducibility of the AFLP profiles.

Table 5.1 Plant material used in the study. Vouchers deposited at PERTH and NSW Herbarium.

Species	Herbarium voucher	DNA No.	Locality and coordinates (decimal degrees, GDA94)
L. conostephioides s.s.	PERTH 08281823	LconossCNS_G00129	Lesueur National Park, Cockshell Gully Road. 0.5 km from S of Coorow - Green Head Road (-30.071°, 115.121°).
		Lconoss_CNS_G00131	
		Lconoss_CNS_G00132	
		Lconoss_CNS_G00142	
		Lconoss_CNS_G00143	
		Lconoss_CNS_G00144	
		Lconoss_CNS_G00145	
		Lconoss_CNS_G00146	
		Lconoss_CNS_G00148	
		Lconoss_D2294	
		Lconoss_D2295	
		Lconoss_D2296	
		Lconoss_D2297	
	PERTH 08282684	Lconotyp_CNS_G00125	5.2 km along The Mount Lesueur Loop Road (-30.175°, 115.189°).
		Lconotyp_CNS_G00141	
		Lconotyp_CNS_G00157	

Species	Herbarium voucher	DNA No.	Locality and coordinates (decimal degrees, GDA94)
		Lconotyp_CNS_G00159	
		Lconotyp_CNS_G00160	
		Lconotyp_D2299	
Leucopogon sp. short style (S. Barrett 1578)	PERTH 08282269	LshortHH_D2292	Top of Hamilla Hill Nature Reserve (-34.286°, 117.703°).
		LshortHH_D2293	
	PERTH 08281904	LshortHV_D2281a	Hi Vallee property (D. and J. Williams) along eastern track in the main valley locality of Warradarge (-30.106°, 115.402°).
		LshortHV_D2282a	
		LshortHV_D2283a	
		LshortHV_D2284a	
		LshortHV_D2285a	
		LshortHV_D2287a	
		LshortHV_D2288a	
		LshortHV_D2289a	
		LshortHV_D2290a	
		LshortHV_D2291a	
L. sp. Bifid Eneabba (M. Hislop 1927)	PERTH 05510465	LBifid_CNS_G00151	Hi Vallee property (D. and J. Williams) Warradarge, upland to north of main valley (-30.099°, 115.402°).
		LBifid_CNS_G00152	
		LBifid_CNS_G00153	

Species	Herbarium voucher	DNA No.	Locality and coordinates (decimal degrees, GDA94)
		LBifid_CNS_G00155	
		LBifid_D2298	
Leucopogon sp. Cockleshell Gully (J.M. Powell 1749)	PERTH 08281726	Lcockel_CNS_G00180	Lesueur National Park, 1.8 km along the Loop Road (-30.184°, 115.159°).
		Lcockel_CNS_G00181	
		Lcockel_CNS_G00182	
		Lcockel_CNS_G00183	
		Lcockel_CNS_G00184	
		Lcockel_CNS_G00186	
		Lcockel_CNS_G00187	
		Lcockel_CNS_G00190	
		Lcockel_CNS_G00191	
		Lcockel_CNS_G00192	
		Lcockel_CNS_G00193	
		Lcockel_D2300	
		Lcockel_D2301	
		Lcockel_D2302	

Table 5.2 Selective primers tested for AFLP analysis. Fluorescent labels 6FAM, VIC, NED or PET used for Hind3 primers are also listed. Selective primers labelled with PET were tested but none of them yielded suitable AFLP profiles. The primer combinations that were chosen for selective amplification of all the samples are indicated in bold.

Selective bases on Mse1 primers	Selective bases and Fluorescent label for Hind3 primers
M – CAA	ACA 6FAM
M – CAA	AGC VIC
M – CAA	AAC NED
M – CAA	ACG PET
M – CAA	ACC 6FAM
M – CAA	AAG VIC
M – CAA	ACT NED
M – CAC	ACA 6FAM
M – CAC	AGC VIC
M – CAC	**AAC NED**
M – CAC	ACG PET
M – CAC	ACC 6FAM
M – CAC	**AAG VIC**
M – CAC	ACT NED
M – CGA	ACA 6FAM
M – CGA	AGC VIC
M – CGA	AAC NED
M – CGA	ACG PET
M – CGA	ACC 6FAM

M – CGA	AAG VIC
M – CGA	ACT NED
M – CAG	ACA 6FAM
M – CAG	AGC VIC
M – CAG	AAC NED
M – CAG	ACG PET
M – CAG	ACC 6FAM
M – CAG	AAG VIC
M – CAG	ACT NED
M – CTG	ACA 6FAM
Selective bases on Mse1 primers	**Selective bases and Fluorescent label for Hind3 primers**
M – CTG	AAC NED
M – CTG	ACG PET
M – CTG	**ACC 6FAM**
M – CTG	AAG VIC
M – CTG	ACT NED
M – CTA	ACA 6FAM
M – CTA	AGC VIC
M – CTA	AAC NED
M – CTA	ACG PET
M – CTA	ACC 6FAM
M – CTA	AAG VIC
M – CTA	ACT NED
M– CTC	**ACA 6FAM**
M– CTC	**AGC VIC**
M– CTC	**AAC NED**

M– CTC	ACG PET
M– CTC	ACC 6FAM
M– CTC	AAG VIC
M– CTC	ACT NED
M – CAT	ACA 6FAM
M – CAT	AGC VIC
M – CAT	AAC NED
M – CAT	ACG PET
M – CAT	ACC 6FAM
M – CAT	AAG VIC
M – CAT	ACT NED
M– CTT	ACA 6FAM
M– CTT	AGC VIC
M– CTT	AAC NED
M– CTT	ACG PET
M– CTT	ACC 6FAM
M– CTT	AAG VIC
M– CTT	ACT NED

5.2.4 Data analysis

The AFLP banding pattern was scored semi-automatically as presence or absence of a band at a particular position using the software Genemarker, version 1.7 (SoftGenetics, State College, PA, USA). Only samples with reliable AFLP profiles were included in the analysis. When individuals were recalcitrant in the amplification of one of the selected primer combinations, data were scored as missing. Scored fragment sizes ranged from 88 to 450 nucleotides.

5.2.3 NeighborNet analysis

In order to detect incompatible or ambiguous phylogenetic signal within the data set, a NeighborNet analysis was performed based on Nei and Li distances (1979) using SplitsTree4, version 4.10. In split networks parallel edges are used to represent the splits computed from the data and detect incompatible and ambiguous signal in the data set (Hudson and Bryant, 2006).

5.2.4 Bayesian cluster analysis

The Bayesian clustering approach as implemented in the software STRUCTURE 2.3.1 was used to identify distinct genetic groups and the presence of admixed individuals. Analyses were run assuming Hardy-Weinberg equilibrium and unlinked loci at linkage equilibrium. The absence of a band (0) was defined as the recessive state, and the genotype possibly underlying the dominant state (1) was randomly sampled by the Markov chain Monte Carlo (MCMC) according to its probability in each iteration as described in Falush *et al.* (2007). The admixture model was applied under the assumption of correlated allele frequencies among populations by using the F-model (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). The number of groups (K) was estimated by running STRUCTURE analyses based on different values of K = 1 – 10. Each analysis was performed four times for two hundred thousand generations, excluding the first 200 000 generations as the burn-in phase.

The number of groups (K) that best fitted the data was also estimated using the Evanno *et al.* (2005) approach as implemented in STRUCTURE harvester. This method uses an ad hoc quantity that is based on the second order rate of change of the likelihood function with respect to K (ΔK). The modal value of the resulting ΔK distribution indicates the K that best fits the data.

5.2.5 Phylogenetic analysis

Neighbor joining analysis (NJ) based on the Nei-Li distance measure was performed using PAUP* (Swofford, 2002). Statistical support for nodes was estimated using bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

Maximum parsimony analysis (MP) was conducted using PAUP* (Swofford, 2002), with the following search parameters specified: heuristic search with 1000 random addition sequence replicates, branch swapping via tree-bisection-reconnection (TBR) and all most parsimonious trees saved (MULTREES on). A strict consensus was generated from the most parsimonious trees and statistical support for nodes was estimated using bootstrap analysis with 1000 replicates and TBR branch swapping (Felsenstein 1985). The trees were rooted on *L.* sp. Bifid Eneabba, which was resolved as sister to all the other taxa within the *L. conostephioides* complex (Figure 2.1).

5.3 Results

The AFLP profiles of 52 accessions generated with six primer combinations yielded 1311 characters, of which 725 were parsimony informative. Independent DNA extractions and restriction ligation reactions produced for 15% of the accessions indicated an error rate of 6 % on the AFLP profiles. The number of scored characters per primer combination ranged from 106 to 274, and fragment sizes ranged from 88 to 450 base pairs.

Within the total data set, pairwise Nei–Li distances ranged from 0.031 (within *L.* sp. short style) to 0.363 (between *L. conostephioides s.s.* (CNS00158) and *L.* sp. short style (D2291a). Within populations, the range of Nei–Li distances was smallest in *L.* sp. Bifid Eneabba (0.082–0.145), and highest in *L.* sp. short style

(0.031–0.197) (Table 5.3).

Table 5.3 Range of intraspecific Nei and Li (1979) distances of 52 accessions, representing four putative taxa within the *L. conostephioides* species complex. The AFLP matrix derived from six primer pair combinations comprised 1311 characters in total.

Putative taxon	**Range of intraspecific Nei–Li distances**
Leucopogon sp. Bifid Eneabba	0.063
Leucopogon. sp. Cockleshell Gully	0.094
Leucopogon conostephioides s.s.	0.123
Leucopogon sp. short style	0.167

5.3.1 NeighborNet analysis

The NeighborNet analysis of the total AFLP data set revealed four main clusters that correspond to the four putative taxa (

Figure 5.2): *L. conostephioides* s.s., *L.* sp. short style, *L.* sp. Bifid Eneabba and *L.* sp. Cockelshell Gully respectively. Within *L.* sp. short style, two subgroups that correspond to the northern and southern populations emerge from the base.

5.3.2 Bayesian cluster analysis - STRUCTURE

Simulation runs specifying 1 to 10 genetic groups using an 'independent allele frequency' model were unable to detect a population structure. In all analyses the four taxa were shown as one homogeneous population. Analyses using the alternate 'correlated allele frequency' model revealed evidence of population structure within the complex (Figure 5.4). The most appropriate number of genetic groups was determined upon the likelihood values, L(K), of each simulation assuming number of populations/genetic groups (K) = 1 – 10. Simulations assuming four groups received the highest likelihood values in comparison to simulations assuming more or less than four populations. Under the Evanno *et al.* (2005) method, the highest ΔK value was achieved when K = 2 (873.26) and the second highest ΔK value when K = 4 (185.87) (Table 5.4). ΔK decreased substantially and stabilized beyond K = 4 (Figure 5.3). Because the L(K) was highest at K = 4 and ΔK stabilized only after this point, more extensive analyses were run based on K=4.

The more extensive analyses assuming four populations received likelihood values between -12717.3 and -12752.8 and revealed the same four groups (Figure 5.3). The four groups suggested by the STRUCTURE analyses were congruent with the four clusters detected in the NeighborNet analysis (Figure 5.2). The first cluster comprised *L. conostephioides s.s.* samples from both populations and corresponded well with the same cluster found in the NeighborNet analysis. The second cluster included *L.* sp. short style from both south and north populations and was concordant with the *L.* sp. short style group detected in the NeighborNet analysis. The third and fourth clusters corresponded to the *L.* sp. Bifid Eneabba and *L.* sp. Cockelshell Gully populations. The STRUCTURE analysis revealed that at least one individual from each cluster has a proportion of genetic admixture (above 20%). *Leucopogon conostephioides s.s.* is the group which contained the greatest number of highly admixed individuals (8), and *L.* sp. Bifid Eneabba and *L.* sp. Cockleshell Gully contained the least (1) (Figure. 5.4).

5.3.3 Phylogenetic analyses

Neighbor joining and parsimony analyses produced very similar topologies and support values. Using *L.* sp. Bifid Eneabba as the outgroup, three major clades corresponding to the following putative taxa were resolved: *L.* sp. Cockleshell Gully clade (NJ bootstrap = 99/MP bootstrap = 100), *L. conostephioides s.s.* (54/70), and *L.* sp. short style (100/99). The support value for these three groups together is 100/83, and indicates that they are distinct from *L.* Bifid Eneabba. Within *L.* sp. short style, the northern and southern populations form two distinct clades with support values of 99/61 and 100/100 respectively (Figure **5.5**). Besides *L.* sp. short style, resolution within the clades was poor.

5.4 Discussion

The phylogenetic analyses presented in Chapter 2 using chloroplast DNA (cDNA) and nuclear encoded ribosomal DNA (nrDNA) sequences evince the monophyly of the *L. conostephioides* species complex (Group VIII) and its sister relationship with Group IX (*Stomarrhena*) (Figure 2.1). The *L. conostephioides* complex is also a well-defined group morphologically with very distinctive pollen attributes (Chapter 4; Figure 4.7).

The results of the Bayesian STRUCTURE clustering analysis conducted on the whole dataset indicates that the most likely number of distinct genetic entities is K=4, representing the four different putative taxa sampled (Figure 5.4). Although the Evanno *et al.* (2005) method suggests that K=2 ($\Delta K = 873.26$) best fit the data, the other analyses (NeighborNet, NJ and MP) did not give any indication that the populations were divided in two genetic clusters and it was not possible to determine which taxa would belong to each of the two hypothesized clusters.

The Evanno *et al.* (2005) method finds the breakpoint in the slope of the distribution of L(K) at the "true" K, where L(K) is an estimate of the posterior probability of the data for a given *K*. When the L(K) values were plotted against the different K, the expected breakpoint at the "true" K was not evident (Figure 5.3) so interpretations about the best K based on this method could not be drawn with confidence. Several studies have reported that the Evanno's method tends to underestimate K and give the highest ΔK at K=2 when there is hierarchical structure in the analysed data set. This may explain why even though K=2 does not fit the

observed pattern of variation in the *L. conostephioides* complex, it is supported by the highest ΔK. Other possible factors that might influence these results are that the model of discrete admixed populations does not represent the data set or that the number of individuals/populations sampled is too small. Even though it is not possible to test K=1 using ΔK values, the fact that when ΔK was plotted against K, the slope was not flat as expected when the 'true' K=1, as there should be no K which L(K) rises substantially from K-1 to K, suggests that the populations sampled do not form one genetic group.

