

Evolution of Pollination in *Prostanthera* Labill. (Lamiaceae)

Trevor Craig Wilson



THE UNIVERSITY OF
SYDNEY

**School of Biological Sciences
University of Sydney
New South Wales
Australia**

July 2010

**Submitted in fulfilment of the requirements
of the degree of Doctor of Philosophy**

Declaration

I hereby declare that the work of this thesis is my own and contains the results of an original investigation. This work was carried out while I was enrolled as a student for the degree of Doctor of Philosophy in the School of Biological Sciences at the University of Sydney and at the Botanic Gardens Trust, Sydney, Australia. This thesis has not been previously submitted for examination at any other university.

Trevor C. Wilson

July 2010

Acknowledgements

I would like to thank my supervisors Murray Henwood and Barry Conn. Together, they provided me with encouragement, skill, inspiration, fun, and enthusiasm, which helped me grow my scientific mind.

I thank the University of Sydney and the Australian government for making my candidacy possible through the Endeavour International Postgraduate Research Scholarship. Funding thanks also goes to the Australian Biological Resources Study (Hansjörg Eichler Scientific Research Fund) and Linnean Society of N.S.W. (Joyce Vickery Scientific Research Grant).

I thank my rugged field volunteers Augusta Supple, Jan Allen, Barry Conn, Louisa Murray, Ido Issler, Emma McIntosh, Anja Klingeböck, Andrew Perkins, Lauren Dukas, Fran Daniels, Marjan Medhat, Robin McAlpine, Mahtab Amjadi, Mark Norman, Rob Smith, Ben Hinton, and Endymion D. Cooper, who braved the wilds to seek the true beauty of nature.

My colleagues at the University of Sydney and Royal Botanic Gardens have been supportive and inspiring, both professionally and personally. At the university I would especially like to thank the plant systematics lab (Endymion D. Cooper, Kerry Gibbons, Dr. Matthew Pye, Andrew Perkins, Camilla Ip, Kirsten Proft, Dan Clark) for ideas, inspiration, help with the thesis and always a good coffee time. Glenda Wardle and Yvonne Davila have always been helpful, especially with pollination ecology. The administrative staff consisting of Susan Ramsey, Semra Yetke, and Susan Thomas has also always been a big help. At the Royal Botanic Gardens, I would especially like to thank Carolyn Porter, Hannah McPherson, Louisa Murray, Ifeanna Tooth, Belinda Araghi, Lisa Woods, Marny Innis, Karen Rinkel, Helen Stevenson as well as staff at all the Botanic Gardens trust: Mount Tomah, Sydney and Mt Annan.

For specimens I would like to thank individuals and their associated herbaria/gardens: Amanda Shade (Kings Park Botanic Gardens), Sarah Fethers (ANBG), Jenny Liney (Eurobodella Botanic Gardens), Stephen Kingdon (Adelaide Botanic Gardens), Helen Vonow (AD), Katherine Tuft, Jaynia Tarnawski (Australian Museum), Brice Ebert, Emma Williams, Adrienne Grant, and Sam Clayman.

I thank those at National Parks for keeping me well equipped and safe, and in particular, Sean Keenan and Felicity Brooke (Mt Buffalo N.P.), Leigh Granger (Cocoparra N.P.), Jessica Herder (Royal N.P.), Aileen Bell (Warrumbungles N.P.), and Darren Pitt (Mt Kaputar N.P.).

I thank Michael Bately (Australian Museum) for native bee identifications and Allan Jones for help with microscopy.

For thanks with postgrad life and help with the thesis, I thank Leonie Whiffen, Peter Oxley, Jacquie Herbert, Bridget Murphy, Lou Pastro, Anke Frank, Will Armour, Julianne Pople, Nikki Curthoys, Miguel Bedoya-Perez, Tony Popic, Lindsey Gray, Matt Renner, and Fiona Powell. Thanks to Su Hanfling and Helen Conn for taking care of my supervisors, who took care of me.

A warm thanks to my dear friends Barbara Bryce and Ricardo Ramirez for their support, encouragement, and guidance on life and the PhD.

I owe deep gratitude to my dear friends and colleagues Jan Allen and Rob Smith, who helped me in every way with this thesis.

I would like to thank my parents, Ron and Stephanie, and my sisters, Natalie and Breeanne, for their unending support.

My deepest thanks goes to Augusta Supple: your courage, enthusiasm, and level of greatness are an inspiration to me and helped me achieve this. Your warmth and sweetness kept me sane and loved along the way.



Prostanthera lasianthos visited by *Phylotocus* sp. (Cetoniinae).

“A traveller should be a botanist,
for in all views plants form the
chief embellishment.”

Charles Darwin

Abstract

Prostanthera Labill. (Lamiaceae) is traditionally divided into section *Klanderia* (F. Muell.) Benth. and section *Prostanthera* based on floral characteristics that correspond to putatively ornithophilous or entomophilous pollination syndromes. To better understand these pollination syndromes and how they evolved, the phylogenetic relationships, floral morphology, and pollination of *Prostanthera* were investigated. Maximum parsimony and Bayesian phylogenies for 66 (~75%) species of *Prostanthera* were constructed using chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) genomes. In all cases *Prostanthera* was found to be paraphyletic with respect to *Wrixonia*. *Prostanthera* section *Prostanthera* was revealed to be paraphyletic relative to section *Klanderia*. A multivariate morphometric analysis of floral characteristics was also congruent with the molecular phylogenies and identified a single putatively ornithophilous group and two putatively entomophilous groups. Quantitative analyses of pollinator visitation further supported hypotheses derived from the analysis of nucleotide data and multivariate morphometrics and identified functional pollinator groups for each clade. Ancestral state reconstruction using squared-change parsimony of geometric morphometric landmark data provided insights into the ancestral pollination syndrome and evolutionary changes necessary to optimise corolla morphology for each clade. It is concluded that ancestral *Prostanthera* was likely pollinated by a diverse range of insects and possibly even birds. The evolution of an ornithophilous clade and a generalist entomophilous clade correspond with separate modifications to the lobes and tube of the corolla. The combination of phylogenetic and morphological data from this study provides a robust understanding of relationships and an insight to the structural changes in the evolution of pollination.

Table of Contents

Declaration	ii
Acknowledgements	iii
Dedication	v
Abstract	vi
Chapter 1: Introduction	1
The importance of pollination.....	2
The pollination syndrome.....	2
Phylogenetics	4
Morphometrics.....	6
<i>Prostanthera</i>	7
Project aims	10
Chapter 2: Molecular data reveal the paraphyly of <i>Prostanthera</i> section <i>Prostanthera</i>	11
Introduction.....	11
Materials and methods	14
Taxon sampling	14
Identification of series	14
Molecular methods	19
Phylogenetic reconstructions.....	22
Results.....	24
Compositional homogeneity	24
Incongruence length difference test.....	24
Maximum parsimony and Bayesian analysis	26
<i>ndhF-rpl32</i> data set	26
<i>trnT-F</i> data set.....	29
ETS data set	29
Combined (<i>trnT-F</i> , <i>ndhF-rpl32</i> , and ETS) data set	36
Discussion.....	37
Classification in the Westringieae	38
Classification in <i>Prostanthera</i>	39
Conclusions.....	41

**Chapter 3: Floral characters and shape support measurable
pollination syndromes in *Prostanthera* 42**

Introduction..... 42
Materials and Methods..... 44
 Specimen collection..... 44
 Measurement of phenetic data 46
 Measurement of geometric data..... 52
 Statistical analysis..... 52
Results..... 54
 Phenetic data 54
 Geometric data..... 65
Discussion 70
 Interpretation of phenetic analysis..... 70
 Interpretation of geometric analysis 72
 Interpretation of pollination syndromes..... 74
Conclusions..... 77

**Chapter 4: Investigation of pollination syndromes in
Prostanthera..... 78**

Introduction..... 78
Materials and methods 81
 Specimens and sites 81
 Observation of phenology..... 82
 Stigmatic receptivity 83
 Visitor censuses 83
 Statistical methods 85
 Flower number..... 87
 Nectar collection..... 87
Results..... 88
 Floral development 88
 Female receptivity 95
 Visitor observations 96
 Multivariate analysis of visitors..... 100
 Multivariate analysis of pollinators 105
 Analysis of pollinators and environmental variables..... 105
 Flower number and nectar 108
Discussion..... 108
 Floral biology of *Prostanthera* 112
 Assessment of pollination syndromes in *Prostanthera* 115
 Environmental influence on pollination 117
 Influence of rewards on pollination..... 118
 Visitation by *Apis mellifera* 119

Conclusions.....	119
Chapter 5: The evolution of pollination in <i>Prostanthera</i> inferred from morphological and phylogenetic analyses.....	121
Introduction	121
Materials and methods.....	123
Specimens.....	123
Phylogenetic analysis.....	124
Morphometric analysis	124
Ancestral state reconstruction.....	126
Results.....	127
Phylogenetic analysis.....	127
Morphometric analysis	130
Evolutionary analysis.....	133
Discussion.....	137
Phylogenetic analysis.....	137
Morphological analysis and interpretation of pollination syndromes	137
Evolution of pollination.....	139
Conclusions.....	142
Chapter 6: Discussion and conclusions	143
Phylogenetic considerations for <i>Prostanthera</i>	144
Assessment of relative warps analysis.....	150
Floral morphology and pollination of <i>Prostanthera</i>	151
Pollination in <i>Prostanthera</i>	153
Evolution of pollination in <i>Prostanthera</i>	156
Suggestions for future research.....	157
Improving the resolution of phylogenies.....	157
A new dimension for geometric morphometrics	159
Quantifying pollinator effectiveness.....	160
Conclusions.....	161
References	162