The estimation of K is often complex. Its biological interpretation is not always straightforward, and should not be based exclusively on one criterion. As STRUCTURE procedure for estimating K generally works well in data sets with a small number of populations, Pritchard and Wen (2003) recommend for cases like this one to follow L(K) itself and to choose the K where the likelihood, L(K), stops making large improvements, and to combine this with prior biological knowledge. In this study, STRUCTURE simulations assuming four groups received the highest likelihood values in comparison to simulations assuming more or less than four populations (Table **5.4**). Therefore K=4 was considered to better represent the observed patterns of variation within the *L. conostephioides* complex and to be the most congruent with the results generated by the NeighborNet and the phylogenetic analyses of the AFLP data.

Figure 5.2 NeighborNet diagram of the four putative taxa sampled from the *L. conostephioides* complex based on the analysis of 1311 AFLP characters derived from six primer pairs of 52 accessions. The scale bar indicates genetic distance based on Nei-Li distances (Nei and Li, 1979).

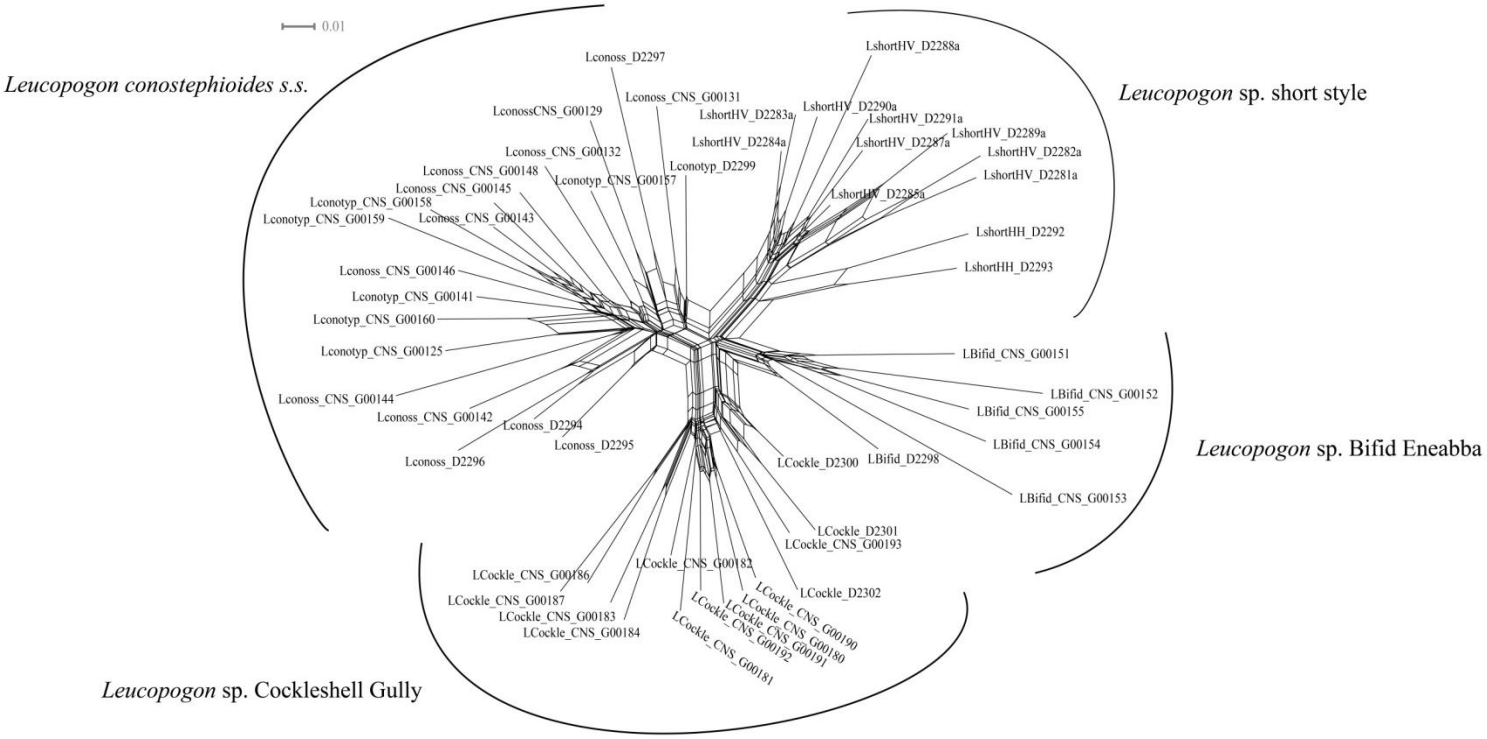
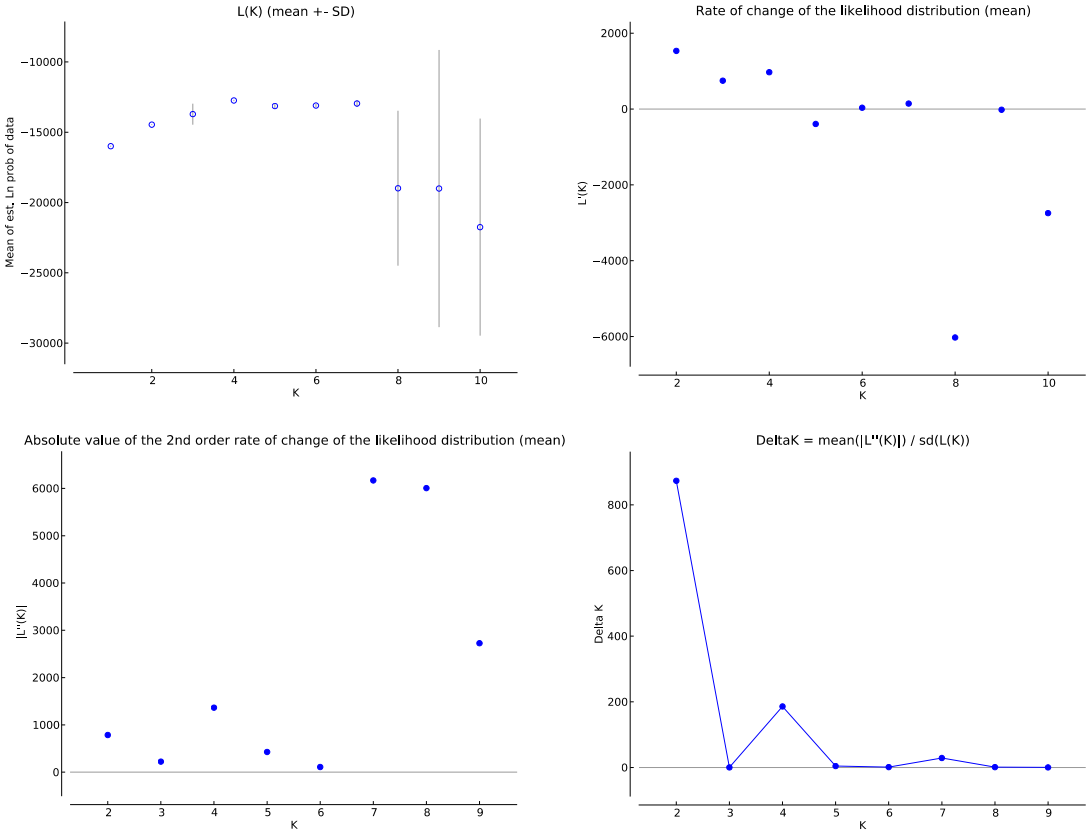


Figure 5.3 Graph of the ΔK statistic for STRUCTURE runs for individuals from *Leucopogon conostephioides s.s., L.* sp. Bifid Eneabba, *L.* sp. Cockleshell Gully and *L.* sp. short style with the number of clusters (K) set between 1 and 10.



The results of the AFLP analyses indicate that the genetic differentiation is congruent with the morphological variation observed in the *L. conostephioides* complex. The NeighborNet (Figure 5.2) analysis of AFLP data revealed four more or less equally distant major genetic groups that correspond to the four putative taxa sampled: *L.* sp. Bifid Eneabba, *L.* sp. short style, *L.* sp. Cockleshell Gully and *L. conostephioides s.s*. As previously discussed, phylogenetic analyses of DNA sequence data placed *Leucopogon* sp. Bifid Eneabba as sister to all the other taxa within the *L. conostephioides* complex so it was used as the outgroup in the AFLP phylogenetic analyses (Figure 2.1). The results of the NeighborNet (

Figure 5.2) and the Bayesian STRUCTURE analyses (Figure. 5.4) confirm that it is a distinct genetic group. *Leucopogon* sp. Bifid Eneabba is a morphologically well-differentiated taxon. It has leaves twisted longitudinally; anthers deeply lobed (i.e. bifid) with filiform, crinkled apices, a character combination which is unique within the *Styphelia-Astroloma* clade. This taxon is restricted to a small area of the Geraldton sandplains where it grows occasionally in fairly close proximity to *L.* sp. short style (Figure 5.1).

In *Leucopogon* sp. short style (100/99) the floral parts are generally smaller in comparison to the other taxa within the complex, and the style is included in, or held at the throat of, the corolla tube (rather than long-exserted). This entity has a scattered, disjunct distribution from the Mount Leuseur area in the north to the Fitzgerald River National Park in the south (Figure 5.1). In the north of its range it grows in the same areas as *L.* sp. Cockleshell Gully and *L. conostephioides s.s*. In the south, it is known to be sympatric with *L*. sp. Coujinup and sometimes to grow in fairly close proximity to *L*. sp. Newdegate. The results of this study indicate that this morphotype has evolved only once, that the northern and southern populations are sisters to each other and that it is best treated as one taxon.

Leucopogon sp. Cockleshell Gully (99/100) has generally the largest floral parts in the complex; bracteoles, and often the sepals, pink vs cream to pale brown like in the other members of the complex. The basal portion of the corolla lobes is subglabrous and the ovary is glabrous. The phylogenetic analyses placed *L.* sp. Cockleshell Gully as sister to *L. conostephioides s.s.,* but this relationship remained unsupported (54/0). Further molecular study is required to confirm its position and phylogenetic relationships within the *L. conostephioides* complex. Its distribution is restricted to Leuseur National Park where it grows in close proximity to *L. conostephioides s.s.*

Although bootstrap support values are moderate for *L. conostephioides s.s.* (54/70), the NeighborNet and Bayesian clustering analyses show it as a well differentiated genetic group that comprises individuals from the two populations sampled (Table 5.1). *Leucopogon conostephioides s.s.* is a morphologically discrete entity characterized by leaves straight and relatively long with respect to the other putative taxa; sepals straight and contracting rather abruptly towards the apex; and a very short, steeply antrorse indumentum on the ovary (very occasionally glabrous); (M. Hislop, pers. comm.). It is widely distributed on the coastal plain from the Leuseur area to the far

south-west corner of Western Australia and occasionally in valleys of the Darling Range (Figure 5.1). The populations sampled for this study occur in close proximity to *L.* sp. Cockleshell Gully and *L.* sp. short style.

Figure. 5.4 Results from Bayesian cluster analysis as implemented in the software STRUCTURE 2.3.1. Bar plots indicate genetic admixture of 52 individuals from the *Leucopogon conostephioides* complex based on 1311 AFLP loci. Analysis were conducted assuming Hardy–Weinberg equilibrium, unlinked loci at linkage equilibrium, applying the admixture model (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007), and with an estimated number of groups (K) = 4.

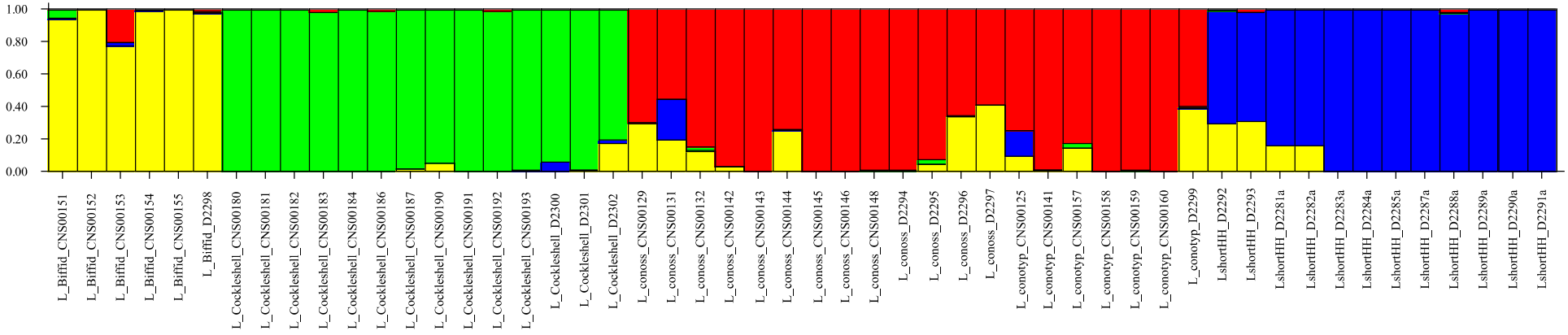


Figure 5.5 Neighbor joining tree based on Nei and Li (1979) distances of 1311 AFLP characters obtained with six primer pair combinations. The tree was rooted on *Leucopogon* sp. Bifid Eneabba. Neighbor joining and parsimony bootstrap values are shown above branches.