List of Figures

Chapter 2: Molecular data reveal the paraphyly of <i>Prostanthera</i> section <i>Prostanthera</i>	11
Figure 2.1. Phylogeny reconstructed from maximum parsimony analysis of <i>ndhF-rpl32</i>	27
Figure 2.2. Phylogeny reconstructed from Bayesian analysis of <i>ndhF-rpl32</i>	28
Figure 2.3. Phylogeny reconstructed from maximum parsimony analysis of <i>trnT-F</i>	30
Figure 2.4. Phylogeny reconstructed from Bayesian analysis of <i>trnT-F</i>	31
Figure 2.5. Phylogeny reconstructed from maximum parsimony analysis of ETS	32
Figure 2.6. Phylogeny reconstructed from Bayesian analysis of ETS.....	33
Figure 2.7. Phylogeny reconstructed from maximum parsimony analysis of combined <i>trnT-F</i> , <i>ndhF-rpl32</i> , and ETS	34
Figure 2.8. Phylogeny reconstructed from Bayesian analysis of combined <i>trnT-F</i> , <i>ndhF-rpl32</i> , and ETS.	35
 Chapter 3: Floral characters and shape support measurable pollination syndromes in <i>Prostanthera</i>	 42
Figure 3.1. Flowers representative of morphological diversity in <i>Prostanthera</i>	44
Figure 3.2. Landmarks and linear morphometrics characters applied to <i>Prostanthera</i> flowers	48
Figure 3.3. Measurements of angle, reflexion, curvature, stigma and anther for <i>Prostanthera</i> flowers	50
Figure 3.4. UPGMA dendrogram derived from floral phenetic data.....	55
Figure 3.5. Box-whisker plots from UPGMA analysis retrieving two groups	56
Figure 3.6. Box-whisker plots from UPGMA analysis retrieving three groups	58
Figure 3.7. Box-whisker plots from UPGMA analysis using <i>a priori</i> groups.....	59
Figure 3.8. SSHMDS of the floral phenetic data	60
Figure 3.9. UPGMA dendrogram derived from floral phenetic data excluding male flower stage characters	62
Figure 3.10. Box-whisker plots from UPGMA analysis for three groups using floral phenetic data and excluding male flower characters	63
Figure 3.11. SSHMDS plots of the floral phenetic data excluding the male flower stage characters	64
Figure 3.12. Ordination derived from relative warps analysis.....	66

Figure 3.13. Thin-plate splines derived from relative warps analysis	67
Figure 3.14. Consensus shape for group 1, group 2a, and group 2b.....	68
Figure 3.15. UPGMA dendrogram derived from geometric data	68
Figure 3.16. Box-whisker-plots derived from UPGMA analysis of geometric data	69
Figure 3.17. SSHMDS plots of the geometric data	71

Chapter 4: Investigation of pollination syndromes in *Prostanthera*..... 78

Figure 4.1. Staining for stigmatic receptivity	83
Figure 4.2. Anthetic stages of the <i>P. striatiflora</i> flower	89
Figure 4.3. Anthetic stages of the <i>P. lasianthos</i> , <i>P. monticola</i> , and <i>P. sieberi</i> flower ...	91
Figure 4.4. Photographs of <i>Prostanthera</i> and <i>Westringia</i> flowers	94
Figure 4.5. Visitors to <i>Prostanthera</i> flowers	97
Figure 4.6. Percent visitation for section <i>Prostanthera</i> , section <i>Klanderia</i> , and <i>P. lasianthos</i>	101
Figure 4.7. UPGMA and whisker box plots derived from visitation data	102
Figure 4.8. SSHMDS plot for visitation data excluding	103
Figure 4.9. SSHMDS plots for visitation or pollinator data for section <i>Prostanthera</i>	104
Figure 4.10. UPGMA and whisker box plots derived from pollinator data	106
Figure 4.11. SSHMDS plot using pollinator data	107
Figure 4.12. CCA of environmental and visitation data for four species of <i>Prostanthera</i>	109
Figure 4.13. Estimated number of flowers per plant.....	110
Figure 4.14. Nectar volume of standing crop per flower.....	111
Figure 4.15. Estimated nectar volume per plant	112

Chapter 5: The evolution of pollination in *Prostanthera* inferred from morphological and phylogenetic analyses..... 121

Figure 5.1. Phylogenies reconstructed from maximum parsimony and Bayesian analyses for combined <i>trnT-F</i> , <i>ndhF-rpl32</i> , and ETS.....	129
Figure 5.2. Relative warps ordination of flower shape	131
Figure 5.3. Thin-plate splines from relative warps analysis	132
Figure 5.4. Relative warps ordination mapped onto phylogenies.....	134
Figure 5.5. Evolutionary principal components analysis.....	135

List of Tables

Chapter 2: Molecular data reveal the paraphyly of <i>Prostanthera</i> section <i>Prostanthera</i>	11
Table 2.1. <i>Prostanthera</i> , Westringieae and Chloanthaeae used for the phylogeny	15
Table 2.2. Primer sequences for <i>trnT-F</i> , <i>ndhF-rpl32</i> , and ETS	20
Table 2.3. Taxa information, base frequencies, and parsimony statistics for maximum parsimony analyses and prior probability statistics for Bayesian analyses ..	25
Chapter 3: Floral characters and shape support measurable pollination syndromes in <i>Prostanthera</i>	42
Table 3.1. <i>Prostanthera</i> used for phenetic and geometric analysis	45
Table 3.2. Floral characteristics measured for phenetic analysis.....	47
Chapter 4: Investigation of pollination syndromes in <i>Prostanthera</i>.....	78
Table 4.1. <i>Prostanthera</i> observed for floral phenology and floral visitation.....	81
Table 4.2. Results for peroxidase and esterase tests	95
Chapter 5: The evolution of pollination in <i>Prostanthera</i> inferred from morphological and phylogenetic analyses.....	121
Table 5.1. <i>Prostanthera</i> and <i>Westringia</i> used for phylogenetic and morphological analyses.....	125
Table 5.2. P-values for the incongruence length difference test	128
Table 5.3. Base frequencies from the <i>trnT-F</i> , <i>ndhF-rpl32</i> and ETS data sets	128
Table 5.4. Statistics for maximum parsimony and Bayesian analysis	128

Chapter 1

Introduction



Flowers of *Prostanthera monticola*.

The importance of pollination

Sexual reproduction enhances evolutionary rates and, therefore, enables species to change and adapt to their environment. The restriction for movement in plants, specifically the plant sporophyte, presents a barrier for individuals to exchange genetic information. Pollination in seed plants is one solution that has enabled breeding biology to occur in terrestrial systems and has enabled plants to inhabit every continent on earth (Crepet 2008). Pollination refers to the transfer of pollen grains (male gametophyte) from their source to the female flower parts where they may germinate and then fertilise the egg cells of the female gametophyte. This transfer can be abiotic (e.g. wind and water) but most pollination (>91%) relies on animals (Buchmann and Nabhan 1996) and is found in over 86% of angiosperm genera (Renner and Ricklefs 1995). Most plants of terrestrial systems are angiosperms and therefore pollination is the most common breeding system (Crane *et al.* 1995; Crepet and Niklas 2009).

The pollination syndrome

An outbreeding species requires pollinators to be able to visit at least twice in order to ensure pollen is transmitted from the stamens to stigma. Complete pollinator fidelity leads to the evolution of flowers with peculiar features such as orchids that attract male wasps by resembling female wasps or corpse flowers that attract flesh flies because they smell like rotting carcasses (Proctor *et al.* 1996). These features were noticed as early as the 1700's when biologists began examining the relationships between animals and plants. Structures such as the long nectar spur of *Angraecum sesquipedale* Thou. (Orchidaceae) began to be used to infer the types of animals that visited flowers (Darwin 1862). A suite of flower characteristics correlated with characteristics of the pollinator was established and this relationship was labelled as a pollination syndrome

(Vogel 1954). A further treatment and modification of this concept describes several specialised or generalised interactions between a plant and its pollinator(s) (Faegri and Van der Pijl 1979). The most common biotic pollination syndromes are insect-pollination (entomophily) and bird-pollination (ornithophily). Short and wide corolla tubes, floral scent, small quantities of concentrated nectar, landing pads, and shorter wavelength light reflection allow an entomophilous flower to attract a wide range of insect pollinators. More specific pollination syndromes can also be identified within entomophily, such as melittophily (bee pollination) or psychophily (butterfly pollination). Ornithophilous flowers usually have long narrow corolla tubes, lack a floral scent, have large quantities of dilute nectar, lack insect landing pads, and are usually coloured red.

Not all characteristics must be present for a syndrome to exist. However, incorrect estimations of pollinators are usually made when based on only a few characteristics. For example, the entomophilous features of *Microlooma sagittatum* remove any suspicion that it is ornithophilous (Ollerton 1998), and the ornithophilous features of *Peraxilla sp.* do not indicate that they are pollinated by bees (Robertson *et al.* 2005). These syndromes become exceedingly difficult to apply to systems when multiple pollinators are involved (Waser *et al.* 1996), which brings to question whether pollination syndromes actually exist. There have been very few tests of pollination syndromes (Hingston and McQuillan 2000; Ollerton and Watts 2000; Consiglio and Bourne 2001; Kay and Schemske 2003; Hargreaves *et al.* 2004; Wilson *et al.* 2004; Zhang *et al.* 2005; Valdivia and Niemeyer 2006; Wester and Claßen-Bockhoff 2006b; Wolfe and Sowell 2006; Martén-Rodríguez *et al.* 2009; Ollerton *et al.* 2009b) (Hingston and McQuillan 2000; Ollerton and Watts 2000; Consiglio and Bourne 2001; Kay and

Schemske 2003; Hargreaves *et al.* 2004; Wilson *et al.* 2004; Zhang *et al.* 2005; Valdivia and Niemeyer 2006; Wolfe and Sowell 2006; Martén-Rodríguez *et al.* 2009; Ollerton *et al.* 2009a), and they offer a varying range of support. Yet pollination syndromes are continuously used as a method for determining pollinators and they are often a method for introducing pollination biology. This emphasises the need to improve the ways in which we understand the relationships between plants and pollinators.

Pollination syndromes are an excellent example of convergent evolution, since they have evolved independently across many different plant families. For example, the long tubular flower that typifies nemestrid fly pollination is observed in the Lamiaceae, Iridaceae, Orchidaceae, and Plumbaginaceae (Goldblatt and Manning 1996; Potgieter and Edwards 2001; Manning and Goldblatt 2005; Combs and Pauw 2009; Ferrero *et al.* 2009), and ornithophily is observed in several different genera of the Lamiaceae (Whitten 1981; Raju and Reddi 1989; Vos *et al.* 1994; Lindqvist and Albert 2002; Wester and Claßen-Bockhoff 2006b; Wester and Claßen-Bockhoff 2006a; Scheen and Albert 2009). Sometimes the convergence upon a particular syndrome can be so similar that incorrect taxonomic conclusions have been made when floral morphology has been used in a phylogeny (Crisp 1996).

Phylogenetics

Phylogenetics provide a tool for exploring morphological evolution in addition to demonstrating relationships amongst taxa. They have been used to infer the evolution in a vast diversity of structures from dragonfly wings (Bybee *et al.* 2008) to nephridial systems of rotifers (Riemann and Ahlrichs 2010). Inferring the evolution of structures

permits a greater understanding about the evolution of complex life history strategies, such as the origins of specialised herbivory (JAMESON *et al.* 2007), host switches for parasites (Bert *et al.* 2008), or the evolution of dioecy in plants (Weller *et al.* 1998). Phylogenies may be constructed by using morphological and/or molecular data as characters for tree building algorithms based on various optimality criteria. The problem with morphological data sets is that they may contain unknown levels of homoplasy based on convergent evolution. One of the advantages of nucleotide sequence data is that it is not subject to the evolutionary pressures acting upon the phenotype. Molecular phylogenies are demonstrating that some characteristics from earlier cladistic studies are homoplastic, such as the plumage pattern in the woodpecker genera *Veniliornis* and *Picoides* (Moore *et al.* 2006), or the pollination syndromes in *Disa* (Johnson *et al.* 1998).

Nucleotide sequence data can be composed of separate data sets from different genomes such as the mitochondrial (mtDNA), chloroplast (cpDNA), and nuclear (nrDNA) DNA. The cpDNA and nrDNA non-coding regions are commonly used for lower taxonomic studies in plants since their rate of evolution is much faster (Tay *et al.* 2010). Each genome is susceptible to biparental inheritance (nrDNA) or hybridisation (cpDNA and nrDNA) that may distort the resolution of the tree. This underlines why most phylogenetic studies of plants now include markers from both genomes to reduce the error of phylogenetic inference (Mort *et al.* 2007). The lower cost and time for sequencing has increased the rate at which full plastid genomes can be sequenced and has advanced the identification of better cpDNA markers (Shaw *et al.* 2007). Furthermore, it has made it easier to provide additional markers so that phylogenetic inferences may be more rigorous.

Morphometrics

Multivariate analysis is a useful application for investigating habitat structure (Kooyman and Rossetto 2006), species diversity (Peeters 2002), and taxonomy (Jiménez-Pérez and Lorea-Hernández 2009). It has recently been used to test pollination syndromes (Wilson *et al.* 2004; Martén-Rodríguez *et al.* 2009; Ollerton *et al.* 2009a) since it analyses a wide diversity of measures such as flower colour, nectar quantity, and structural measurements. It is also used in multivariate morphometrics, which is a method used to quantify shape.

Multivariate morphometrics has traditionally combined several linear measurements to provide a quantitative description and interpretation of biological shape (Rohlf 1990). The quantification of shape can have severe weaknesses since linear measurements do not incorporate depth, their representation of form is uneven, and they often refer to total lengths rather than distances between homologous points (Strauss and Bookstein 1982). Recent applications termed ‘geometric morphometrics’ (Rohlf and Marcus 1993; Mitteroecker and Gunz 2009), such as elliptic fourier shape analysis (Kuhl and Giardina 1982; Rumpunen and Bartish 2002), or landmark analysis (Kendall 1986; Rohlf 1990; Bookstein 1991; Rohlf and Marcus 1993), attempt a less biased description of shape. Relative warps analysis is a more recent development of landmarks analysis (Rohlf and Marcus 1993) that ordines the deviations calculated between homologous landmarks amongst different shapes. The advantage that this technique offers over other landmark analyses is that it implements a thin-plate spline to describe the distortions in shape in the ordination (Bookstein 1989). The spline is a modification of Thompsons’ (1942) cartesian grid and the bending and distortions allow a visualisation of the differences

between different shapes. The advances in digital imaging and computational processing speed over the past two decades have dramatically reduced the cost of data acquisition and calculations. The use of this technique originated in anthropology (Bookstein *et al.* 2003) and has recently been used for a variety of zoological investigations such as the identification of subspecies in fresh water flagellates using scale shape (Neustupa and Němcová 2007) and of trophic ecology of cichlid fishes based on body shape (Rüber and Adams 2001). The remarkably few applications in botany (Jensen *et al.* 2002; Gómez *et al.* 2006; Jacques and Zhou 2010) has left open a vast opportunity to explore the variation in plant form.

Prostanthera

The Lamiaceae consists of many taxa demonstrative of transitions from insect pollination to bird pollination (Huck 1992b). This is a well supported clade (Cantino 1992; Wagstaff and Olmstead 1997; Wagstaff *et al.* 1998) and is considered ancestrally insect-pollinated. At least seven genera are confirmed to have subgroups that have evolved bird-pollination and there are many more species that have floral morphology indicative of ornithophily (Whitten 1981; Raju and Reddi 1989; Huck 1992b; Vos *et al.* 1994; Lindqvist and Albert 2002; Wester and Claßen-Bockhoff 2006a; Ford and Johnson 2008). Their ornithophilous features are akin to ornithophilous morphologies typical of other plant families, such as the Scrophulariaceae (Wilson *et al.* 2007; Cronk and Ojeda 2008). This makes taxa in the Lamiaceae a stable framework in which to investigate evolutionary transitions between entomophily and ornithophily. *Salvia* (Lamiaceae) itself comprises two convergent pathways to ornithophily, either by the Trochilidae in the Americas (Wester and Claßen-Bockhoff 2006b), or the Nectarinidae in Africa (Wester and Claßen-Bockhoff 2006a).

The Prostantheroideae are one of seven subfamilies placed in the Lamiaceae and are composed of two endemic Australian tribes, the Chloantheae and the Westringieae (Harley *et al.* 2004). A robust molecular phylogeny has been provided for the former tribe (Conn *et al.* 2009), but there is little understanding about the relationships within the Westringieae.

The Westringieae appear to represent numerous evolutionary paths towards a bird-pollinated morphology (Conn 2004). Amongst six genera (*Hemiandra*, *Hemigenia*, *Microcorys*, *Prostanthera*, *Westringia*, and *Wrixonia*) the first four listed contain at least one species that appears to be ornithophilous (Keighery 1980; Guerin 2005). *Prostanthera* is the largest genus and contains a highly diverse set of species in terms of morphological diversity and geographic distribution (Conn 1984; 1988). Species are woody evergreen shrubs or sub-shrubs, although *P. lasianthos* can grow into a small tree. Their distribution across temperate Australia includes climates as diverse as rainforest to desert (Conn 1992a).

The inflorescences of *Prostanthera* are anthotelic (Briggs and Johnson 1979) and are considered lateral (axillary) (Conn 1984). In *Prostanthera* section *Klanderia*, one flower is regarded as the uniflorescence (Briggs and Johnson 1979), or partial inflorescence (Troll 1964; Weberling 1989). Many species of section *Prostanthera* (such as *P. rotundifolia* and *P. lasianthos*) have bracteose racemiform conflorescences, which superficially appear to be elaborations of a botryoidal inflorescence (Conn 1984). The flowers of *Prostanthera* have a 5-merous corolla that consists by five basally fused petals to form a corolla tube, with free distal ends forming five lobes. The abaxial lobe

and the two lateral lobes are separated from the adaxial lobes such that they often appear to form a distinctive abaxial corolla lip, a character of the family. The two adaxial lobes, which compose the adaxial lip of the flower, are almost completely fused together and are referred to as the adaxial median lobe-pair (Conn 1984). The flower of *Prostanthera* is distinguished from all other Westringieae by its bilobed calyx and four functional bilocular anthers (Cantino 1992). Each anther bears what is believed to be an extension of the connective tissue between each theca, and is referred to as an anther appendage (Conn 1984). These appendages might have a function in pollination similar to other superficially similar structures associated with the anthers of other Lamiaceae (Claßen-Bockhoff *et al.* 2004).

Prostanthera was earlier separated into two genera (*Cryphia* R. Brown and *Prostanthera* Labill. by Brown 1810) but Bentham (1870) reduced *Cryphia* to the synonymy of *Prostanthera*, recognising two sections, namely section *Klanderia* (F.v. Muell.) Benth. and section *Prostanthera* Benth. (as sect. 'Euprostanthera'). This classification has been followed by subsequent authors. Section *Klanderia* was circumscribed as having long, narrow, and tubular corollas whereas those of section *Prostanthera* are short and wide. *Prostanthera aspalathoides* (sect. *Klanderia*), for example, is a widespread, small subshrub of the Australian semi-arid inland with flowers bearing red long-tubular corollas. *Prostanthera lasianthos* (sect. *Prostanthera*) is a small tree of eastern Australian rainforests with flowers bearing white gullet-shaped corollas. Therefore, according to corolla characteristics, section *Klanderia* are considered ornithophilous and section *Prostanthera* are considered entomophilous. However, very little quantified visitation or pollination data are available for any species (Keighery 1982; Conn 1984).

Project aims

The primary purpose of this study is to understand the evolution of pollination in *Prostanthera* by examining floral morphology and pollinators within a phylogenetic context. Since there is poor understanding of the infrageneric relationships of *Prostanthera*, the priority is to construct the first phylogeny for the genus using nucleotide sequence data, which is the focus of Chapter 2. Then, the floral morphology and other characteristics relating to pollination syndromes are quantified to examine the diversity of pollination syndromes in Chapter 3. Observations of the pollination biology and quantification of visitors are used to test the putative pollination syndromes in Chapter 4. The data of the previous chapters are then combined in an analysis (Chapter 5) to infer the evolution of pollination in *Prostanthera*.