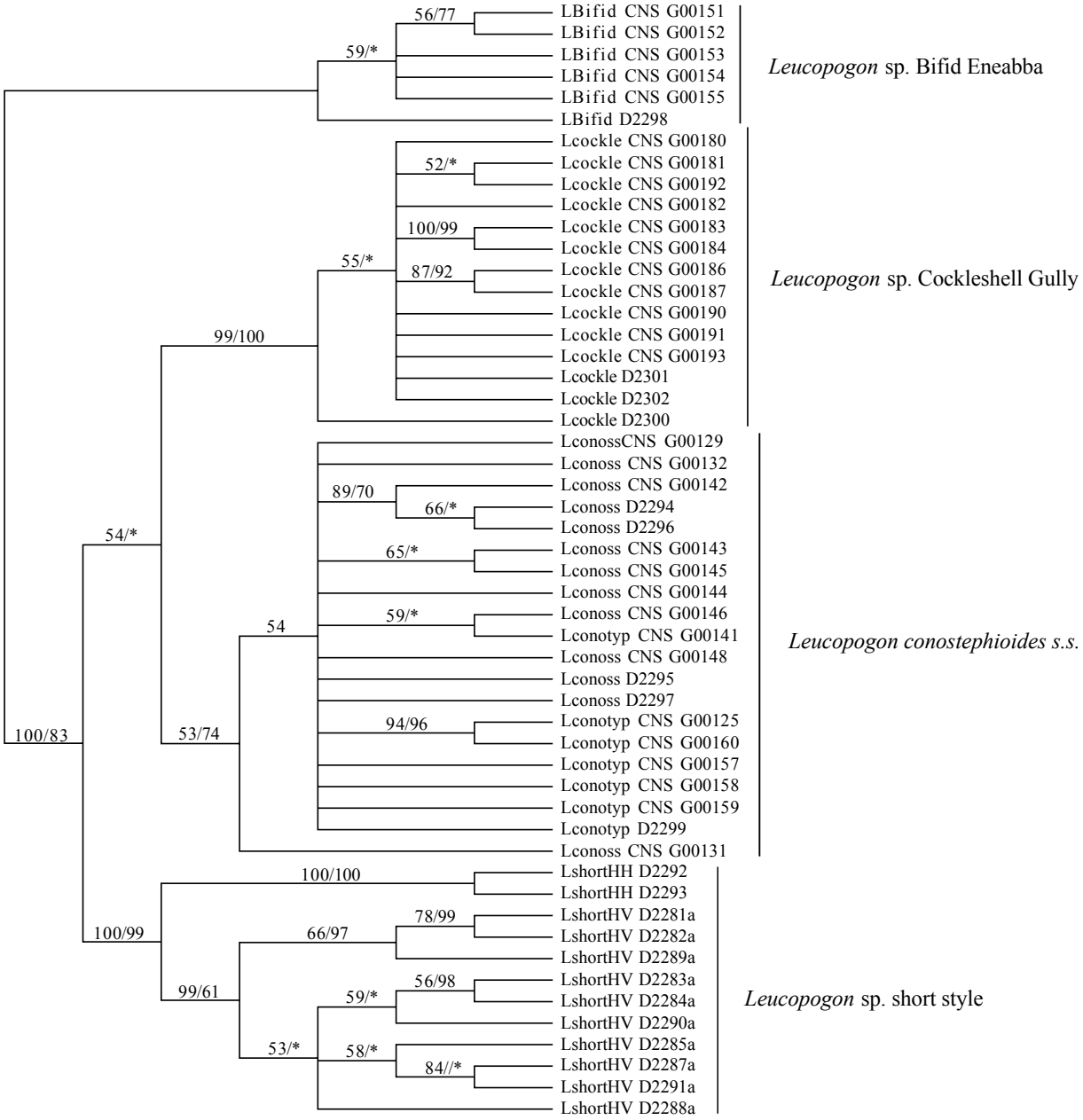


Table 5.4 Mean LnP (K) and ΔK statistics for STRUCTURE simulations with the number of clusters (K) set between 1 and 10 for individuals from *Leucopogon conostephioides s.s., L.* sp. Bifid Eneabba, *L.* sp. Cockleshell Gully and *L.* sp. short style. In bold are the values for K = 2, 4. N/A: not available.

# K	Mean LnP(K)	ΔK
1	-15994.5	N/A
2	**-14461.2**	**873.259259**
3	-13713.8333	0.314079
4	**-12742.7333**	**185.873204**
5	-13136.8	4.325226
6	-13102.7333	1.242696
7	-12959.0667	28.871365
8	-18984.7333	1.097302
9	-19003.8333	0.277672
10	-21749.5333	N/A

The moderate bootstrap support values for *L. conostephioides s.s.* in the NJ and MP analyses are likely to have been influenced by the presence of individuals that contain genetic elements from other populations, mainly from *L.* sp. Bifid Eneabba and *L.* sp. short style (Figure 5.4). This creates character states that group them with the other clades (i.e. *L.* sp. Bifid Eneabba and *L.* sp. short style) and results in low support values. Two possible hypotheses would explain the occurrence of admixed individuals: ongoing gene flow between these populations or recent genetic isolation. Ongoing gene flow is usually associated with the incidence of individuals with intermediate morphologies, but such individuals have not been observed in the field to date (M. Hislop, pers. comm.). Also, the fact that individuals with genetic admixture do not appear at the intersection of two splits on the NeighborNet (each connecting it to one of the two parental taxa) suggests that these are not the result of ongoing gene flow.

Alternatively, the retention of ancestral genetic elements as a consequence of recent divergence and genetic isolation seems more likely. Emergent reproductive barriers and divergent ecological adaptations could be driving speciation in these populations. According to preliminary field observations and morphological examinations, possible factors involved in the genetic and

ecological differentiation in the *L. conostephioides* complex are differences in flowering time, structural changes in floral morphology, and soil type preferences.

Differences in flowering times are a common prezygotic reproductive barrier in angiosperms, especially for species occurring in sympatry. Different flowering times have been recorded for some of the putative taxa within the *L. conostephioides* complex. The most noticeable differences are in *L.* sp. Bifid Eneabba and *L.* sp. Carnamah, which come into flower in late spring (October – December) while the other members of the complex usually begin flowering from autumn to early winter (March – July) (M. Hislop, pers. observation). More detailed field observations are required to assess the possible association of flowering phenology with the hypothesized recent divergence of these populations.

The association between floral morphology and pollination syndromes is well known (Judd *et al.*1999). Species of *Leucopogon* are generally thought to be insect-pollinated, probably by bees, long-tongued flies, and Lepidoptera because of their small tubular flowers (Ford *et al.* 1979). But as it has been shown here (Chapter 2) and in previous studies, the current concept of *Leucopogon* includes several independent lineages and consequently the floral characters that have been used to define this polyphyletic taxon have evolved separately, possibly in response to similar pollinator adaptations. Given their direct role in reproduction, pollination syndromes can drive speciation by directly affecting gene flow patterns and by exerting divergent selection pressures on populations. Accordingly, recently diverged species can show important differences in pollinator interactions associated with small morphological modifications (e.g. shifts in guilds or pollen attachment sites within guilds). Differences in size (e.g. *L.* sp. short style with floral parts generally smaller, and *L.* sp. Cockleshell Gully with the largest flowers in the complex), colour (e.g. *L.* sp. Cockleshell Gully with bracteoles and sepals often pink rather than cream to pale brown), shape (e.g. *L.* sp. Bifid Eneabba with deeply lobed anthers, with filiform, crinkled apices) and the position of some floral parts (*L.* sp. short style with style included in the corolla tube rather than long-exserted) occur within the *L. conostephioides* complex. The potential impact of these floral morphological discrepancies observed between the four putative taxa sampled on their pollination syndromes and how they could be driving speciation can only be established with adequate field experiments. The need for further research on the pollination biology in the *Styphelia-Astroloma* clade is once again accentuated.

Field observations suggest that there are subtle differences in the edaphic preferences between the members of the complex in the Geraldton sandplains. *Leucopogon conostephioides s.s*. mostly occurs on deep sand relatively low in the landscape, *L.* sp. Bifid Eneabba and *L.* sp. Cockleshell Gully grow higher in the landscape in shallow sand over laterite, and *L.* sp. short style appears to grow on more loamy soils often in rocky sites with laterite at or very close to the surface (M. Hislop, pers. comm.). Although these observations are limited and the edaphic characteristics of the localities where specimens were collected are not readily available, there appears to be some congruence between distributional pattern and soil type.

In comparison to other Styphelieae, the species from the *L. conostephioides* complex (Group VIII) have rather dry drupes. Since the successful radiation of the Styphelieae may be partly a consequence of their possession of fleshy fruit, it could be conjectured that the dispersal ability of the members of the *L. conostephioides* complex is limited as they are less likely to be transported by animals such as birds. Soil preferences may restrict populations' distribution and affect their individual physiological response, and combined with the potential drifting constraints may operate as a barrier for gene flow and subsequently lead to speciation.

Differences in soil preferences evoke possible variation in mycorrhizal associations. Mycorrhizal types have broadly been associated with ecosystem and soil environment characteristics (Read, 1991). Examples of correlation between soil types, the assembly structure and occurrence of mycorrhizal associations, and plant community composition have been documented in Borneo and in the Australian woodlands and sclerophyll forests. Edaphic factors are major determinants of the vegetation composition in shrublands, in which the Styphelieae are most diverse in Australia, and where soils are poor in nutrients and plants have developed competent mechanisms to enhance nutrient uptake. The ability of plants to efficiently obtain nutrients is highly dependent on their mycorrhizal associations with fungi and has a direct effect on their fitness. It could be therefore hypothesised that these mutualistic interactions are one of the factors that drive speciation in plant shrubland communities. As they are under strong selective pressure, they promote ecological divergence in plant populations and can potentially differ between recently diverged species.

The successful adaptation and diversification of the Styphelieae in nutrient-poor, acid soils has been generally attributed to their mycorrhizal associations (Read, 1983). Although little is

known about their functional significance, preliminary evidence suggests that these symbiotic relationships may be host-specific and consequently be an important factor influencing their diversity along edaphic gradients in shrublands. Further investigation on the mycorrhizal fungi interactions within the *L, conostephioides* complex would lead to a better appreciation of the impact of these interactions, if any, on the divergence patterns within the complex.

A good understanding of the complexity of the biotic interactions along with an accurate estimation of the taxonomic diversity is critical for effective conservation strategies, which are most needed for several species within the *Styphelia-Astroloma* clade that are highly threatened or known only from few localities (http://florabase.dec.wa.gov.au/).

Notwithstanding the accuracy of the estimated patterns of genetic diversity in the *L. conostephioides* complex are subject to the sampling limitations of this study, the analyses of AFLP data support the hypothesis that *Leucopogon conostephioides s.s., L.* sp. Bifid Enebba, *L.* sp. Cockleshell Gully, and *L.* sp. short style are distinct in their morphology and their genetics, and merit recognition at species level.

Because of the taxonomic turmoil and diversity in the Styphelieae, the majority of studies in the tribe have focused on broad scale questions and few studies have addressed species level issues. Hence, further research in the *Styphelia-Astroloma* clade should concentrate on lower taxonomic levels. Within the *L. conostephioides* complex, the taxonomic status of *L.* sp. Coujinup and *L.* sp. Newdegate remains subject of further research as they are widely distributed entities with a continuous range of morphological variation among populations.

5.5 Conclusions

Leucopogon conostephioides s.s., L. sp. Bifid Enebba, *L.* sp. Cockleshell Gully, and *L.* sp. short style are morphologically distinct entities with divergent evolutionary paths. Analyses of AFLPs data evinced four genetic groups that correspond to the four putative taxa sampled within the complex. While the morphological differences between these taxa are discrete, genetic differentiation is not complete and some individuals present genetic admixture. Retention of ancestral genetic elements as a consequence of recent divergence and genetic isolation appears to be the most suitable hypothesis to explain this result. According to preliminary field observations

and morphological examinations, possible factors involved in the genetic and ecological differentiation in the *L. conostephioides* complex are differences in flowering time, structural changes in floral morphology, and soil type preferences. The first two would have an important impact on the pollination syndrome of each taxon, and the third one would physically delimit their distribution and promote ecological divergence by determining the assembly structure and occurrence of their mycorrhizal associations. The congruence between the genetic differentiation and the morphological variation observed in the complex suggest that these putative taxa should be considered at the species level.

Chapter 6 General conclusions

6.1 Phylogenetics and historical biogeography

The principal aim of this dissertation was to provide a strong foundation for a phylogenetic classification of the Styphelieae, focusing on the *Styphelia-Astroloma* clade, and using four different approaches: phylogenetics, biogeography, palynology and population genetics.

Firstly, an extensively sampled multigene phylogeny was generated to estimate the evolutionary relationships within the *Styphelia-Astroloma* clade. For practical purposes, the majority of taxa that belong to this clade were arranged in twelve robust groups. Groups I, II, III, IV, VI, VIII, IX, XI and XII are morphologically distinct and can be diagnosed by different character combinations (Table 6.1). Groups V, VII and X proved to be most challenging to address. Taxa from Groups V and VII are uniform in their morphology and not clearly diagnosable by any combination of characters. Group X is the largest and most morphologically heterogeneous and inconsistent of the groups. It comprises several smaller, often well-supported and morphologically discrete sub-clades, and *Croninia kingiana,* a clearly distinctive species for which a monotypic genus was erected. *Styphelia pulchella, S. hainesii, S. exarrhena, Leucopogon esquamatus,* and *Coleanthera myrtoides* remain ungrouped either because their phylogenetic relationships are not clear or because they do not exhibit evident morphological affinities with any of the groups.

These results reflect the difficulties that previous workers have had in assigning members of the *Styphelia-Astroloma* clade to genera, particularly the species of *Astroloma, Leucopogon,* and *Styphelia*. Two alternatives are possible prioritizing the principle of monophyly: 1) circumscribe the *Styphelia-Astroloma* clade as a large and very diverse single genus; or 2) erect further segregate genera that generally correspond to the groups presented here. Further targeted work, particularly on morphology, is necessary to formally propose a generic classification that resolves the previous inconsistencies and provides a predictive and stable taxonomic framework. Thus no formal taxonomic recommendations are included in this dissertation.

Table 6.1 Morphological character combinations for Groups I to XII (except V, VII and X). Abbreviation: *s.l.: sensu lato; s.s.: sensu stricto; p.p.: pro parte.*

Group	Potential diagnostic character combinations
I: *Astroloma s.s.*	Filaments linear or narrowly elliptic in section; anthers partially included within the corolla tube; corolla red, pink or orange, to cream and light green, corolla lobes erect in basal two thirds to three quarters, spreading or recurved above or rarely more or less throughout; external surface of corolla lobes glabrous, bitextured; presence of basal hair tufts within the corolla tube.
II: *Styphelia s.l.*	Leaves glabrous, flat or concave, more or less smooth; corolla cream; fruit distinctively ovoid that tapers to a more or less acute apex.
III: *Styphelia s.l.*	Revolute leaf margins, grooved, hairy abaxial leaf surfaces; corolla white; and fruit globose-ellipsoid with an obtuse apex.
IV: *Leucopogon rotundifolius* + *L. cuneifolius*	Corolla tube hairy (below the lobes), lobes spreading from the base and recurved, but not revolute; ovoid fruit that tapers to a more or less acute apex.
VI: *Styphelia s.s.*	Anthers strongly exserted from the corolla tube, corolla lobes typically revolute and strongly coiled abaxially, hairs in tufts at the base of the corolla tube
VIII: *Leucopogon conostephioides* complex	Leaves pungent, usually adaxially concave; flowers pendulous (excluding *L. hispidus*), nectary of partite scales, stigma unexpanded and undifferentiated from the style, style long-exserted from the corolla tube, ovary variously hairy in most taxa, locules 2 or 3 (4 or 5 in *L.* sp.

	Coujinup), sepals acute or acuminate and longer than the corolla tube (except shorter in *L*. sp. Coujinup), dry drupe.
IX: '*Stomarrhena'*	Terete filaments, basal hair tufts in the corolla tube absent (except in *A. stomarrhena*), corolla white (except the red flowered *A. stomarrhena*), lobes spreading from the base and recurved or revolute throughout, inner corolla tube variously hairy below the throat, sepals at least as long as the corolla tube and corolla lobes spreading from the base.
XI: *Leucopogon blepharolepis* + *L.* sp. Moore River	Leaf-like flattened fruit
XII: *Styphelia s.l.* (New Caledonia)	Leaves apex acute, flowers turbinate-shaped, included anthers, corolla tube and ovaries glabrous.

Notwithstanding these remaining impediments, this thesis provides a background to answer questions related to the origins and evolutionary history of the Styphelieae implementing a molecular dating approach. The New Zealand Styphelieae were chosen as a case study because they exemplify the controversies on the origins and evolutionary relationships of the New Zealand biota, which are important questions for biogeographic theory in general.

Parsimony, maximum likelihood and Bayesian phylogenetic analyses showed that each of the eight extant species of New Zealand Styphelieae sampled are a distinct lineage that is nested within an Australian clade. With the exception of *Acrothamnus colensoi,* of which the sister group occurs in New Guinea, their closest relatives are all from Tasmania and/or the east coast of mainland Australia. Molecular dating analyses indicate that all of the New Zealand lineages diverged from their non-New Zealand sisters within the last 7 Ma. The fact that the minimum estimated age of the fossil *Cyathodophyllum novae-zelandiae* (20-23 Ma) does not coincide with the range of the estimated ages of the extant New Zealand lineages suggests that the fossil and the extant Styphelieae in New Zealand are not related. The results from the relative dating analysis

indicate that to accept that *C. novae-zelandiae* belongs to one of the extant New Zealand lineages it would be necessary to accept that Styphelieae arose in the early-mid Mesozoic (210-120 Ma), which contradicts multiple lines of evidence on the age of the Ericales and indeed the angiosperms.

A possible historical biogeographical scenario that explains these results is that the lineage to which *C. novae-zelandiae* belongs went extinct in New Zealand, and the extant New Zealand Styphelieae are derived from Australian lineages that recolonised (presumably by long distance dispersal) no earlier than the late Miocene to Pliocene. Since the Styphelieae possess fleshy fruit and birds have been documented as possible dispersal agents, (McIntyre *et al.* 1995; Metcalfe, 1996; Young and Bell, 2010) zoochory appears to be a potential dispersal mechanism. The conditions that facilitated the recolonization of New Zealand might be associated with the emergence of alpine and subalpine environments and the development of subarid areas during the Pliocene (5 – 2 Ma).

6.2 Evolution and taxonomic significance of pollen types and morphology

In the search for new morphological synapomorphies to support the groups identified in Chapter 2, a representative pollen survey was conducted within the Styphelieae, broadly sampling the *Styphelia-Astroloma* clade, and the evolutionary patterns of pollen type and morphological diversity were investigated. Three different pollen types were found in the Styphelieae: 1) pseudomonads, present in all the species sampled within the *Styphelia-Astroloma* clade as well as in *Monotoca, Oligarrhena* and *Leucopogon s.s.*; 2) A-type (permanent tetras with variable sterility), observed in *Acrothamnus, Acrotriche, Conostephium, Leptecophylla, Pentachondra involucrata, Stenanthera* and *Needhamiella pumilo;* and 3) T-type (regular tetrads), present in *Brachyloma, Lissanthe,* and *Pentacondra pumila*. The pollen type in the tribes Epacrideae, Cosmelieae, Prionoteae and Richeeae is regular tetrads. True regular monads were not recorded in Styphelieae.

Since pseudomonads are universally present in the *Styphelia-Astroloma* clade, the taxonomic utility of pollen type in this clade is limited. In contrast, pollen morphological characters are variable. Six different types of ornamentation were documented in the *Styphelia-Astroloma* clade: perforate, granulate, gemmate, areolate, rugulate, and verrucate. The number of apertures varies from three to more than six, and the size ranges from 15 to 110 μm. This variation is

consistent with the phylogenetic groups and show promising taxonomic utility.

The three different clades currently assigned to *Astroloma* - *Stenanthera,* outside the *Styphelia-Astroloma* clade, Groups I (*Astroloma s.s.*) and IX (*Stomarrhena*) – differ mainly in pollen type, number of apertures and size. Species of *Stenanthera* have A-Type pollen with no apertures. Group I has large (45 – 110 μm) pseudomonads, psilate to perforate, always with six apertures, and sometimes with a thickened annulus. Species from Group IX have smaller pseudomonads (45 – 60 μm), psilate to granulate, annulus always absent, and six or more apertures. *Styphelia s.s.* (Group VI) has large pseudomonads (45 – 80 μm), with gemmate + granulate ornamentation while the *Styphelia* segregates (Groups II, III, *S. exarrhena, S. hainesii*) have smaller pseudomonads (20 – 50 μm) with gemmate + verrucate, areolate, or perforate ornamentation. The leucopogonoids (Groups IV, V, VII, VIII, X and XI) also show important differences in pollen morphology, by which they can be distinguished from each other. Groups IV, V and VII have six apertures and similar size ranges, but species from Group VII exhibit psilate to granulate ornamentation and always a thickened annulus. Groups VIII (*L. conostephioides* complex) and XI (*L. blepharolepis*) are the only ones with rugulate ornamentation, but species from Group VIII have six apertures with no annulus while *L. blepharolepis* has only four apertures with a very conspicuous annulus. Group X is also very diverse in exine ornamentation and presence/absence of annulus. It is the only group within the *Styphelia-Astroloma* clade however that comprises species with 3 – 4 (very rarely 5) pollen apertures, for which can be readily recognized from the other leucopogonoids (see Table 4.1, Figure 4.1– 4.14). Pseudomonads have a single origin in the *Styphelia-Astroloma* clade while the different character states for all the morphological characters examined have arisen multiple times (
Figure **4.17**– 4.21).

6.3 Genetic variation at shallow phylogenetic levels

The results of the phylogenetic analyses raised questions about the taxonomic significance of the observed morphological variation in floral and vegetative characters, and its correlation with the genetic diversity inside the groups. These questions led to the assessment of genetic diversity at shallow phylogenetic levels with more sensitive molecular markers (i.e. AFLPs). Group VIII (the *Leucopogon conostephioides* complex) and its phylogenetic relationships were well resolved, but the most appropriate taxonomic status for the taxa belonging to this group was not sufficiently clear

from their morphology alone. Further evidence that the observed variation between populations was genetically founded and not an artifact of environmental factors was required before making any taxonomic treatment.

The analyses of the AFLP data evinced four genetic groups that correspond to the four putative taxa sampled within the complex: *Leucopogon conostephioides s.s., L.* sp. Bifid Enebba, *L.* sp. Cockleshell Gully, and *L.* sp. short style. The genetic differentiation between these groups however is not complete and some individuals exhibit genetic admixture, possibly due to the retention of ancestral genetic elements as a consequence of recent divergence. Preliminary field observations and morphological examinations suggest that the potential factors involved in the genetic and ecological differentiation in the *L. conostephioides* complex are differences in flowering time, structural changes in floral morphology, and soil type preferences. These factors could be associated or determined by mutualistic interactions (i.e. pollination syndrome and mycorrhizal associations) of which little is known and further investigation is critically needed. Both morphology and genetic structure within the *L. conostephioides* complex suggest that these groups are evolutionarily distinct and they merit recognition at species level.

6.4 Future directions

A phylogenetic framework and alternative morphological attributes found in pollen are proposed in this dissertation to support a taxonomic revision of the polyphyletic and morphologically heterogeneous genera *Astroloma, Leucopogon* and *Styphelia*. A phylogenetic circumscription of these three genera is essential for a proper understanding and estimation of the diversity of the Ericaceae in Australia, and for the formal description of new endangered species.

The comprehensively sampled multigene phylogeny presented here together with distributional data obtained from herbarium records, provides the basis for further research on the spatial distribution of phylogenetic diversity and endemism within the *Styphelia-Astroloma* clade. Such studies may address questions regarding historical evolutionary processes and identify novel areas of evolutionary importance and hence of conservation significance. Predictive modelling of distributional ranges could be undertaken in order to explore the possible impact of future environmental change, and may lead to more effective conservation strategies based on sound science.

Our understanding of the evolution and diversification of the *Styphelia-Astroloma* clade would improve with a greater appreciation of the factors driving the speciation processes in the clade. Several analytical tools to estimate diversification parameters and explore correlations with specific traits (e.g. BayesRates, Silvestro *et al.* 2011) are currently available. With this type of analysis, the potential impact of the evolution of specific morphological traits (e.g. fleshy fruit, number of pollen apertures) on speciation rates in the *Styphelia-Astroloma* clade could be investigated.

The pollen survey presented here provides a good estimation of the pollen morphological diversity and its evolutionary patterns. The utility of these findings is not restricted to the taxonomy of extant taxa, it also offers new information for the identification of fossil taxa, which could lead to a more accurate estimation of the past diversity. For this purpose, previously identified pollen fossils should be re-examined along with undetermined fossils that may now prove to be identifiable.

The results presented here, taken together with previous research, indicate that many of the floral morphological traits used to define current taxa are likely to have undergone convergent evolution. As it has been shown in several groups of plants, high variation in floral morphology is often associated with differences in pollination syndrome. The need for studies in pollination biology was here identified several times (Chapters 3 and 4). An improved knowledge of the pollinators, their interactions and the level of specificity in each of the twelve groups in the *Styphelia-Astroloma* clade could explain part of the pollen and floral diversity, which would allow a better understanding of their evolutionary drivers, history and ecology.

The preliminary results shown in Chapter 5 suggest that another mutualistic interaction of potentially great influence in the speciation processes within the *Styphelia-Astroloma* clade is mychorrizal symbiosis. Given the important role of mychorrizae in the adaptation of the Ericaceae to poor soils, it would be worth investigating the level of specificity of these interactions and patterns of evolution in a phylogenetic framework. Pollination by animals and mycorrhizal symbiosis are vital and complex plant interactions that are vulnerable to environmental threats such as climate change (Waterman *et al.* 2011). The complexity and effect of these interactions on epacrid plant species and communities must be investigated in order to permit the implementation

of sound conservation strategies.

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Appendix 2.1 GenBank accession numbers. * Sequence not generated.

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Tribe Styphelieae					
Acrothamnus colensoi (Hook.f.) Quinn	JQ667370	*	AY372573	AY372573	*
Acrothamnus hookeri (Sond.) Quinn	JQ305848	AY372641	JQ310786	AY971370	*
Acrothamnus maccraei (F.Muell.) Quinn	JQ305849	AY372644	JQ310787	AF208778	KC197093
Acrothamnus montanus (R.Br.) Quinn	JQ667222	AY372576	*	*	*
Acrothamnus spathaceus (Pedley) Quinn	JQ667385	AY372656	KC411601	AY372580	*
Acrothamnus suaveolens (Hook.f.) Quinn	JQ667471	JQ667207	KC411507	AY372589	KC197079
Acrotriche affinis DC.	JQ667440	JQ667189	*	AY372541	KC197137
Acrotriche cordata (Labill.) R.Br.	JQ667442	*	KC411502	AY372545	KC197071
Acrotriche dura (Benth.) Quinn	JQ667362	*	*	*	KC197087
Acrotriche patula R.Br.	JQ667234	*	KC411501	AY372542	KC197070
Agiortia pedicellata (C.T.White) Quinn	JQ667390	JQ667165	*	AY372577	KC197077
Astroloma acervatum ms.	KC479107	*	JQ310801	JQ257003	KC197171
Astroloma baxteri A.Cunn. ex DC.	KC479116	KC479125	KC411624	AY372543	KC197161
Astroloma chloranthum ms.	KC479115	*	*	*	KC197160
Astroloma ciliatum (Lindl.) Druce	JQ305852	AY372599	JQ310790	AF208748	KC197120
Astroloma compactum R.Br.	JQ667250	JQ667092	KC411570	*	KC197114

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Astroloma drummondii Sond.	*	*	*	*	KC197165
Astroloma epacridis (DC.) Druce	JQ667251	JQ667096	KC411568	AY372546	KC197113
Astroloma foliosum Sond.	JQ667252	*	KC411520	KC494225	KC197094
Astroloma glaucescens Sond.	KC479111	*	KC411621	KC494227	KC197156
Astroloma humifusum (Cav.) R.Br.	U80433	AY372602	KC411545	AF155866	KC197097
Astroloma macrocalyx Behr and F.Muell. ex Sond.	*	JQ667210	KC411567	AY372547	KC197112
Astroloma microcalyx Sond.	JQ667249	*	KC411573	KC494228	KC197122
Astroloma microdonta Benth.	KC479101	*	KC411612	KC494229	KC197148
Astroloma oblongifolium ms	JQ667246	JQ667098	KC411574	*	*
Astroloma pallidum R.Br	JQ667248	JQ667091	KC411569	AY372548	*
Astroloma pinifolium (R.Br.) Benth.	JQ305853	JQ305828	JQ310791	AY372549	KC197119
Astroloma prostratum R.Br.	JQ305854	JQ305829	JQ310792	JQ286180	KC197121
Astroloma recurvum A.J.G. Wilson	JQ305855	JQ305830	JQ310793	JQ286181	KC197123
Astroloma serratifolium (DC.) Sond.	KC479112	*	KC411622	*	KC197157
Astroloma serratifolium (DC.) Sond. 'Northern variant'	JQ667264	JQ667101	KC411521	*	*
Astroloma sp. Baal Gammon (B.P.Hyland 10341)	*	AY372608	*	AY372552	KC197103
Astroloma sp. Cataby (E.A.Griffin 1022)	*	*	*	KC494226	*
Astroloma sp. Eneabba (N.Marchant s.n.)	KC479106	*	KC411616	KC494230	KC197152
Astroloma sp. Galena (G.Phelan and A.Chant 9)	JQ667271	JQ667107	KC411599	KC494231	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Astroloma sp. Kalbarri (D. and B.Bellairs 1368)	JQ667273	*	KC411607	*	KC197143
Astroloma sp. Nannup (R.D.Royce 3978)	JQ667270	JQ667106	KC411524	*	*
Astroloma sp. Narrogin (R.D. Royce 8158)	JQ667262	JQ667100	KC411575	*	KC197124
Astroloma sp. sessile leaf (J.L.Robson 657)	JQ667272	JQ667108	KC411600	KC494233	*
Astroloma stomarrhena Behr and F.Muell. ex Sond.	JQ667291	*	KC411608	AY372550	KC197144
Astroloma tectum R.Br.	JQ667266	AY372606	KC411571	AY372551	*
Astroloma xerophyllum (DC.) Sond.	JQ667275	JQ667110	KC411631	AY372554	KC197168
Brachyloma concolor (F.Muell.) Benth.	JQ305856	JQ305831	JQ310794	JF437581	KC197129
Brachyloma daphnoides (Sm.) Benth.	JQ667474	JQ667206	KC411547	AF155859	KC197098
Brachyloma ericoides (Schltdl.) Sond.	JQ667284	*	KC411582	JF437582	KC197134
Brachyloma mogin Cranfield	*	*	KC411539	*	*
Brachyloma nguba Cranfield	*	*	*	*	KC197126
Brachyloma pirara Cranfield MS	JQ667285	JQ667119	*	*	*
Brachyloma preisii Sond.	JQ305857	JQ305832	JQ310795	AY372555	KC197128
Brachyloma preissii var. *brevifolium* Sond.	JQ667276	JQ667111	*	*	*
Brachyloma saxicola J.T.Hunter	*	*	KC411552	*	KC197106
Brachyloma sp. Forrestania White (M.Hislop and F.Hort MH2591)	JQ667283	JQ667118	KC411514	KC494235	*
Brachyloma sp. Murchison (A.P.Brown 312)	JQ667286	JQ667120	*	*	*
Coleanthera myrtoides Stschegl.	KC479100	*	KC411576	AY372556	KC197125