Chapter 2

Molecular data reveal the paraphyly of *Prostanthera* section *Prostanthera*

Introduction

Floral morphology is currently used to distinguish two sections within *Prostanthera*: *Prostanthera* section *Klanderia* and *Prostanthera* section *Prostanthera* (Bentham 1870; Conn 1984). The red, curved tubular flowers of section *Klanderia* are so distinct from the flowers of section *Prostanthera* that each section was originally recognised as a separate genus (Brown 1810). Each section, however, has members with characteristics that conflict with its original description. For instance, although flowers of section *Klanderia* were identified as having small to absent anther appendages, some recently described members have large anther appendages (Conn 1984). The only traits that consistently distinguish section *Klanderia* from section *Prostanthera* are corolla shape and colour. These are distinctive characteristics of ornithophily and entomophily and suggest that the sectional classification of *Prostanthera* does not represent natural relationships. This is because pollination syndromes describe similarities of pollinator selection pressure and not necessarily phylogenetic relationships. For example, red, tubular flowers have evolved in a plethora of genera within the Lamiaceae (Whitten 1981; Raju 1989; Raju and Reddi 1989; Huck 1992; Vos *et al.* 1994; Lindqvist and Albert 2002; Guerin 2005; Wester and Claßen-Bockhoff 2007; Ford and Johnson 2008). Molecular evidence and careful morphological study support the conclusion that

unrelated species have independently evolved this morphology even within a genus such as *Salvia* (Walker *et al.* 2004).

Prostanthera is a member of the Westringieae, which contains *Hemiandra*, *Hemigenia*, *Microcorys*, *Prostanthera*, *Westringia*, and *Wrixonia* (Conn 1992b). *Eichlerago tysoniana* used to also be included until it was reclassified as *Prostanthera tysoniana* (Carrick 1977; Conn 1992b). This tribe is well supported by molecular and morphological data but is not well resolved (Cantino 1992; Conn 1992a; Guerin 2008). These data suggest that *Microcorys*, *Hemiandra*, and *Westringia* nest within *Hemigenia* (Guerin 2008). *Wrixonia*, *Prostanthera* and *P. tysoniana* form a group sister to these genera (Cantino 1992; Conn 1992b). *Wrixonia* appears to be the most closely allied genus to *Prostanthera* and is distinguished from it because its adaxial pair of anthers are reduced to staminodes (Carrick 1976; Cantino 1992). In addition to the sectional classification, three morphological series were recognised within section *Prostanthera* when it was described (Bentham 1870). A member of the *Racemosae* has an inflorescence composed of flowers in terminal racemes whereas the *Subconcaevae* and *Convexae* have axillary flowers. A member of the *Subconcaevae* is distinguished from the *Convexae* because its flower has an anther-appendage twice as long as the theca. Recent descriptions of new species appear to conflict with this classification. For example, *P. linearis* is a member of *Convexae* yet it has flowers in terminal racemes. This supports Hillson's (1959) conclusions that macro-morphological features likely not reliable indicators of phylogeny. Furthermore, *P. behriana*, *P. baxteri*, *P. canaliculata*, and *P. eurybioides* are members of the *Subconcaevae* yet their appendage is equal or less than the length of the theca which conflicts with their placement (Conn

1988). Therefore better characters are needed to investigate the relationships within *Prostanthera*.

The relationships in *Prostanthera* are investigated in this Chapter in order to understand how putative pollination syndromes have evolved and if floral morphology correlates with phylogenetic relationships. A phylogeny is constructed using molecular data since morphological characteristics might be a result of convergent evolution based on the selection pressures by pollinators. The phylogeny is constructed from chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) markers using maximum parsimony and Bayesian analysis. The *trnT-F* marker was employed since the results from phylogenetic analysis of *Microcorys* and *Hemigenia* provide high infrageneric resolution (Guerin 2008). Another chloroplast marker (*ndhF-rpl32*) was used since it has a reportedly high number of potentially informative characters (Shaw *et al.* 2007). The nuclear ribosomal external transcribed spacer (ETS) was used to complement the chloroplast data. Although ITS has been used in other Lamiaceae (Steane *et al.* 1999; Prather *et al.* 2002; Jamzad *et al.* 2003; Steane *et al.* 2004; Trusty *et al.* 2005; Edwards *et al.* 2006; Barber *et al.* 2007; Ford and Johnson 2008; Conn *et al.* 2009), ETS was used since it has been shown to be more variable (Baldwin and Markos 1998).

All putatively ornithophilous and entomophilous species that have been discovered since the last complete description of *Prostanthera* (Bentham 1870) have been classified as either section *Klanderia* or section *Prostanthera*, respectively. However, new members of section *Prostanthera* have not been given a series membership. In this study, they are placed into the *Convexae*, *Racemosae*, or *Subconcavae* according to the

original classification system. The groups formed by this classification are then compared with the molecular phylogeny.

Materials and methods

Taxon sampling

DNA was collected for 66 species of *Prostanthera*, eight species in the Westringieae, and seven species in the Chloantheae (Table 2.1). Samples were collected in the field, from living collections at botanic gardens, and from herbarium sheets. Fresh leaf material was desiccated in silica gel and stored at 4 °C (Chase and Hills 1991). All voucher samples were deposited at the National Herbarium of NSW, Sydney, Australia (NSW).

Identification of series

All species of section *Prostanthera* that have not been formally placed in Bentham's (1870) series were studied from herbarium sheets and published descriptions and are identified in Table 2.1. The following key, adapted from Bentham (1870), was used to categorise these species into the *Convexae*, *Racemosae*, or *Subconcaevae*. The length of the anther appendage is employed in the original key but is abandoned here since the classification of many species in the original series is inconsistent with their appendage length. The results and references for the conclusion of each species are supplied in Appendix 2.1.

Table 2.1 Species of *Prostanthera*, tribe Westringieae and tribe Chloantheae used for phylogenetic analysis. += amplified; N= not amplified; B.G.= Botanic Garden; N.P.= National Park; N.R.= Nature Reserve; S.F.= State Forest; *= not allocated to series by Bentham (1870).

Species	Section	Series	Locality	State	Accession	<i>trnT-F</i>	<i>rpl32-ndhf</i>	ETS
<i>Prostanthera althoferi</i> B.J.Conn	<i>Prostanthera</i>	<i>Convexae</i> *	Plumridge Lakes, Great Victoria Desert	WA	NSW808470	+	+	+
<i>P. askania</i> B.J.Conn	<i>Prostanthera</i>	<i>Racemosae</i> *	Strickland S.F.	NSW	NSW717011	+	+	+
<i>P. aspalathoides</i> A.Cunn.ex Benth.	<i>Klanderia</i>	-	Gubbata N.R.	NSW	NSW803944	+	+	+
<i>P. baxteri</i> Benth.	<i>Prostanthera</i>	<i>Subconcaevae</i>	ANBG living collection	WA	CANB8910156	+	+	+
<i>P. behriana</i> Schltldl.	<i>Prostanthera</i>	<i>Convexae</i>	Monarto	SA	AD20050034	+	+	+
<i>P. caerulea</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Blue Mountains N.P.	NSW	NSW4210209	+	+	+
<i>P. calycina</i> F.Muell. ex Benth.	<i>Klanderia</i>	-	Eyre Peninsula	SA	AD20040496	+	+	+
<i>P. carrickiana</i> B.J.Conn	<i>Klanderia</i>	-	Esperence	WA	NSW803848	+	+	+
<i>P. chlorantha</i> (F.Muell.) Benth.	<i>Klanderia</i>	-	Kangaroo Island	SA	NSW743683	+	+	+
<i>P. cineolifera</i> R.T.Baker&H.G.Sm.	<i>Prostanthera</i>	<i>Racemosae</i> *	Pokolbin S.F.	NSW	NSW791484	+	+	+
<i>P. cruciflora</i> J.H.Willis	<i>Prostanthera</i>	<i>Racemosae</i> *	Mt. Kaputar N.P.	NSW	AE922944	+	+	+
<i>P. cryptandroides</i> subsp. <i>euphrasioides</i> (Benth.)B.J.Conn	<i>Prostanthera</i>	<i>Subconcaevae</i>	Warialda	NSW	NSW784616	+	+	+
<i>P. cuneata</i> Benth.	<i>Prostanthera</i>	<i>Convexae</i>	Mt. Buffalo N.P.	VIC	NSW844231	+	+	+
<i>P. decussata</i> F.Muell.	<i>Prostanthera</i>	<i>Convexae</i>	Kosciuszko N.P.	VIC	NSW791485	+	+	+
<i>P. densa</i> A.A.Ham.	<i>Prostanthera</i>	<i>Convexae</i> *	Port Stephens	NSW	NSW789038	+	+	+
<i>P. denticulata</i> R.Br.	<i>Prostanthera</i>	<i>Convexae</i>	Ganoo N.P.	NSW	NSW844199	+	+	+
<i>P. discolor</i> R.T.Baker	<i>Prostanthera</i>	<i>Convexae</i> *	Wollemi N.P.	NSW	NSW619945	+	+	+
<i>P. eurybioides</i> F.Muell.	<i>Prostanthera</i>	<i>Subconcaevae</i>	Monarto	SA	AD20060793	+	+	+
<i>P. ferricola</i> B.J.Conn&K.A.Sheph	<i>Prostanthera</i>	<i>Subconcaevae</i>	Robinson Ranges	WA	NSW746276	+	+	+
<i>P. florifera</i> B.J.Conn	<i>Klanderia</i>	-	O'Connor Dam, Kimba	SA	CANB9901279	+	+	+
<i>P. galbraithiae</i> B.J.Conn	<i>Prostanthera</i>	<i>Convexae</i> *	Holey Plains State Park	VIC	NSW808463	+	+	+
<i>P. granitica</i> Maiden&Betche.	<i>Prostanthera</i>	<i>Racemosae</i> *	Warrumbungles N.P.	NSW	NSW844192	+	+	+
<i>P. grylloana</i> F.Muell.	<i>Klanderia</i>	-	Jaurdi Station	WA	NSW808472	+	+	+
<i>P. hindii</i> B.J.Conn	<i>Prostanthera</i>	<i>Racemosae</i> *	Gardens of Stone N.P.	NSW	NSW5022365	+	+	+