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Conostephium preissii Sond.	KC479113	*	*	*	KC197158
Croninia kingiana (F.Muell.) J.M.Powell	KC479092	KC479126	*	AF208750	KC197140
Cyathopsis albicans (Brongn. and Gris) Quinn	JQ667230	*	KC411509	AY636039	KC197082
Leptecophylla abietina (Labill.) C.M.Weiller	*	AY372618	*	AY372561	*
Leptecophylla divaricata (Hook.f.)	*	AY372619	*	AY372562	*
Leptecophylla juniperina (J.R.Forst. and G.Forst.) C.M.Weiller (NZ)	*	AY372621	*	AY372563	*
Leptecophylla juniperina (J.R.Forst. and G.Forst.) C.M.Weiller (TAS)	*	AY372622	*	AY372564	*
Leptecophylla juniperina subsp. *oxycedrus* (Labill.) C.M.Weiller	*	AY372623	*	AY372565	*
Leptecophylla juniperina subsp. *parvifolia* (R.Br.) C.M.Weiller	*	AY372624	*	AY372566	*
Leptecophylla pendulosa (Jarman) C.M.Weiller	*	AY372625	*	AY372567	*
Leptecophylla robusta (Hook.f.) C.M.Weiller	*	*	*	AY372568	EF635442
Leptecophylla tameiameiae (Cham. and Schltdl.) C.M.Weiller	*	AY372626	*	AY372569	*
Leptecophylla abietina (Labill.) C.M.Weiller	AY372561	AY372618	*	AY372561	*
Leptecophylla divaricata (Hook.f.) C.M.Weiller	*	AY372619	*	AY372562	*
Leptecophylla juniperina (J.R.Forst. and G.Forst.) C.M.Weiller (NZ)	*	AY372621	*	AY372563	EF635454
Leptecophylla juniperina (J.R.Forst. and G.Forst.) C.M.Weiller (TAS)	*	AY372622	*	AY372564	*
Leptecophylla juniperina subsp. *oxycedrus* (Labill.) C.M.Weiller	*	AY372623	*	AY372565	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leptecophylla pendulosa (Jarman) C.M.Weiller	*	AY372625	*	AY372567	*
Leptecophylla robusta (Hook.f.) C.M.Weiller	AY372568	*	*	AY372568	*
Leptecophylla tameiameiae (Cham. and Schltdl.) C.M.Weiller	*	AY372626	*	AY372569	*
Leucopogon sp. 'Koolyanobbing'	JQ667391	*	KC411591	*	*
Leucopogon aff. *marginatus* W.Fitzg.	JQ667423	JQ667183	KC411563	KC494237	*
Leucopogon allittii F.Muell.	JQ667292	AY372627	*	AF208753	JF437571
Leucopogon alternifolius R.Br.	JQ305858	AY372628	JQ310796	AF208754	*
Leucopogon amplexicaulis (Rudge) R.Br.	JQ305859	JQ305833	JQ310797	AF208755	*
Leucopogon apiculatus R.Br.	JQ667294	JQ667125	KC411565		*
Leucopogon appresus R.Br.	JQ305860	JQ305834	JQ310798	AF208756	*
Leucopogon assimilis R.Br.	JQ667297	JQ667126	KC411553	AF208757	*
Leucopogon australis R.Br.	JQ305861	*	JQ310799	AF208758	*
Leucopogon blepharolepis (F.Muell.) Benth.	JQ667300	JQ667128	KC411559	AY372571	*
Leucopogon bossiaea (F.Muell.) Benth.	JQ305862	JQ305835	JQ310800	AF208759	*
Leucopogon carinatus R.Br.	*	*	KC411605		*
Leucopogon concinnus Benth.	JQ305863	JQ305836	*	*	*
Leucopogon conostephioides DC.	KC479098	*	KC411610	*	KC197146
Leucopogon cordatus Sond.	JQ667307	AY372633	KC411548	AF208760	*
Leucopogon cordifolius Lindl.	JQ667309	JQ667132	KC411630	*	KC197167

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leucopogon crassiflorus (F.Muell.) Benth.	JQ667311	JQ667134	KC411594	*	*
Leucopogon crassifolius Sond.	JQ667312	AY372634	KC411561	AF208764	KC197110
Leucopogon cuneifolius Stschegl.	*	AY372635	*	AF208765	JF437573
Leucopogon cymbiformis A.Cunn. ex DC.	JQ667314	*	KC411533	AF208766	*
Leucopogon denticulatus W.Fitzg.	KC479099	*	KC411611	*	KC197147
Leucopogon distans R.Br.	JQ667315	JQ667135	KC411555	KC494238	*
Leucopogon elegans Sond.	KC479123	*	*	*	KC197153
Leucopogon ericoides (Sm.) R.Br.	JQ667317	*	KC411546	KC494239	*
Leucopogon esquamatus R.Br.	JQ667320	AY372638	KC411556	AF208769	JF437577
Leucopogon extremus Hislop and Puente-Lel	JQ305865	JQ305837	JQ310802	*	*
Leucopogon fasciculatus (G.Forst.) A.Rich.	JQ667425	JQ667185	KC411505	*	KC197075
Leucopogon fraseri A.Cunn. ex DC. (NSW)	JQ667330	JQ667138	KC411572	AF208771	KC197118
Leucopogon fraseri A.Cunn. ex DC. (NZ)	JQ667331	JQ667139	KC411550	AF208772	*
Leucopogon fraseri A.Cunn. ex DC. (TAS)	JQ667329	JQ667137	KC411585	*	*
Leucopogon gibbosus Stschegl.	JQ667334	*	*	AF155863	*
Leucopogon gilbertii Stschegl.	JQ667335	*	KC411557	KC494240	*
Leucopogon glabellus R.Br.	*	AY372620	*	AF208773	*
Leucopogon glaucifolius W.Fitzg.	JQ667337	JQ667142	KC411544	KC494241	*
Leucopogon hispidus E.Pritz.	JQ667338	JQ667143	KC411593	*	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leucopogon juniperinus R.Br.	JQ667343	JQ667145	KC411551	AF208775	KC197105
Leucopogon lanceolatus R.Br. var. *lanceolatus*	JQ667344	AY372642	*	AF208776	*
Leucopogon lavarackii Pedley	JQ667398	JQ667167	KC411603	*	*
Leucopogon leptanthus Benth.	JQ667346	JQ667146	*		*
Leucopogon leptospermoides R.Br.	JQ667347	AY372643	*	AF208777	*
Leucopogon margarodes R.Br.	JQ667352	JQ667148	KC411504	AY372574	*
Leucopogon melaleucoides A.Cunn. ex DC.	JQ667290	*	*	*	*
Leucopogon microphyllus (Cav.) R.Br.	JQ667354	AY005097	*	AF155862	*
Leucopogon muticus R.Br.	JQ305866	AF015638	*	AF155864	*
Leucopogon neoanglicus F.Muell. ex Benth.	*	AY372646	*	AF208779	*
Leucopogon nutans E.Pritz.	JQ305867	JQ305838	JQ310803	AF208780	*
Leucopogon obtectus Benth.	JQ305868	JQ305839	JQ310804	JQ257004	*
Leucopogon opponens (F.Muell.) Benth.	KC479109	*	KC411619	*	KC197154
Leucopogon ovalifolius Sond.	JQ667358	JQ667151	KC411554	AF208781	KC197108
Leucopogon oxycedrus Sond.	JQ667359	JQ667152	*	AF208782	*
Leucopogon parviflorus (Andrews) Lindl.	JQ305870	*	JQ310805	AF208783	KC197076
Leucopogon pendulus R.Br.	JQ667365	JQ667153	KC411538	AF208784	KC197150
Leucopogon planifolius Sond.	KC479110	JQ667154	KC411620	*	KC197155
Leucopogon plumuliflorus (F.Muell.) F.Muell. ex Benth.	JQ667369	AY372651	*	AF208785	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leucopogon propinquus R.Br.	KC479105	AY372654	KC411615	AF208788	*
Leucopogon pubescens S.Moore	JQ667373	JQ667157	KC411587	*	*
Leucopogon racemulosus DC.	JQ667374	JQ667158	KC411588	KC494243	*
Leucopogon rotundifolius R.Br.	JQ667376	JQ667159	KC411564	*	*
Leucopogon ruscifolius R.Br.	JQ667378	JQ667160	KC411602	*	*
Leucopogon setiger R.Br.	JQ667379	AY372655	*	AF208790	KC197107
Leucopogon sonderensis J.H.Willis	JQ667323	*	KC411506	*	KC197078
Leucopogon sp. 'Tarin Rock'	JQ667415	JQ667179	KC411527	*	*
Leucopogon sp. 'Wilson'	JQ667404	JQ667171	KC411525	*	*
Leucopogon sp. Arrino (M.Hislop 2675)	JQ667409	JQ667175	KC411526	*	*
Leucopogon sp. Badgingarra (R. Davis 421)	JQ667408	*	KC411596	*	*
Leucopogon sp. Bifid Eneabba (M.Hislop 1927)	*	*	KC411604	*	KC197139
Leucopogon sp. Bindoon (F.Hort 2766)	JQ667413	*	KC411589	KC494244	*
Leucopogon sp. Bremer Bay (K.R.Newbey 4667)	JQ667414	JQ667178	KC411597	*	*
Leucopogon sp. Brookton (K.Kershaw and L.Kerrigan KK2192)	JQ667391	*	KC411530	*	*
Leucopogon sp. ciliate Eneabba (F.Obbens and C.Godden s.n. 3/7/2003)	JQ667412	JQ667177	KC411541	*	*
Leucopogon sp. Coomallo (R.J. Cranfield 1457)	KC479104	*	KC411614	KC494245	KC197151
Leucopogon sp. Coujinup (M.A.Burgman 1085)	KC479117	*	KC411625	*	KC197162
Leucopogon sp. Forrestania (G.F. Craig 2386)	JQ667426	*	KC411531	*	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leucopogon sp. Gunapin (F.Hort 808)	KC479093	*	*	*	KC197141
Leucopogon sp. Howatharra (D. and N.McFarland 1046)	JQ667382	JQ667161	KC411543	*	*
Leucopogon sp. Kalbarri (J.M.Powell 1695)	KC479094	*	KC411535	*	*
Leucopogon sp. Kau Rock (M.A.Burgman 1126)	JQ667387	JQ667164	KC411517	KC494247	KC197090
Leucopogon sp. Margaret River (J.Scott 207)	*	*	KC411542	*	*
Leucopogon sp. Mid West (J.S.Beard 7388)	KC479122	*	KC411633	*	KC197170
Leucopogon sp. Mondurup (K.F.Kenneally 11445)	JQ667421	JQ667182	KC411536	*	KC197096
Leucopogon sp. Moore River (M.Hislop 1695)	JQ667402	*	KC411529	*	*
Leucopogon sp. Mt Heywood (M.A.Burgman 1211)	JQ667393	*	KC411537	KC411537	*
Leucopogon sp. Murdoch (M.Hislop 1037)	KC479102	*	KC411613	*	KC197149
Leucopogon sp. Newdegate (M. Hislop 3585)	KC479119	*	KC411627	*	KC197163
Leucopogon sp. Northern ciliate (R. Davis 3393)	KC479120	*	KC411629	*	KC197166
Leucopogon sp. Northern Scarp (M.Hislop 2233)	JQ667419	*	*	KC494248	*
Leucopogon sp. Ongerup (A.S.George 16682)	JQ667407	JQ667174	KC411540	*	*
Leucopogon sp. Port Gregory (C.Page 33)	JQ667395	JQ667166	KC411592	*	*
Leucopogon sp. short style (S.Barrett 1578)	KC479118	*	KC411628	*	KC197164
Leucopogon sp. Southern Granite (E.D.Middleton EDM266)	JQ667405	JQ667172	*	*	*
Leucopogon sp. Tathra (M.Hislop 2900)	JQ667397	*	KC411586	KC494249	*
Leucopogon sp. Walpole (R.J.Cranfield 10940)	JQ667396	*	KC411528	*	*

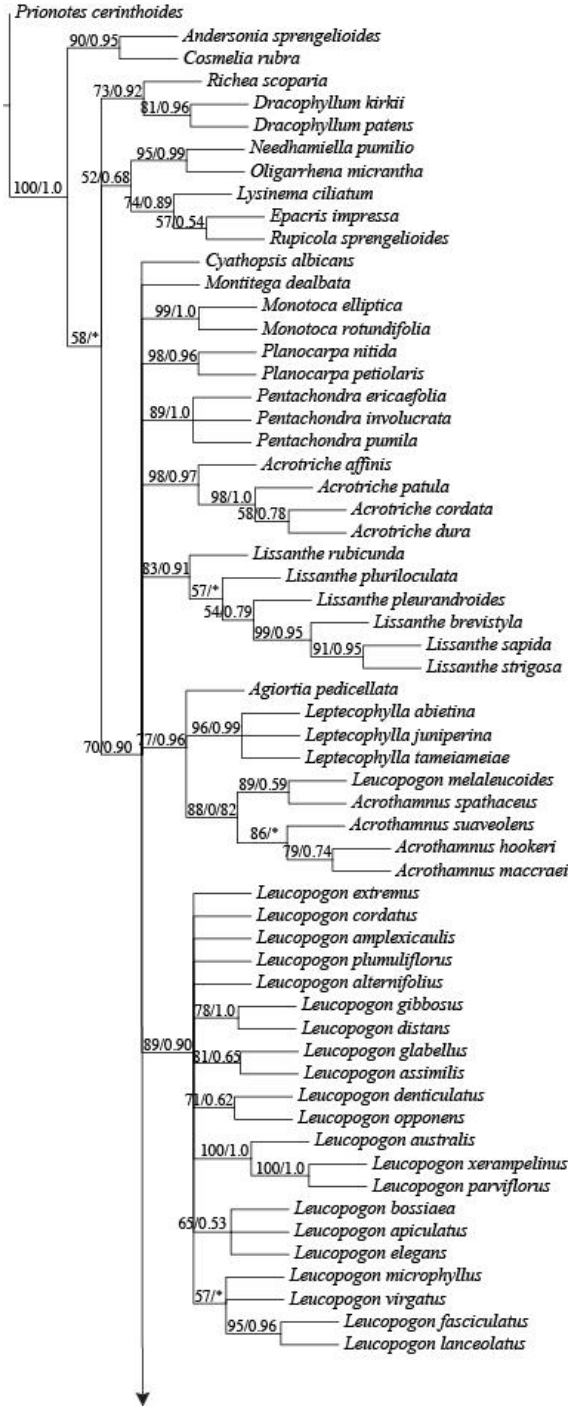
Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Leucopogon sp. Warradarge (M.Hislop 1908)	KC479096	*	KC411609	*	KC197145
Leucopogon sp. Wheatbelt (S.Murray 257)	JQ667420	*	KC411532	*	KC197095
Leucopogon sp. Yanchep (M.Hislop 1986)	JQ667406	JQ667173	*	*	*
Leucopogon sp. Yandanooka (M.Hislop 2507)	JQ667418	*	KC411590	*	*
Leucopogon sp. Yanneymooning (F.Mollemans 3797)	JQ667392	*	KC411598	*	*
Leucopogon strictus Benth.	JQ667424	JQ667184	KC411562	AF208791	KC197111
Leucopogon tamminensis E.Pritzel	JQ667427	*	KC411558	AF208792	*
Leucopogon virgatus (Labill.) R.Br.	JQ305871	JQ305840	JQ310806	*	*
Leucopogon woodsii F.Muell.	KC479108	*	KC411618	*	*
Leucopogon xerampelinus de Lange, Heenan and M.I.Dawson	JQ667298	JQ667127	*	JX993989	KC197074
Leucopogon yorkensis Pedley	JQ667429	*	KC411626	*	*
Lissanthe brevistyla A.R. Bean	JQ305872	JQ305841	JQ310807	AY372581	KC197073
Lissanthe pleurandroides (F.Muell.) Crayn and Hislop	JQ667333	JQ667141	KC411515	*	KC197086
Lissanthe pluriloculata (F.Muell.) J.M.Powell, Crayn and E.A.Br.	JQ667224	*	*	AF208786	*
Lissanthe rubicunda (F.Muell.) J.M.Powell, Crayn and E.A.Br.	JQ305873	JQ305842	JQ310808	AY372579	KC197072
Lissanthe sapida R.Br.	JQ667386	JQ667163	*	AF208793	KC197100
Lissanthe strigosa (Sm.) R.Br.	JQ667388	AY372658	*	AF208794	KC197101
Melichrus erubescens A.Cunn. ex DC.	JQ667446	JQ667191	KC411519	*	KC197092
Melichrus procumbens (Cav.) Druce	JQ305874	JQ305843	JQ310809	AF155856	KC197117

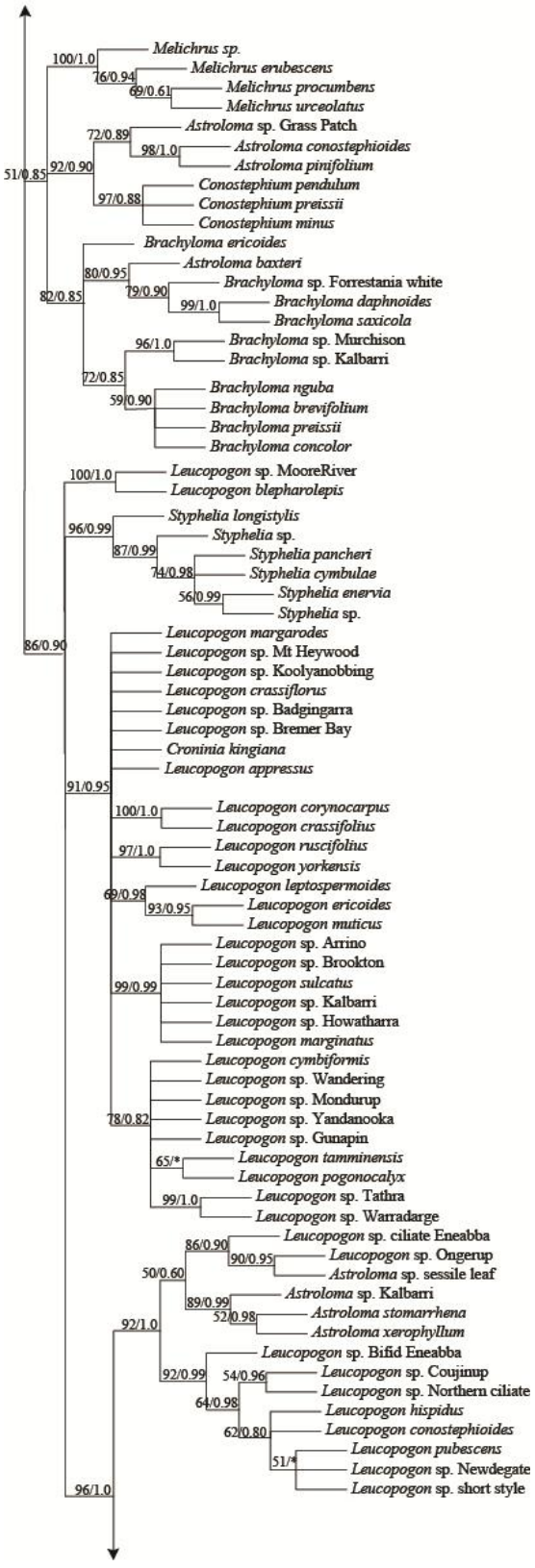
Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Monotoca elliptica (Sm.) R.Br.	JQ305876	AY005099	*	AY005085	KC197099
Monotoca empetrifolia R.Br.	JQ305877	GU732295	*	GU121405	*
Monotoca rotundifolia J.H.Willis	U80422	AF539984	*	AF155853	*
Monotoca scoparia (Sm.) R.Br.	JQ667432	AF015640	*	AF155857	*
Montitega dealbata (R.Br.) C.M.Weiller	U80423	AF539985	*	AF155854	*
Pentachondra dehiscens Cherry	*	AY005109	*	AY005093	*
Pentachondra ericifolia Hook.f.	*	AY005104	*	AY005090	*
Pentachondra involucrata R.Br.	*	AY005101	*	AY005087	*
Pentachondra pumila (J.R.Forst. and G.Forst.) R.Br. (AU)	*	AY005103	*	AY005089	*
Pentachondra pumila (J.R.Forst. and G.Forst.) R.Br. (NZ)	*	AY005104	*	AY005090	*
Planocarpa nitida	*	AY372663	*	AY372593	*
Planocarpa petiolaris (DC.) C.M.Weiller	*	AY372594	*	AY372664	*
'Pseudactinia' sp.	*	AY372665	*	AY372596	JF437567
Styphelia adscendens R.Br.	JQ667377	JQ667209	KC411583	KC494250	KC197135
Styphelia cymbulae Spreng.	JQ667231	*	KC411511	KC494251	*
Styphelia enervia (Guillaumin) Sleumer	JQ667472	JQ667205	*	KC494252	KC197081
Styphelia exarrhena (F.Muell.) F.Muell.	*	AY372666	*	AY372587	JF437575
Styphelia hainesii F.Muell.	JQ305878	JQ305845	JQ310811	JQ286183	KC197089
Styphelia intertexta A.S.George	*	JQ667203	KC411518	KC494253	KC197091

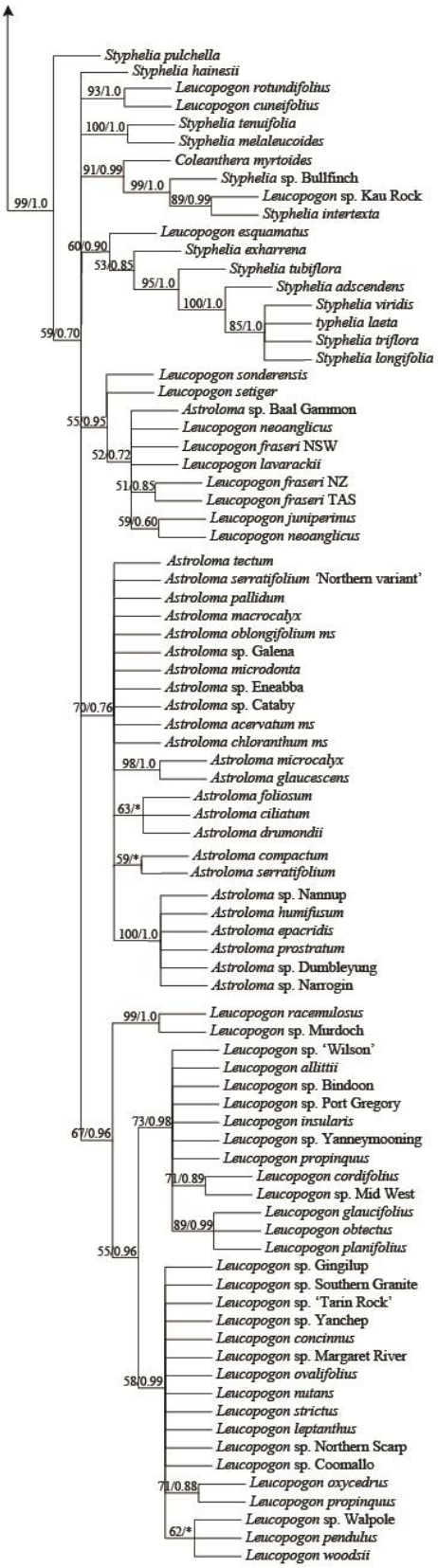
Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Styphelia longifolia R.Br.	JQ667467	JQ667201	KC411584	KC494254	KC197136
Styphelia longistylis (Brongn. and Gris) Sleumer	JQ667470	*	KC411508	KC494255	KC197080
Styphelia melaleucoides A.Cunn. ex F.Muell.	KC479114	*	KC411623	*	KC197159
Styphelia pancheri (Brongn. and Gris) F.Muell.	JQ667229	JQ667083	KC411510	KC494256	KC197083
Styphelia pulchella (Sond.) F.Muell.	*	JQ667089	KC411577	KC494257	KC197127
Styphelia sp.	JQ667376	*	KC411512	KC494258	KC197084
Styphelia sp.	JQ667473	*	KC411513	KC494259	KC197085
Styphelia sp. Bullfinch (M.Hislop 3574)	KC479095	*	KC411606	*	KC197142
Styphelia tenuifolia Lindl.	JQ305880	JQ305847	JQ310813	AY372590	KC197116
Styphelia triflora Andrews	JQ667265	JQ667102	KC411580	KC494260	KC197132
Styphelia triflora Andrews	JQ667375	JQ667208	KC411581	AY372587	KC197133
Styphelia tubiflora Sm.	JQ667253	JQ667094	KC411579	AY372591	KC197130
Styphelia viridis subsp. *viridis* Andrews	JQ305881	AY005105	JQ310814	AF155865	KC197104
Tribe Cosmelieae					
Andersonia sprengelioides R.Br.	U79742	AF015631	*	AF155843	*
Cosmelia rubra R.Br.	GQ392894	GQ392946	*	AF155842	*
Sprengelia incarnata Sm.	U80421	AF015645	*	AF155841	*
Tribe Epacrideae					
Epacris impressa Labill.	*	AF015636	*	AF155849	*

Species	***rbc*L**	***mat*K**	***trn*H-*psb*A**	***atp*B-*rbc*L**	**ITS**
Epacris sinclairii Hook f.	*	*	*	JX993992	*
Lysinema ciliatum R.Br.	*	AY372615	*	AY372559	*
Rupicola sprengelioides Maiden and Betche	U80427	AF015643	*	AF155851	*
Tribe Oligarrheneae					
Needhamiella pumilio (R.Br.) L.Watson	*	AY005100	*	AY005086	*
Oligharrhena micrantha R.Br.	*	AY005101	*	AY005087	*
Tribe Prionoteae					
Prionotes cerinthoides (Labill.) R.Br.	U79743	AF015642	*	AF155838	*
Tribe Richeeae					
Dracophyllum kirkii Berggr.	GQ392905	GQ392957	*	*	AY649410
Dracophyllum longifolium R.Br. ex Roem. and Schult.	GQ392907	GQ392959	*	AF155845	AF419809
Dracophyllum patens W.R.B.Oliv.	GQ392917	GQ392969	*	*	AY649412
Richea pandanifolia Hook.f.	GQ392936	GQ392988	*	AF155844	*
Richea scoparia Hook.f.	GQ392938	GQ392990	*	*	*