Species	Section	Series	Locality	State	Accession	<i>trnT-F</i>	<i>rpl32-ndhf</i>	ETS
<i>P. hirtula</i> Benth.	<i>Prostanthera</i>	<i>Racemosae</i>	Mt. Buffalo N.P.	VIC	NSW844197	+	+	+
<i>Prostanthera howelliae</i> Blakely	<i>Prostanthera</i>	<i>Convexae</i> *	Sackville	NSW	NSW799701	+	+	+
<i>P. incana</i> A.Cunn ex.Benth.	<i>Prostanthera</i>	<i>Racemosae</i>	Blue Mountains N.P.	NSW	NSW803914	+	+	+
<i>P. incana</i> A.Cunn ex.Benth.	<i>Prostanthera</i>	<i>Racemosae</i>	Buddawang N.P.	NSW	NSW844193	+	+	+
<i>P. incisa</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Blue Mountains N.P.	NSW	NSW862999	+	+	+
<i>P. incurvata</i> B.J.Conn	<i>Klanderia</i>	-	Norseman region	WA	CANB9900248	+	+	+
<i>P. junonis</i> B.J.Conn	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Somersby	NSW	NSW749548	+	+	+
<i>P. lanceolata</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i> *	Upper Corindi	NSW	NSW799705	+	+	+
<i>P. lasianthos</i> Labill.	<i>Prostanthera</i>	<i>Racemosae</i>	Mount Tomah Botanic Gardens	NSW	NSW799707	+	+	+
<i>P. lasianthos</i> Labill.	<i>Prostanthera</i>	<i>Racemosae</i>	Bruny Island	TAS	NSW860451	+	+	+
<i>P. linearis</i> R.Br.	<i>Prostanthera</i>	<i>Convexae</i>	Brisbane Waters N.P.	NSW	NSW784600	+	+	+
<i>P. lithospermoides</i> F.Muell.	<i>Prostanthera</i>	<i>Subconcaevae</i>	ANBG living collection	QLD	CANB9808063	+	+	+
<i>P. magnifica</i> C.A.Gardner	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Kings Park living collection	WA	NSW803846	+	+	+
<i>P. marifolia</i> R.Br.	<i>Prostanthera</i>	<i>Convexae</i>	Manly, Sydney	NSW	NSW743858	+	+	+
<i>P. melissifolia</i> F.Muell.	<i>Prostanthera</i>	<i>Racemosae</i>	Otway N.P.	VIC	NSW803972	+	+	+
<i>P. monticola</i> B.J.Conn	<i>Klanderia</i>	-	Mount Buffalo N.P.	VIC	NSW803943	+	+	+
<i>P. nivea</i> Benth. var. <i>induta</i>	<i>Prostanthera</i>	<i>Subconcaevae</i>	Warrumbungles N.P.	NSW	NSW803949	+	+	+
<i>P. nivea</i> Benth. var. <i>nivea</i>	<i>Prostanthera</i>	<i>Subconcaevae</i>	Central Tilba	NSW	NSW844232	+	+	+
<i>P. ovalifolia</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Whian Whian S.F.	NSW	NSW4200941	+	+	+
<i>P. palustris</i> B.J.Conn	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Bundjalung N.P.	NSW	NSW863020	+	+	+
<i>P. petraea</i> B.J.Conn	<i>Prostanthera</i>	<i>Racemosae</i> *	Bald Rock N.P.	NSW	NSW791461	+	+	+
<i>P. phyllicifolia</i> F.Muell.	<i>Prostanthera</i>	<i>Convexae</i>	Deua N.P.	NSW	Eurobodella B.G.#2888	+	+	+
<i>P. porcata</i> B.J.Conn	<i>Klanderia</i>	-	Deua N.P.	NSW	Eurobodella B.G.#35	+	+	+
<i>P. prunelloides</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Blue Mountains N.P.	NSW	NSW799703	+	+	+
<i>P. rhombea</i> R.Br.	<i>Prostanthera</i>	<i>Convexae</i>	Pokolbin S.F.	NSW	NSW803849	+	+	+
<i>P. ringens</i> Benth.	<i>Klanderia</i>	-	Goonoo N.P.	NSW	NSW844195	+	+	+
<i>P. rotundifolia</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	East Gippsland	VIC	NSW494368	+	+	+
<i>P. rotundifolia</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Upper Scamander	TAS	NSW862995	+	+	+
<i>P. rugosa</i> Benth.	<i>Prostanthera</i>	<i>Convexae</i>	Joadja N.R.	NSW	NSW844198	+	+	+

Species	Section	Series	Locality	State	Accession	trnT-F	rpl32-ndhf	ETS
<i>P. saxicola</i> var. <i>montana</i> A.A.Ham.	<i>Prostanthera</i>	<i>Subconcaevae</i>	Blue Mountains N.P.	NSW	NSW803916	+	+	+
<i>Prostanthera saxicola</i> var. <i>major</i> Benth.	<i>Prostanthera</i>	<i>Subconcaevae</i>	Gibraltar Ranges N.P.	NSW	NSW784611	+	+	+
<i>P. scutellarioides</i> (R.Br.)Briq.	<i>Prostanthera</i>	<i>Convexae</i> *	Nightcap N.P.	NSW	NSW785872	+	N	N
<i>P. scutellarioides</i> (R.Br.)Briq.	<i>Prostanthera</i>	<i>Convexae</i> *	Castlereagh N.R.	NSW	NSW799702	+	+	+
<i>P. sejuncta</i> M.L.Will.	<i>Prostanthera</i>	<i>Convexae</i> *	Fortis Creek N.P.	NSW	NSW791458	+	+	+
<i>P. semiteres</i> B.J.Conn	<i>Klanderia</i>	-	Newdegate	WA	NSW808468	N	N	+
<i>P. serpyllifolia</i> (R.Br.)Briq. subsp. <i>microphylla</i>	<i>Klanderia</i>	-	Pulletop N.R.	NSW	NSW813913	+	+	+
<i>P. sieberi</i> Benth.	<i>Prostanthera</i>	<i>Racemosae</i>	Illawarra Escarpment N.R.	NSW	NSW844227	+	+	+
<i>P. sp.E sensu</i> B.J.Conn	<i>Prostanthera</i>	<i>Racemosae</i> *	Morton N.P.	NSW	NSW749547	+	+	+
<i>P. sp.</i> "Wiseman's Ferry"	<i>Prostanthera</i>	<i>Racemosae</i> *	Wiseman's Ferry	NSW	NSW749554	+	+	+
<i>P. spinosa</i> F.Muell.	<i>Prostanthera</i>	<i>Convexae</i>	Kangaroo Island	SA	NSW799708	+	+	+
<i>P. staurophylla</i> F.Muell.	<i>Prostanthera</i>	<i>Convexae</i> *	Mt. Mackenzie N.R.	NSW	NSW785873	+	+	+
<i>P. stenophylla</i> B.J.Conn	<i>Prostanthera</i>	<i>Convexae</i> *	Wollemi N.P.	NSW	NSW619740	+	+	+
<i>P. striatiflora</i> F.Muell.	<i>Prostanthera</i>	<i>Convexae</i>	Mount Yardi	SA	NSW449546	+	+	+
<i>P. suborbicularis</i> C.T.White&W.D.Francis	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Quilpie-Thargomindah Rd toward Eulo	Qld	CANB741662	+	+	+
<i>P. teretifolia</i> Maiden&Betche	<i>Prostanthera</i>	<i>Convexae</i> *	Torrington	NSW	NSW714620	+	+	+
<i>P. tysoniana</i> (Carrick)B.J.Conn	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Austin, Byro Station	WA	NSW205148	N	N	+
<i>P. violacea</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Buddawang National Park	NSW	NSW615474	+	+	+
<i>P. walteri</i> F.Muell.	<i>Klanderia</i>	-	Mount Ellery	VIC	NSW808464	+	+	+
<i>P. wilkeana</i> F.Muell.	<i>Prostanthera</i>	<i>Subconcaevae</i> *	Little Sandy Desert	WA	NSW594892	N	+	+
Outgroup taxa								
<i>Brachysola halganiacea</i> (F.Muell.&Tate)Rye	-	-	Southern Cross region	WA	CANB602366	+	+	+
<i>Brachysola coerulea</i> (F.Muell.)Rye	-	-	Coolgardie	WA	NSW480339	+	+	+
<i>Cyanostegia lanceolata</i> Turcz.	-	-	Highbury	WA	NSW494623	+	+	+
<i>Cyanostegia corifolia</i> Munir	-	-	Eneabba	WA	NSW494553	+	+	+
<i>Dicrastylis exsuccosa</i> (F.Muell.)Druce	-	-	Kings Park living collection, Perth	WA	KPBG 20000486	+	+	N
<i>Dicrastylis lewellinii</i> (F.Muell.)F.Muell.	-	-	North Western Plains	WA	KPBG 20001266	+	+	+
<i>Hemiandra coccinea</i> O.H.Sarg.	-	-	Wongan Hills N.R.	WA	CANB602355	+	+	+
<i>Hemigenia purpurea</i> R.Br.	-	-	Royal National Park	NSW	NSW803917	+	+	+

Species	Section	Series	Locality	State	Accession	<i>trnT-F</i>	<i>rpl32-ndhf</i>	ETS
<i>Microcorys tenuifolia</i> Benth.	-	-	Koolbabbie Farm	WA	CANB9709567	+	+	+
<i>Newcastelia bracteosa</i> F.Muell.	-	-	North-east of Norseman	WA	NSW494660	N	N	+
<i>Westringia lucida</i> B.Boivin	-	-	Kosciuszko N.P.	NSW	NSW618148	+	+	+
<i>Westringia longifolia</i> R.Br.	-	-	Tahmoor	NSW	NSW4166185	+	+	+
<i>Westringia senifolia</i> F.Muell.	-	-	Mt. Buffalo N.P.	NSW	NSW799706	N	N	+
<i>Wrixonia prostantheroides</i> F.Muell.	-	-	Paynes Find	NT	CANB602334	+	N	+
<i>Wrixonia schultzii</i> (Tate) Carrick	-	-	West Macdonnells N.P.	NT	NSW413681	+	+	+

- 1 Flowers in terminal racemes, leaves of the inflorescence all or mostly reduced to membranous or broad acuminate or very deciduous bracts *Racemosae*
- 1 Flowers axillary, the floral leaves similar to the stem-ones or rather smaller ...2
- 2 Leaves convex or with revolute margins *Convexae*
- 2 Leaves concave or with incurved margins or flat, the margins never recurved
..... *Subconcavae*

Molecular methods

Double-stranded DNA was extracted using the DNeasy Plant Mini kit (Qiagen 2001). Material was ground dry or in an Eppendorf tube with pre-heated lyses buffer (Qiagen 2001).