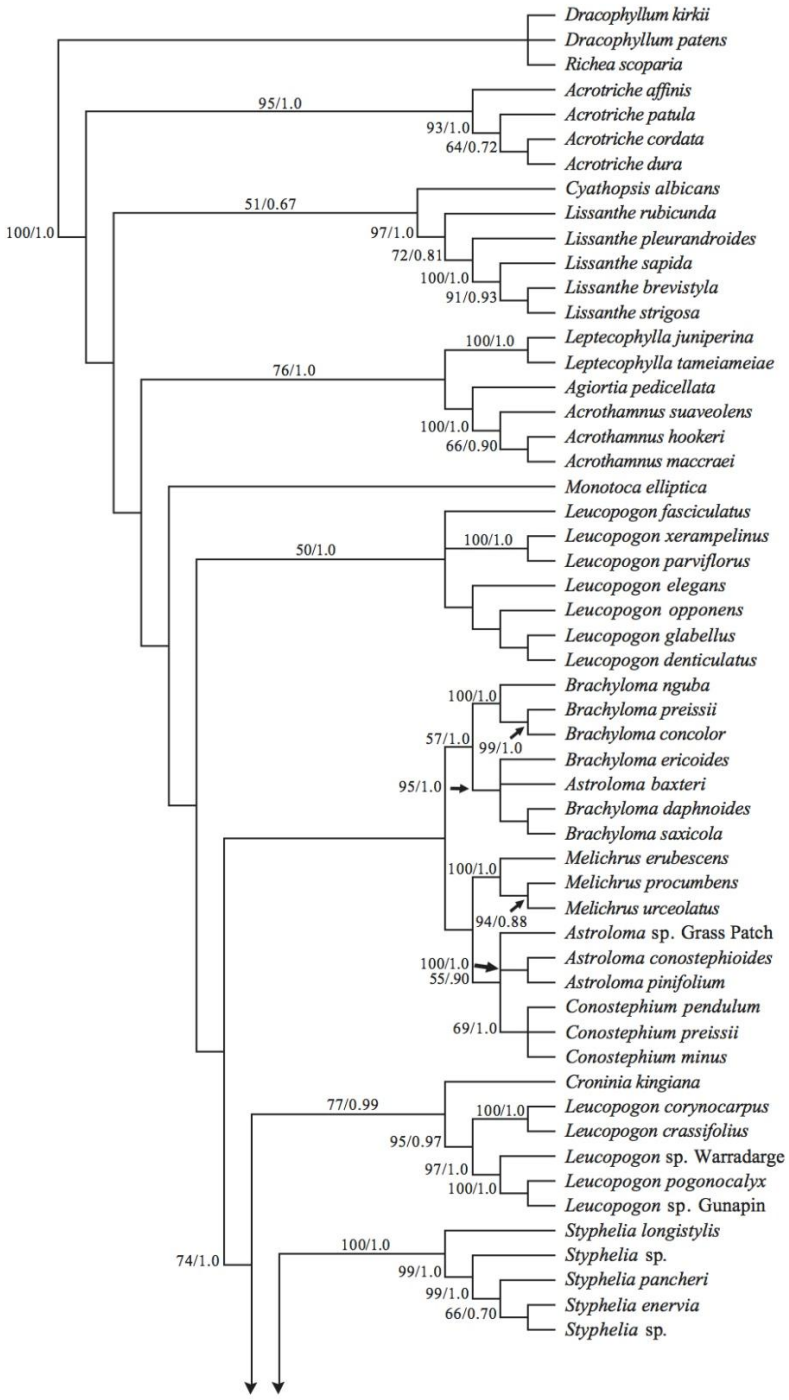
Appendix 2.2 One of the 10000 equally parsimonious trees obtained from the analyses of the combined chloroplast regions *rbc*L, *mat*K, *trn*H-*psb*A, and *atp*B-*rbc*L. Branch support values are on top of the branches in the following order: MP Jacknife/BI posterior probability. Tree length=2479. consistency index (CI) = 0.63, retention index (RI) = 0.85, rescaled consistency index (RC) = 0.54. *corresponds to support values below 50/0.50.

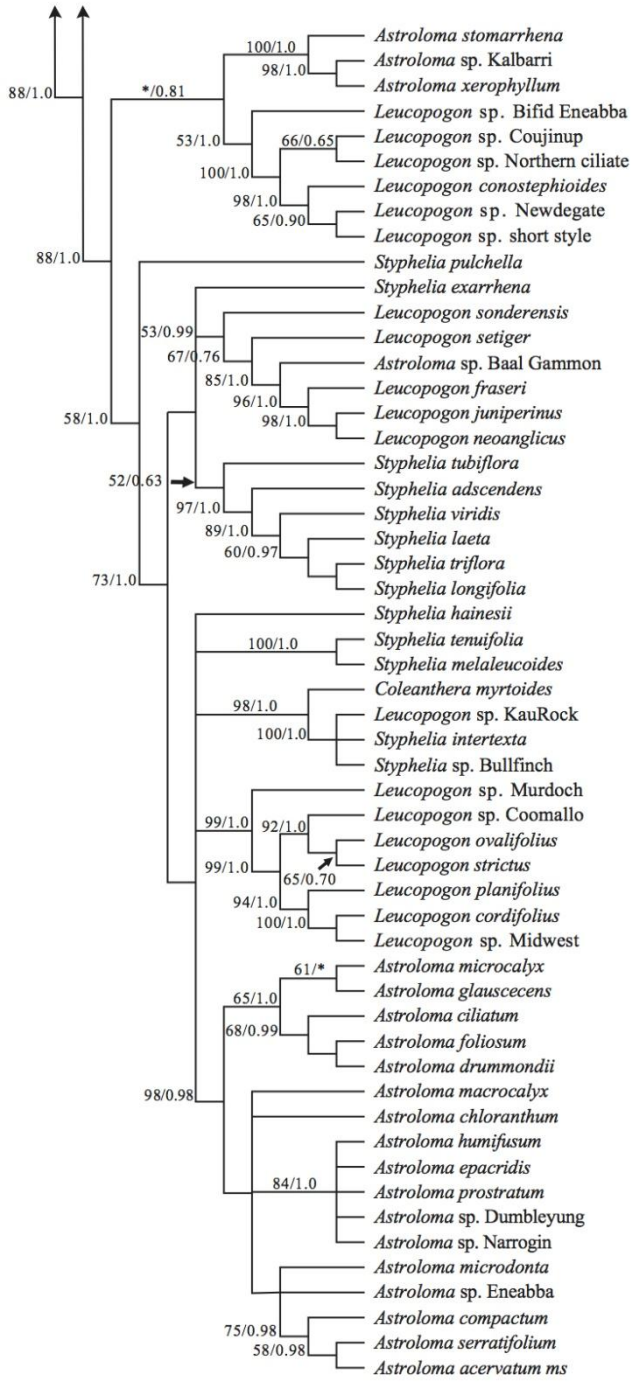



Melichrus sp.
Melichrus erubescens
Melichrus procumbens
Melichrus urceolatus
Astroloma sp. Grass Patch
Astroloma conostephioides
Astroloma pinifolium
Conostephium pendulum
Conostephium preissii
Conostephium minus
Brachyloma ericoides
Astroloma baxteri
Brachyloma sp. Forrestania white
Brachyloma daphnoides
Brachyloma saxicola
Brachyloma sp. Murchison
Brachyloma sp. Kalbarri
Brachyloma nguba
Brachyloma brevifolium
Brachyloma preissii
Brachyloma concolor
Leucopogon sp. MooreRiver
Leucopogon blepharolepis
Styphelia longistylis
Styphelia sp.
Styphelia pancheri
Styphelia cymbulae
Styphelia enervia
Styphelia sp.
Leucopogon margarodes
Leucopogon sp. Mt Heywood
Leucopogon sp. Koolyanobbing
Leucopogon crassiflorus
Leucopogon sp. Badgingarra
Leucopogon sp. Bremer Bay
Croninia kingiana
Leucopogon appressus
Leucopogon corynocarpus
Leucopogon crassifolius
Leucopogon ruscifolius
Leucopogon yorkensis
Leucopogon leptospermoides
Leucopogon ericoides
Leucopogon muticus
Leucopogon sp. Arrino
Leucopogon sp. Brookton
Leucopogon sulcatus
Leucopogon sp. Kalbarri
Leucopogon sp. Howatharra
Leucopogon marginatus
Leucopogon cymbiformis
Leucopogon sp. Wandering
Leucopogon sp. Mondurup
Leucopogon sp. Yandanooka
Leucopogon sp. Gunapin
Leucopogon tamminensis
Leucopogon pogonocalyx
Leucopogon sp. Tathra
Leucopogon sp. Warradarge
Leucopogon sp. ciliate Eneabba
Leucopogon sp. Ongerup
Astroloma sp. sessile leaf
Astroloma sp. Kalbarri
Astroloma stomarrhena
Astroloma xerophyllum
Leucopogon sp. Bifid Eneabba
Leucopogon sp. Coujinup
Leucopogon sp. Northern ciliate
Leucopogon hispidus
Leucopogon conostephioides
Leucopogon pubescens
Leucopogon sp. Newdegate
Leucopogon sp. short style
100/1.0
76/0.94
69/0.61
72/0.89
98/1.0
51/0.85
92/0.90
97/0.88
80/0.95
79/0.90
82/0.85
99/1.0
96/1.0
72/0.85
59/0.90
100/1.0
96/0.99
87/0.99
74/0.98
56/0.99
86/0.90
91/0.95
100/1.0
97/1.0
69/0.98
93/0.95
99/0.99
78/0.82
65/*
99/1.0
86/0.90
90/0.95
50/0.60
89/0.99
52/0.98
92/1.0
92/0.99
54/0.96
64/0.98
62/0.80
51/*
96/1.0


Styphelia pulchella
Styphelia hainesii
93/1.0
Leucopogon rotundifolius
Leucopogon cuneifolius
100/1.0
Styphelia tenuifolia
Styphelia melaleucoides
Coleanthera myrtoides
91/0.99
Styphelia sp. Bullfinch
99/1.0
99/1.0
89/0.99
Leucopogon sp. Kau Rock
Styphelia intertexta
Leucopogon esquamatus
60/0.90
Styphelia exharrena
53/0.85
Styphelia tubiflora
95/1.0
Styphelia adscendens
100/1.0
Styphelia viridis
85/1.0
typhelia laeta
59/0.70
Styphelia triflora
Styphelia longifolia
Leucopogon sonderensis
Leucopogon setiger
55/0.95
Astroloma sp. Baal Gammon
Leucopogon neoanglicus
52/0.72
Leucopogon fraseri NSW
Leucopogon lavarackii
51/0.85
Leucopogon fraseri NZ
Leucopogon fraseri TAS
59/0.60
Leucopogon juniperinus
Leucopogon neoanglicus
Astroloma tectum
Astroloma serratifolium 'Northern variant'
Astroloma pallidum
Astroloma macrocalyx
Astroloma oblongifolium ms
Astroloma sp. Galena
Astroloma microdonta
Astroloma sp. Eneabba
70/0.76
Astroloma sp. Cataby
Astroloma acervatum ms
Astroloma chloranthum ms
98/1.0
Astroloma microcalyx
Astroloma glaucescens
Astroloma foliosum
63/*
Astroloma ciliatum
Astroloma drumondii
59/*
Astroloma compactum
Astroloma serratifolium
Astroloma sp. Nannup
Astroloma humifusum
100/1.0
Astroloma epacridis
Astroloma prostratum
Astroloma sp. Dumbleyung
Astroloma sp. Narrogin
99/1.0
Leucopogon racemulosus
Leucopogon sp. Murdoch
Leucopogon sp. 'Wilson'
Leucopogon allittii
Leucopogon sp. Bindoon
Leucopogon sp. Port Gregory
73/0.98
Leucopogon insularis
67/0.96
Leucopogon sp. Yanneymooning
Leucopogon propinquus
71/0.89
Leucopogon cordifolius
Leucopogon sp. Mid West
Leucopogon glaucifolius
89/0.99
Leucopogon obtectus
55/0.96
Leucopogon planifolius
Leucopogon sp. Gingilup
Leucopogon sp. Southern Granite
Leucopogon sp. 'Tarin Rock'
Leucopogon sp. Yanchep
Leucopogon concinnus
Leucopogon sp. Margaret River
58/0.99
Leucopogon ovalifolius
Leucopogon nutans
Leucopogon strictus
Leucopogon leptanthus
Leucopogon sp. Northern Scarp
Leucopogon sp. Coomallo
71/0.88
Leucopogon oxycedrus
Leucopogon propinquus
Leucopogon sp. Walpole
62/*
Leucopogon pendulus
Leucopogon woodsii

Appendix 2.3 One of 10000 equally parsimonious trees obtained from the nuclear-encoded ribosomal Internal Transcribed Spacer (ITS) analyses. Branch support values are on top of the branches in the following order: MP Jacknife/BI posterior probability. Tree length=1397. CI = 0.50, RI = 0.85, RC = 0.43




Astroloma stomarrhena
Astroloma sp. Kalbarri
Astroloma xerophyllum
Leucopogon sp. Bifid Eneabba
Leucopogon sp. Coujinup
Leucopogon sp. Northern ciliate
Leucopogon conostephioides
Leucopogon sp. Newdegate
Leucopogon sp. short style
Styphelia pulchella
Styphelia exarrhena
Leucopogon sonderensis
Leucopogon setiger
Astroloma sp. Baal Gammon
Leucopogon fraseri
Leucopogon juniperinus
Leucopogon neoanglicus
Styphelia tubiflora
Styphelia adscendens
Styphelia viridis
Styphelia laeta
Styphelia triflora
Styphelia longifolia
Styphelia hainesii
Styphelia tenuifolia
Styphelia melaleucoides
Coleanthera myrtoides
Leucopogon sp. KauRock
Styphelia intertexta
Styphelia sp. Bullfinch
Leucopogon sp. Murdoch
Leucopogon sp. Coomallo
Leucopogon ovalifolius
Leucopogon strictus
Leucopogon planifolius
Leucopogon cordifolius
Leucopogon sp. Midwest
Astroloma microcalyx
Astroloma glauscecens
Astroloma ciliatum
Astroloma foliosum
Astroloma drummondii
Astroloma macrocalyx
Astroloma chloranthum
Astroloma humifusum
Astroloma epacridis
Astroloma prostratum
Astroloma sp. Dumbleyung
Astroloma sp. Narrogin
Astroloma microdonta
Astroloma sp. Eneabba
Astroloma compactum
Astroloma serratifolium
Astroloma acervatum ms
100/1.0
98/1.0
*/0.81
88/1.0
53/1.0
66/0.65
100/1.0
98/1.0
65/0.90
88/1.0
53/0.99
67/0.76
85/1.0
96/1.0
98/1.0
58/1.0
52/0.63
97/1.0
89/1.0
60/0.97
73/1.0
100/1.0
98/1.0
100/1.0
99/1.0
92/1.0
99/1.0
65/0.70
94/1.0
100/1.0
61/*
65/1.0
68/0.99
98/0.98
84/1.0
75/0.98
58/0.98

2

Appendix 4.1. Character states scored for each taxon included in the survey. Numbers I to XI indicate the group to which they belong according to the results from Chapter 2. NSW: National Herbarium of New South Wales; PERTH: Western Australian Herbarium; AQ: Queensland Herbarium (BRI); LAE: Papua New Guinea National Herbarium; HO: Tasmanian Herbarium; CHR: Allan Herbarium, Landcare Research New Zealand Limited. * Voucher information not available.