Universal or specifically designed primers were used for amplification of double-stranded DNA (Table 2.2). The internal primers AP-TRNL and AP-TRNLr (A. Perkins, National Herbarium of NSW, pers. comm.) were used if the amplification of *trnT-F* was problematic. The reverse primer ETS-PROS2 was designed for use with the forward primer 18S-E (Baldwin and Markos 1998) since the ETS-B primer (Beardsley and Olmstead 2002) had little success. The 26S-IGS and 18S-ETS were used in long PCR to design the ETS-PROS2 primer.

Long PCR amplification was performed using a MyCycler thermal cycler (BioRad Laboratories Inc., Australia). Reactions contained 5 µL of 10X NH₄ buffer, 2.5 µL of 2.4mM MgCl₂, 2 µL of each 0.25mM dNTP, 2.5 µL of each primer, 0.5 µL of BIO-X-ACT™ Long DNA Polymerase (Bioline, www.bioline.com), and 29.1 µL of H₂O. One µL of genomic DNA was added to complete a reaction volume of 51 µL. The long PCR

Table 2.2 Details of primer sequences used for amplification of *trnT-F*, *ndhF-rpl32*, and ETS for taxa in the Westringieae and Chloanthaeae.

Region	Genome	Direction	Primer	Primer sequences (5'-3')	Reference	Designed for
<i>trnT-F</i>	Chloroplast	Forward	AP-TRNL	AGACGCTACGGACTTAAT	Perkins, (pers. comm.)	Apiales
		Reverse	AP-TRNLr	ATTAAGTCCGTAGCGTCT	Perkins, (pers. comm.)	Apiales
		Forward	A50272	ATTTGAACTGGTGACACGAG	Taberlet <i>et al.</i> (1991)	Universal
		Reverse	CAL-TR	GCGATGCTCTAACCTCTGA	Perkins (2001)	Orchidaceae
ETS	Nuclear	Reverse	ETS-PROS2	GCAGCGACGACATCCCAACC	This study	Prostanthereae
		Forward	18S-E	GCAGGATCAACCAGGTAGCA	Baldwin and Markos (1998)	Universal
			26S-IGS	GGATTGTTACCCACCAATAGGGAACGTGAGCTG	Baldwin and Markos (1998)	Universal
			18S-ETS	ACTTACACATGCATGGCTTAATCT	Baldwin and Markos (1998)	Universal
			ETS-B	ATAGAGCGCGTGAGTGGTG	Beardsley and Olmstead (2002)	<i>Mimulus</i>
<i>ndhF-rpl32</i>	Chloroplast	Forward	<i>ndhF</i>	GAAAGGTATKATCCAYGMATATT	Shaw <i>et al.</i> (2007)	angiosperms
		Reverse	<i>rpl32-R</i>	CCAATATCCCTTYTITTTCCAA	Shaw <i>et al.</i> (2007)	angiosperms

protocol consisted of an initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 15 seconds (denaturation) and 68 °C for 5 minutes (annealing and extension of primers), followed by a final 10 minutes at 68 °C.

Standard PCR amplification was performed using a MyCycler thermal cycler (BioRad Laboratories Inc., Australia). The master mix comprised 5 µL of 10X NH₄ buffer, 2.4 µL of 2.4mM MgCl₂, 1 µL of each 0.25mM dNTP, 1 µL of each primer, 0.5 µL of BIOTAQ™ (Bioline, www.bioline.com) and 36.1 µL of H₂O. 1 µL of genomic DNA was added to the master mix to complete a reaction volume of 51 µL. The PCR protocol using *trnT-F* consisted of an initial denaturation step at 95°C for 1 minute, 35 cycles of denaturation (95°C for 30 seconds), annealing (55 °C for 30 seconds), and extension (72 °C for 1 minute), and a final step of 4 minutes at 72 °C. The PCR protocol for ETS was similar except that the annealing temperature was raised to 60 °C.

All PCR products were purified using Sureclean™. The sequencing reactions were performed by Sydney University and Prince Alfred [Hospital] Molecular Analysis Centre (SUPAMAC) using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kits and an Applied Biosystems 3730xl DNA Analyzer (ABI Biosystems, www.appliedbiosystems.com).

Consensus sequences were compiled from forward and reverse sequences for each marker. Electropherograms were checked visually, edited and aligned with the computer programs Sequencher 4.5™ (gene codes corporation), ClustalW (Thompson *et al.* 1997), and BioEdit sequence alignment editor® (Hall 1999). Indels were coded in Seqstate version 1.4 (Müller 2005) using the simple indel coding method described by

Simmons and Ochoterena (2000) and added to the alignment as simple binary characters. Alignments for all three markers are provided in Appendix 2.2-2.4. Alignments for the different combinations of these three markers are also provided in Appendix 2.5-2.8.

Phylogenetic reconstructions

Maximum parsimony analyses were conducted using PAUP* Version 4.0b10 (Swofford 2002) for all three datasets, separately or in concatenated data sets. Combined data analysis included species without missing DNA regions. Heuristic searches were used with the following settings: all nucleotide substitutions and insertion/deletion events weighted equally, random taxon addition starting tree with TBR branch swapping, uninformative characters excluded, and MULTREES in effect. Analyses for each dataset resulted in large numbers of optimal trees that exceeded the available memory and prevented the completion of the analysis. Restricted analyses were, therefore, performed acquiring 1000 replicates and saving 100 trees for each replicate, saving a total of 100000 trees. Gaps were treated as missing and positions where bases were ambiguous were coded as polymorphic rather than uncertain. Identical trees were eliminated using the CONDENSE option and then a strict consensus of all most parsimonious trees was calculated. Length of the shortest tree (L), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated for all data sets.

It has been shown there is a general positive relationship between congruence and phylogenetic accuracy (Cunningham 1997b; a). Therefore congruence between data sets (*trnT-F*, *ndhF-rpl32*, and ETS) was examined using the incongruence length

difference test (ILD) in PAUP* Version 4.0b10. A test was performed for all combinations of data sets. One-thousand ILD replicates were run with the heuristic search settings described above, with the exception of using only 100 random sequence replicates.

A reverse constraint analysis was used to test the results of each heuristic search. The analysis used the same heuristic search settings as above and searched for trees equal to, or less than, the length of the strict consensus.

Branch support was assessed by conducting bootstrap analysis on all data sets (Felsenstein 1985a). Bootstrap analyses ran 1000 replicates using the same heuristic search settings described above. A bootstrap value of 95% - 100% is considered strong support, 75-95% is considered moderate support, and 50-74% is considered weak support.

Bayesian analysis was conducted with Mr. Bayes v3.1.2 (Huelsenbeck and Ronquist 2001) using datasets without indels. All searches were conducted with the transversion model plus gamma 'TVM+G', using the Akaike Information Criterion (AIC) as indicated by Modeltest (Posada and Crandall 1998). The number of substitution types was set to 6 and the rate variation across sites was set as gamma-distributed. Priors were set according to the 'TVM+G' model (Appendix 2.9). Tree space was searched using the Metropolis Coupled Markov Chain Monte Carlo analysis using 4 chains starting at randomly selected trees. The chains were run for 5000000 generations in two simultaneous runs, saving one tree every 100 generations. Most analyses reached a plateau of negative log-likelihood scores after 30000 generations and so trees found

before the chains converged on a narrow likelihood score were discarded by setting the ‘burn-in’ value to 30000. Posterior probability (PP) values ≥ 0.95 are considered to be significant and PP values < 0.90 are not reported in the results (Larget and Simon 1999).

Results

Compositional homogeneity

Mean base frequencies and results of chi-square tests for a dataset of each molecular marker are listed in Table 2.3. The results of a chi-square test on these values indicated that the frequencies did not significantly stray from compositional homogeneity indicating compositional bias does not play a significant function in these data.

Incongruence length difference test

An incongruence length difference (ILD) test indicates significant incongruence ($p < 0.005$) for all combinations of the *trnT-F*, *ndhF-rpl32*, and ETS data sets. Despite this incongruence, there is a high likelihood that the combination of markers may still improve phylogenetic accuracy. For instance, even though Barker and Lutzoni (2002) demonstrate that ILD tests are significantly correlated with a decrease in phylogenetic accuracy, they also show that nearly half of the combined data sets with significant ILD values have an increased accuracy compared with the two separate analyses. This suggests that the test is a relatively poor indicator of data set ‘combinability’ (Barker and Lutzoni 2002). Yoder *et al.* (2001) also demonstrate that incongruence can be detected in combined analyses that are phylogenetically accurate. Therefore, the analysis of the combined data set (*trnT-F*, *ndhF-rpl32*, and ETS) is still presented here as the precise utility of the ILD test is unclear.

Table 2.3. Taxa information, base frequencies, and parsimony statistics for maximum parsimony analyses and prior probability (P.P.) statistics for Bayesian analyses of chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) markers.

	Plastid		Nuclear	All Markers
	<i>trnT-F</i>	<i>ndhF-rpl32</i>	ETS	
Total number of taxa	83	82	86	80
Total number of ingroup	77	76	80	75
Aligned length	1817	750	426	2968
Total characters eliminated	1618	654	187	2479
Parsimony informative substitutions	165	96	239	489
Indels	162	113	76	238
Parsimony informative indels	83	66	49	191
Total parsimony informative characters	248	162	288	680
<i>Base frequencies and X² test</i>				
A	0.3105	0.33962	0.24356	0.31109
C	0.16799	0.15468	0.27307	0.18244
G	0.15904	0.12712	0.28921	0.17216
T	0.35592	0.37858	0.19416	0.33430
X ²	9.967019	31.241007	84.011694	23.159349
degrees freedom	246	243	255	237
p-value	1.00	1.00	1.00	1.00
<i>Parsimony statistics</i>				
Tree length	474	400	1168	1382
Reverse constraint analysis score	475	401	1169	1382
Consistency index	0.597	0.477	0.411	0.469
Retention index	0.838	0.764	0.708	0.736
Rescaled consistency index	0.500	0.365	0.291	0.345
nodes resolved from strict consensus	44	27	60	57
% nodes resolved with Bootstrap values $\geq 75\%$	13	6	28	30
% nodes resolved with P.P. ≥ 0.95	22	16	33	41

Maximum parsimony and Bayesian analysis

Tree information and statistics are listed for the maximum parsimony (MP) and Bayesian inference (BI) analyses performed on all datasets (Table 2.3). The reverse constraint analysis found no trees with the same or shorter number of steps than the most parsimonious trees.

The Westringieae are resolved as a clade and have strong support (BS=100%, PP=1.0) in all MP and BI trees (Figs. 2.1-2.6, node A). All taxa from *Wrixonia* and *Prostanthera* are recovered in a strongly supported clade (BS=100%, PP=1.0). The remaining results for each data set are described separately since the resolution within this clade varies between markers and analyses.

ndhF-rpl32 data set

The clade of *Wrixonia* and *Prostanthera* is poorly resolved in MP and BI trees (clade B, Figs. 2.1 and 2.2). The BI tree (Fig. 2.2) is more resolved and recovers *Wx. schultzii* with *P. carrickiana*, *P. ferricola*, *P. incurvata*, *P. magnifica*, and *P. striatiflora* in clade I. It also recovers *P. aspalathoides*, *P. calycina*, *P. chlorantha*, *P. florifera*, and *P. serpyllifolia* in clade H. Clades I and H have strong Bayesian support (PP=0.96 and 1.0, respectively) and contain most species of section *Klanderia*. Two large clades (E and F) are resolved but do not have strong support. Both clades contain only section *Prostanthera* and each has at least two out of three representatives of Bentham's (1870) series classification.

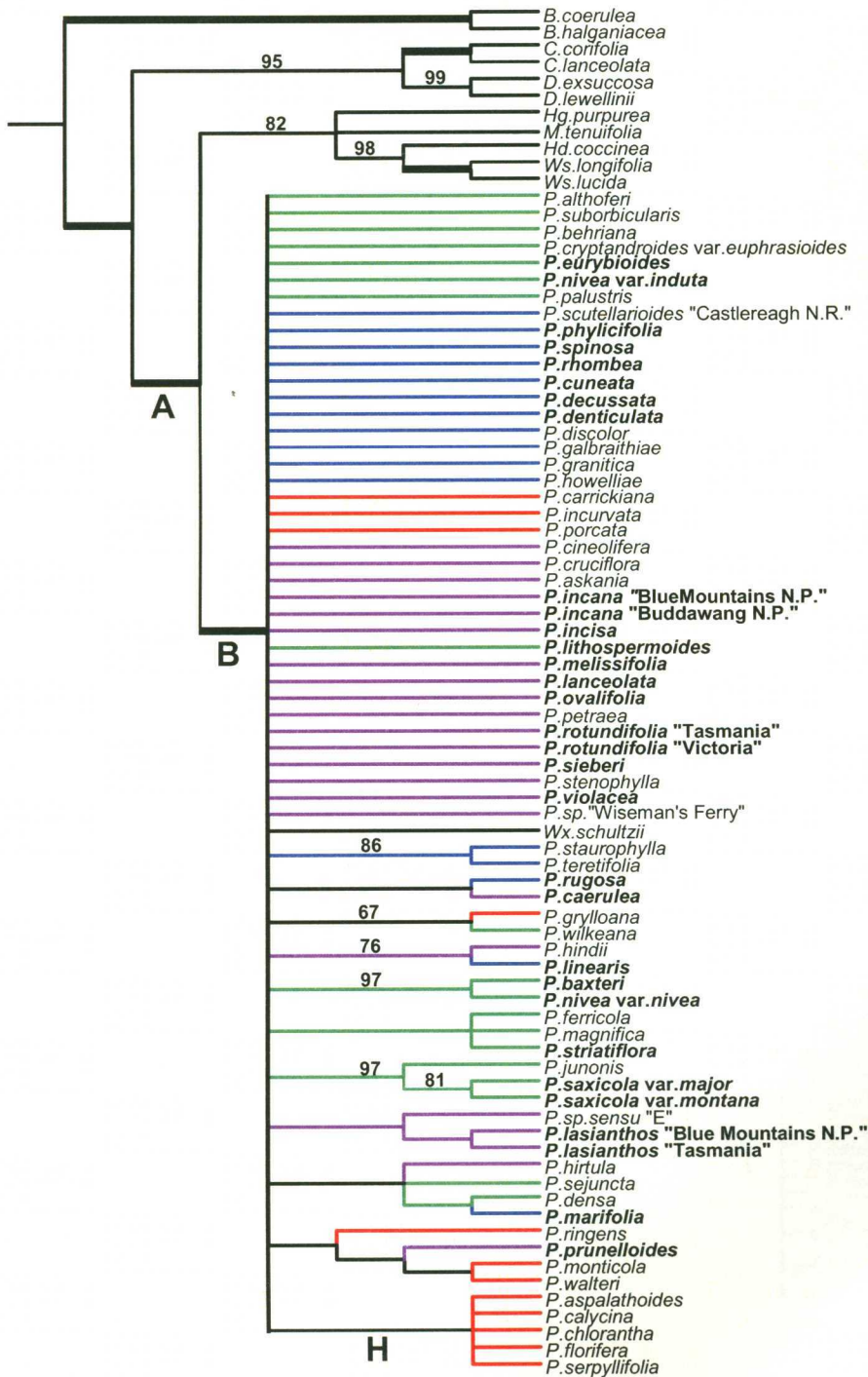


Figure 2.1. The strict consensus of 78794 trees from maximum parsimony (MP) analysis of the *ndhF-rpl32* sequence data for *Prostanthera* and its outgroups. Bootstrap values of 75% are reported above branches and thickened branches received 100%. Coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcavae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold. The abbreviations of genera are as follows: *B*=*Brachysola*; *C*=*Cyanostegia*; *D*=*Dicrastylis*; *Hd*=*Hemiandra*; *Hg*=*Hemigenia*; *M*=*Microcorys*; *P*=*Prostanthera*; *Ws*=*Westringia*; *Wx*=*Wrixonia*.

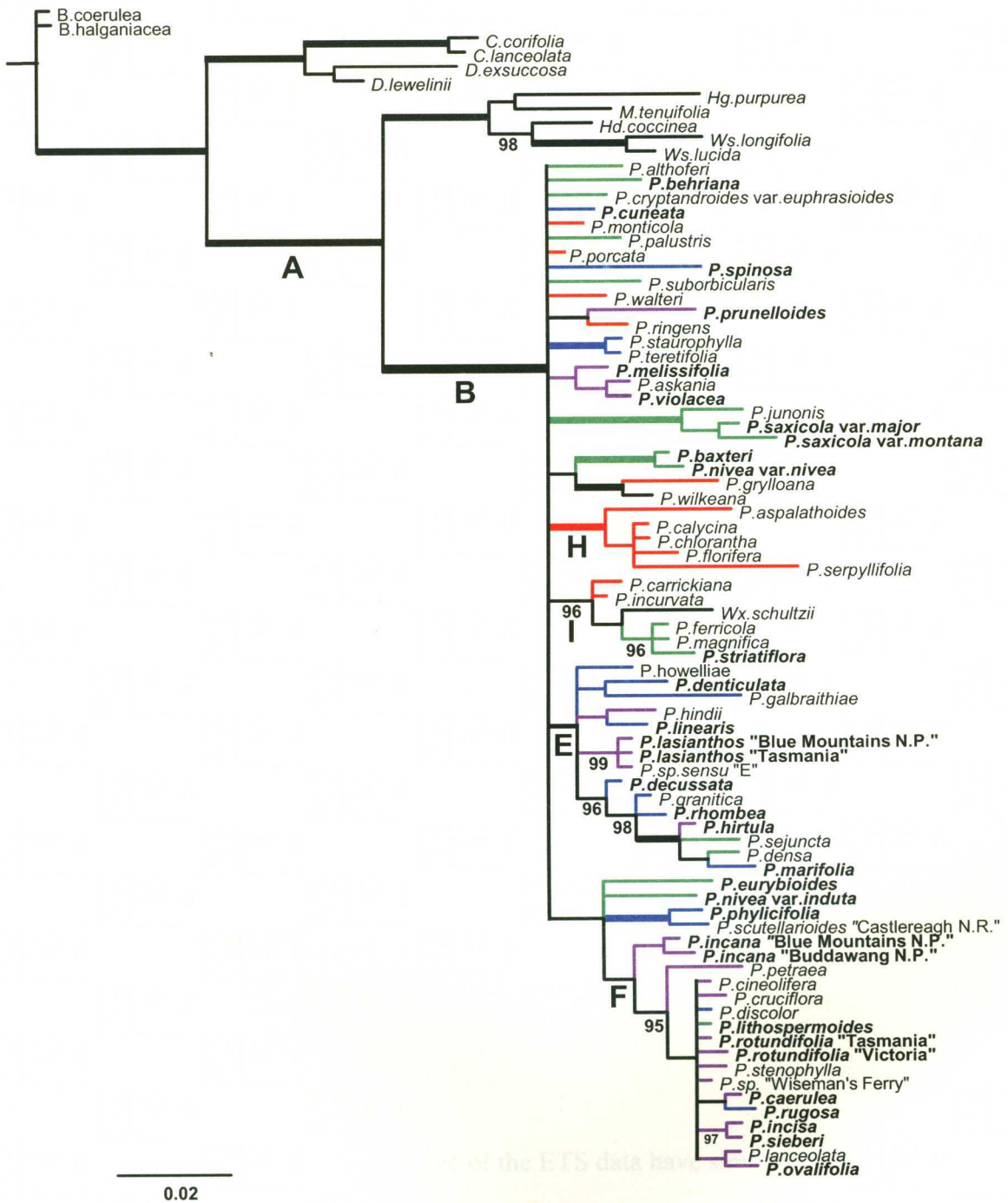


Figure 2.2. The 50% majority rule phylogram from Bayesian analysis of the *ndhF-rpl32* sequence data for *Prostanthera* and its outgroups. Prior probabilities of greater than 0.95 are reported below branches and thickened branches receive 1.00. Values are formatted as a % rather than with a decimal place. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcavae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

***trnT-F* data set**

The strict consensus of the MP tree and the BI tree of the *trnT-F* data set are poorly resolved and have similar topologies (Figs. 2.3 and 2.4). Clade J contains both species of *Wrixonia*, three species of section *Klanderia* (*P. carrickiana*, *P. incurvata*, and *P. porcata*) and three species of section *Prostanthera* (*P. ferricola*, *P. magnifica* and *P. striatiflora*). *Wrixonia schultzii* nests within a moderately supported subclade (BS= 85%, PP= 1.0) with *P. ferricola*, *P. magnifica* and *P. striatiflora*.