Taxa	Pollen type	Ornamentation	No. of apertures	Size (µm)	Annulus	Reference	Voucher
***Styphelia-Astroloma* clade**							
I: *Astroloma* s.s.							
A. ciliatum (Lindl.) Druce	Pseudomonad	Psilate to slightly perforate	6	75-110	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW361523
A. epacridis (DC.) Druce	Pseudomonad	Perforate	6	50-60	Slightly thickened	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	A.R. Chapman 429
A. humifusum (Cav.) R.Br.	Pseudomonad	Psilate	6	65-80	Absent	S. Smith-White, 1955; A. Wilson (unpubl)	*
A. pallidum R.Br.	Pseudomonad	Psilate	6	100-110	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW487715
A. prostratum R.Br.	Pseudomonad	Perforate	6	46-60	Present	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW446375

A. serratifolium (DC.) Sond.	Pseudomonad	Psilate to slightly perforate	6	45-50	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	J.M. Powell 2447
A. sp. Dumbleyung[†] (A.J.G. Wilson 146)	Pseudomonad	Psilate	6	45-50	Present	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW482432
A. sp. Cataby (E.A.Griffin 1022)	Pseudomonad	Psilate	6	80-105	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	PERTH01297562
A. sp. Nannup (R.D.Royce 3978)	Pseudomonad	Perforate	6	55-60	Slightly thickened	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	PERTH06608787
A. macrocalyx Sond.	Pseudomonad	Perforate	6	83-94	Absent	A. Wilson (unpbl.)	*
A. tectum Sond.	Pseudomonad	Perforate	6	75-78	Slightly thickened	S. Smith-White (1995); A. Wilson (unpbl.)	*
II: *Styphelia s.l.*							
S. melaleucoides A.Cunn. ex F.Muell.	Pseudomonad	Verrucate	>6	35-40	Absent	C. Puente-Lelièvre, 2013	PERTH05679281
S. tenuifolia Lindl.	Pseudomonad	Verrucate	>6	44-48	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW265446

[†] *Astroloma recurvum*

III: *Styphelia s.l.*							
Styphelia intertexta A.S.George	Pseudomonad	Perforate	6	20-25	Absent	C. Puente-Lelièvre, 2013	PERTH07016395
IV: *Leucopogon s.l. p.p.*							
L. cuneifolius Stschegl.	Pseudomonad	Psilate to Perforate	6	20-28	Absent	S. Smith-White, 1955; C. Quinn (unpbl.)	NSW446378
V: *Leucopogon s.l. p.p.*							
L. allittii F.Muell.	Pseudomonad	Psilate to Perforate	6	35-40	Absent	C. Quinn (unpbl.)	NSW404170
L. cordifolius Lindl.	Pseudomonad	Perforate	6	40-45	Absent, or slightly thickened	C. Puente-Lelièvre, 2013	NSW414604
L. oxycedrus Sond.	Pseudomonad	Finely Perforate	6	40-50	Absent, or slightly thickened	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW700477
L. ovalifolius Sond.	Pseudomonad	Psilate	6	30-35	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW810901
L. propinquus R.Br.	Pseudomonad	Psilate to Perforate	6	30-35	Absent, or slightly thickened	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW406036

L. pendulus R.Br.	Pseudomonad	Perforate	6	25-30	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW201982
L. strictus Benth.	Pseudomonad	Psilate	6	40-45	Absent, or slightly depressed	S. Smith-White, 1955; C. Quinn (unpbl.)	PERTH05303087
VI: *Styphelia s.s.*							
S. adscendens R.Br.	Pseudomonad	Gemmate, granulate	>6	45-50	Absent	C. Puente-Lelièvre, 2013	NSW265576
S. laeta R.Br.	Pseudomonad	Gemmate, granulate	>6	~60	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW464396
S. longifolia R.Br.	Pseudomonad	Gemmate, granulate	>6	50-60	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW143268
S. triflora Andrews	Pseudomonad	Gemmate, granulate	>6	65-70	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW264274
S. viridis Andrews	Pseudomonad	Gemmate, granulate	>6	70-80	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW237892
VII: *Leucopogon s.l. p.p.*							
A. sp. Baal Gammon (B.P.Hyland 10341)	Pseudomonad	Perforate	6	50-70	Present	C. Puente-Lelièvre, 2013	NSW409420

L. fletcheri Maiden and Betche	Pseudomonad	Granulate	6,>6	38-40	Present	C. Puente-Lelièvre, 2013	NSW436103
L. juniperinus R.Br.	Pseudomonad	Perforate	6?	35-40	Present	S. Smith-White, 1955; C. Quinn (unpbl.)	NSW40559
L. neoanglicus F.Muell. ex Benth.	Pseudomonad	Psilate-Perforate	6	35-45	Present	C. Quinn (unpbl.); C. Puente-Lelièvre, 2013.	AQ670606
L. setiger R.Br.	Pseudomonad	Granulate	6	~30	Present	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW108989
L. sonderensis J.H.Willis	Pseudomonad	Psilate to perforate	6	45-50	Present	C. Puente-Lelièvre, 2013	NSW474001
VIII: *Leucopogon conostephioides* complex							
L. conostephioides DC.	Pseudomonad	Rugulate	6?	24-28	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	PERTH08020833
L. pubescens S.Moore	Pseudomonad	Rugulate	6	20-25	Absent	C. Puente-Lelièvre, 2013	PERTH05704413
L. sp. Newdegate (M. Hislop 3585)	Pseudomonad	Rugulate	6	28-32	Absent	C. Puente-Lelièvre, 2013	PERTH08176361
L. sp. short style (S.Barrett 1578)	Pseudomonad	Rugulate	6?	25-30	Absent	C. Puente-Lelièvre, 2013	PERTH05364787

IX: *Stomarrhena*								
A. stomarrhena Sond.	Pseudomonad	Psilate		6	60-70	Absent, depressed	S. Smith-White, 1955; A. Wilson (unpbl.)	*
A. xerophyllum (DC.) Sond.	Pseudomonad	Granulate		>6	62-68	Absent, depressed	S. Smith-White, 1955; A. Wilson (unpbl.)	NSW201621
L. sp. ciliate Eneabba (F.Obbens and C.Godden s.n. 3/7/2003)	Pseudomonad	Granulate		6	45-60	Absent	C. Puente-Lelièvre, 2013	PERTH08182078
X: *Leucopogon s.l. p.p.*								
L. appressus R.Br.	Pseudomonad	Psilate		3	20-25	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW262140
L. crassiflorus (F.Muell.) Benth.	Pseudomonad	Fossulate		4	36-45	Present	C. Quinn (unpbl.)	NSW405390
L. crassifolius Sond.	Pseudomonad	Fossulate-Perforate		3,4	25-30	Present	C. Quinn (unpbl.)	NSW449306
L. corynocarpus Sond.	Pseudomonad	Psilate to Perforate		3	~25	Absent	C. Quinn (unpbl.)	NSW414352
L. cymbiformis	Pseudomonad	Psilate	to	3	15-20	Absent	C. Quinn (unpbl.)	NSW436232

A.Cunn. ex DC.		Perforate					
L. ericoides (Sm.) R.Br.	Pseudomonad	Granulate	4,5	20-25	Present	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW391618
L. leptospermoides R.Br.	Pseudomonad	Slightly granulate	3	25-30	Absent	C. Puente-Lelièvre, 2013	NSW626484
L. muticus R.Br.	Pseudomonad	Perforate	3	20-25	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	J.M. Powell 2961
L. ruscifolius R.Br.	Pseudomonad	Perforate	3	25-35	Absent	C. Puente-Lelièvre, 2013	NSW451549
Croninia kingiana (F.Muell.) J.M.Powell	Pseudomonad?	Verrucate-slightly verrucate	>6	36- 44	Present	J.M. Powell, 1993; Streiber, 1999; C. Quinn (unpubl.)	NSW263778, Cranfield 6029
XI: *Leucopogon s.l. p.p.*							
L. blepharolepis (F.Muell.) F.Muell. ex Benth.	Pseudomonad	Rugulate	4	30-40	Present	C. Quinn (unpbl.); C. Puente-Lelièvre, 2013.	NSW201182, NSW417707
Ungrouped taxa							
Coleanthera myrtoides Stschegl.	Pseudomonad	Rugulate, perforate	6	25-28	Absent	C. Puente-Lelièvre, 2013	J.M. Powell 2811
Leucopogon	Pseudomonad	Areolate	4,5	35-40	Absent	C. Quinn (unpbl.)	NSW410225

esquamatus R.Br.							
Styphelia exarrhena (F.Muell.) F.Muell.	Pseudomonad	Areolate	5,6	~28	Absent	Streiber, 1999	NSW238193
Styphelia hainesii F.Muell.	Pseudomonad	Areolate	4,5	40-50	Absent	A. Wilson (unpbl.); C. Puente-Lelièvre, 2013	NSW650516
Styphelia pulchella (Sond.) F.Muell.	Pseudomonad	Gemmate, verrucate	>6	~35	Absent	Streiber, 1999.	NSW360998
Outgroup							
Styphelieae							
***Acrothamnus* Quinn**							
A. colensoi (Hook.f.) Quinn	A-type	Psilate	3	30-50	Absent	C. Quinn *et al.* 2005; C. Quinn (unpl).	CHR496541
A. hookeri (Sond.) Quinn	A-type	Psilate	3	30-40	Absent	C. Quinn *et al.* 2005; C. Quinn (unpl).	NSW700935
A. maccraei (F.Muell.) Quinn	A-type	Psilate	3	30-35	Absent	C. Quinn *et al.* 2005; C. Quinn (unpl).	NSW441684
A. suaveolens (Hook.f.) Quinn	A-type	Psilate	3	28-32	Absent	C. Quinn *et al.* 2005; C. Quinn (unpl).	LAE61948
***Acrotriche* R.Br.**							
A. affinis DC.	A-type	Perforate	3	25-35	Absent	C. Quinn (unpbl.);	NSW270671

A. cordata (Labill.) R.Br.	A-type	Perforate	3	30-40	Absent	M. Schneemilch and M. Kokkinn, 2011. C. Quinn (unpbl.); M. Schneemilch and M. Kokkinn, 2011.	NSW270682
A. patula R.Br.	A-type	Perforate	3	30-34	Absent	C. Quinn (unpbl.); M. Schneemilch and M. Kokkinn, 2011.	NSW271014
***Brachyloma* Sond.**							
B. daphnoides (Sm.) Benth.	T-type	Psilate	3	25-30	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	AQ678035
B. scortechinii F.Muell.	T-type; A-type	Psilate	>6	45-55	Absent	S. Smith-White, 1995; Streiber, 1999.	NSW390967
Astroloma baxteri A.Cunn. ex DC.	Pseudomonad	Perforate	>6	~39	Absent	Streiber, 1999.	Pritzel 328
***Conostephium* Benth.**							
C. pendulum Benth.	A-type	Granulate	0	21-27	Absent	C. Quinn (unpbl.)	Cranfield 817/78
***Leptecophylla* C.M. Weiller**							
L. abietina (Labill.)	A-type	Psilate	3	30-35	Absent	C. Quinn (unpbl.)	HO97895

C.M.Weiller							
L. juniperina (J.R.Forst. and G.Forst.) C.M.Weiller	A-type	Psilate	3	30-35	Absent	C. Quinn (unpbl.)	NSW437688
Leucopogon s.s.							
L. amplexicaulis (Rudge) R.Br.	Pseudomonad	Psilate	3	20-25	Absent	S. Smith-White, 1955; C. Quinn (unpbl.)	NSW441320
L. australis R.Br.	Pseudomonad	Psilate	3	20-25	Absent	S. Smith-White, 1955; C. Quinn (unpbl.)	NSW203005
L. bossiaea (F.Muell.) Benth.	Pseudomonad	Psilate	3	25-30	Absent	C. Quinn (unpbl.)	NSW296433
L. virgatus (Labill.) R.Br.	Pseudomonad	Perforate	3?	25-30	Absent	S. Smith-White, 1955; C. Quinn (unpbl.)	NSW423098
***Lissanthe* R.Br.**							
L. pluriloculata (F.Muell.) J.M.Powell, Crayn and E.A.Br.	A-type	Perforate?	3	45-50	Absent	C. Quinn (unpbl.)	Bean 6325, Blaxell 89/233
L. strigosa subsp. *subulata*	T-type	Perforate	3	45-50	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW238918, NSW460981

Monotoca R.Br.							
M. elliptica (Sm.) R.Br.	Pseudomonad	Psilate	3	12-18	Absent	C. Quinn (unpbl.)	J.M. Powell 4573
M. rotundifolia J.H.Willis	Pseudomonad	Psilate	3	12-20	Absent	C. Quinn (unpbl.)	CBG9400399
***Pentachondra* R.Br.**							
P. involucrata R.Br.	A-type	Psilate	3	33-42	Absent	C. Venkata Rao, 1961; C. Quinn (unpbl.)	NSW366406
P. pumila (J.R.Forst. and G.Forst.) R.Br.	T-type	Perforate	3	28-38	Absent	S. Smith White, 1955; C. Venkata Rao, 1961; C. Quinn (unpbl.)	NSW392498
Stenanthera R.Br.							
Astroloma conostephioides (Sond.) F.Muell. ex Benth.	A-type	Striate	0	47-94	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	Whiblet s.n.
Astroloma pinifolium (R.Br.) Benth.	A-type	Psilate	0	64-100	Absent	S. Smith-White, 1955; C. Puente-Lelièvre, 2013	NSW452742
Astroloma sp. Grass	A-type	Psilate, granulate	0	50-72	Absent	C. Puente-Lelièvre, 2013	NSW650517,

Patch (A.J.G.Wilson 110)							PERTH06827713
Cosmelieae							
Andersonia sprengelioides R.Br.	A-type	Psilate	3	?	?	Lemson, 2011; Smith-White, 1955	*
Cosmelia rubra R.Br.	A-type	Psilate	3	?	?	Lemson, 2011; Smith-White, 1955	*
Epacrideae							
Epacris impressa Labill.	T-type	?	3	~62	?	C. Venkata Rao, 1961	*
Lysinema ciliatum R.Br.	T-type	?	?	~40	?	C. Venkata Rao, 1961	*
Rupicola sprengelioides Maiden and Betche	T-type	Reticulate?	3	32-35	?	Australasian pollen and spores atlas (http://apsa.anu.edu.au)	*
Oligarrheneae							
Needhamiella pumilio (R.Br.) L.Watson	A-type	Psilate to Perforate	0	20-26	Absent	C. Quinn (unpbl.)	J.M. Powell 2391
Oligarrhena micrantha R.Br.	Pseudomonad	Psilate to Perforate	3	8.5-11	Absent	C. Quinn (unpbl.)	NSW418386, NSW398414

Prionoteae							
Prionotes cerinthoides (Labill.) R.Br.	T-type	?	?	?	?	C. Venkata Rao, 1961	*
Richeeae							
Dracophyllum kirkii Berggr.	T-type	?	3	?	?	Furness, 2009	*
Dracophyllum patens W.R.B.Oliv.	T-type	?	3	?	?	Furness, 2009	*
Richea scoparia Hook.f.	T-type	?	3	?	?	C. Venkata Rao, 1961	*