The three species of section *Klanderia* listed above form a well supported clade (BS=96%, PP=1.0); there is little phylogenetic resolution for the rest of this section. Much of section *Prostanthera*, however, is recovered in two clades (E and F) in MP and BI trees. Clade E receives weak bootstrap support and Bayesian support (BS= 57%, PP= 1.0) whereas clade F is supported in both trees (BS= 87%, PP=1.0). Both clades contain at least two out of three representatives of Bentham's (1870) series classification.

ETS data set

The MP strict consensus tree and BI tree of the ETS data have similar topologies and are well resolved (Figs. 2.5 and 2.6). *Wrixonia* nests within clade D of *Prostanthera*. This clade has weak bootstrap support but strong Bayesian support (BS= 57%, PP= 0.99). All species of section *Klanderia* are also recovered within clade D. Clade H is a strongly supported (BS= 100%, PP= 1.0) group of section *Klanderia* found in the arid regions of south eastern Australia.

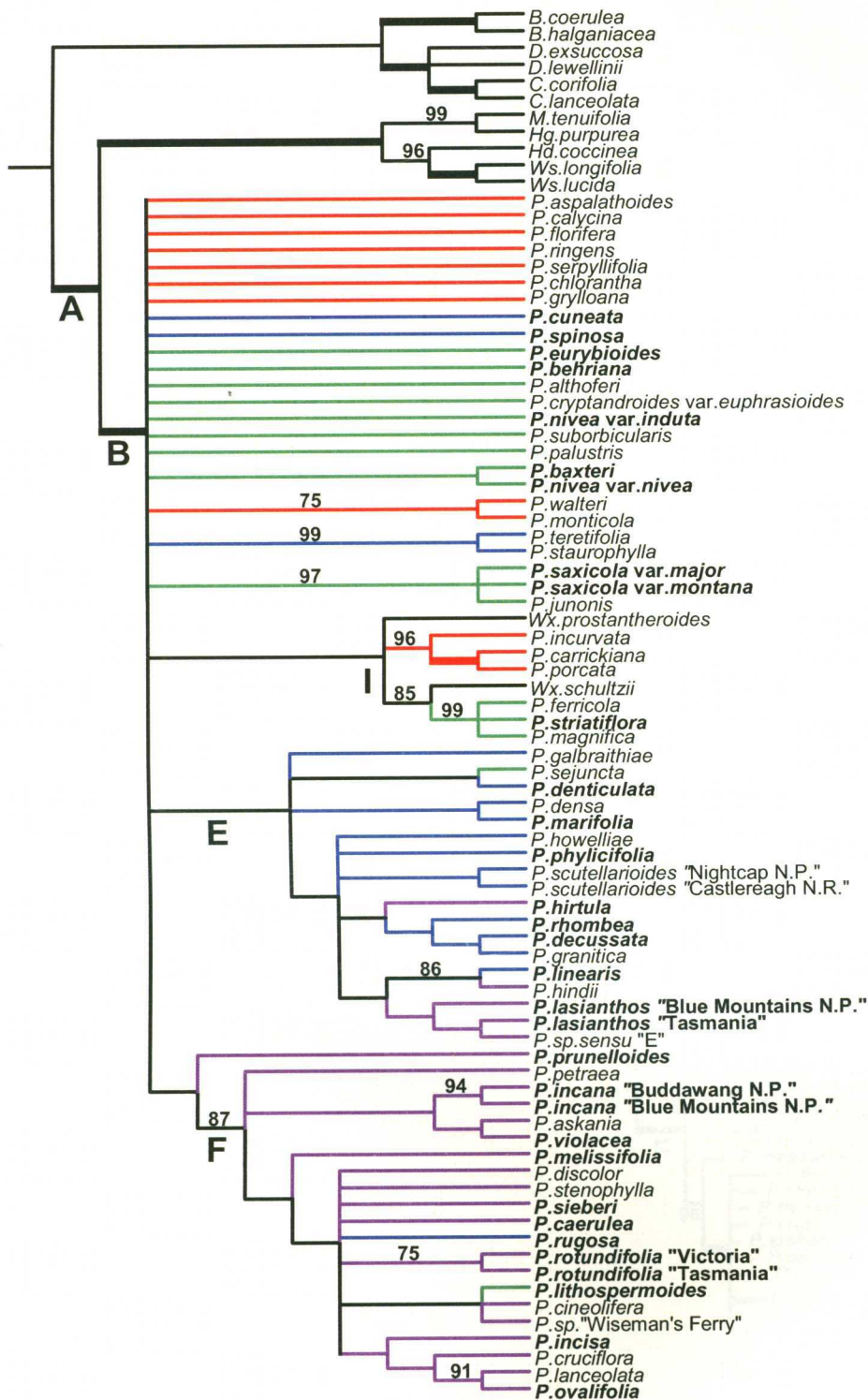


Figure 2.3. The strict consensus of 11095 trees from maximum parsimony (MP) analysis of the *trnT-F* sequence data for *Prostanthera* and its outgroups. Bootstrap values of 75% are reported above branches and thickened branches received 100%. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcaevae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

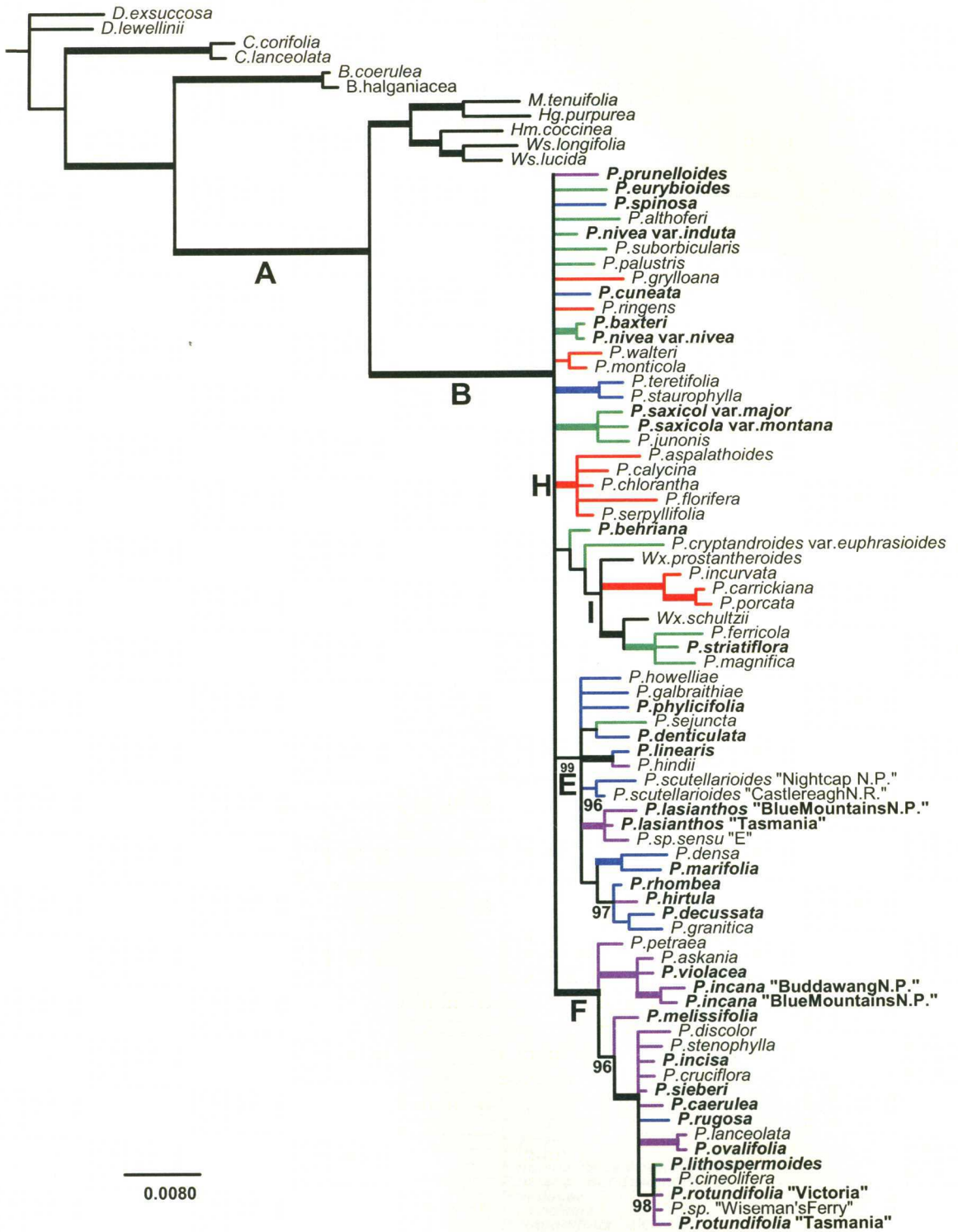


Figure 2.4. The 50% majority rule phylogram from Bayesian analysis of the *trnT-F* sequence data for *Prostanthera* and its outgroups. Prior probabilities of greater than 0.95 are reported below branches and thickened branches receive 1.00. Values are formatted as a % rather than with a decimal place. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcavae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

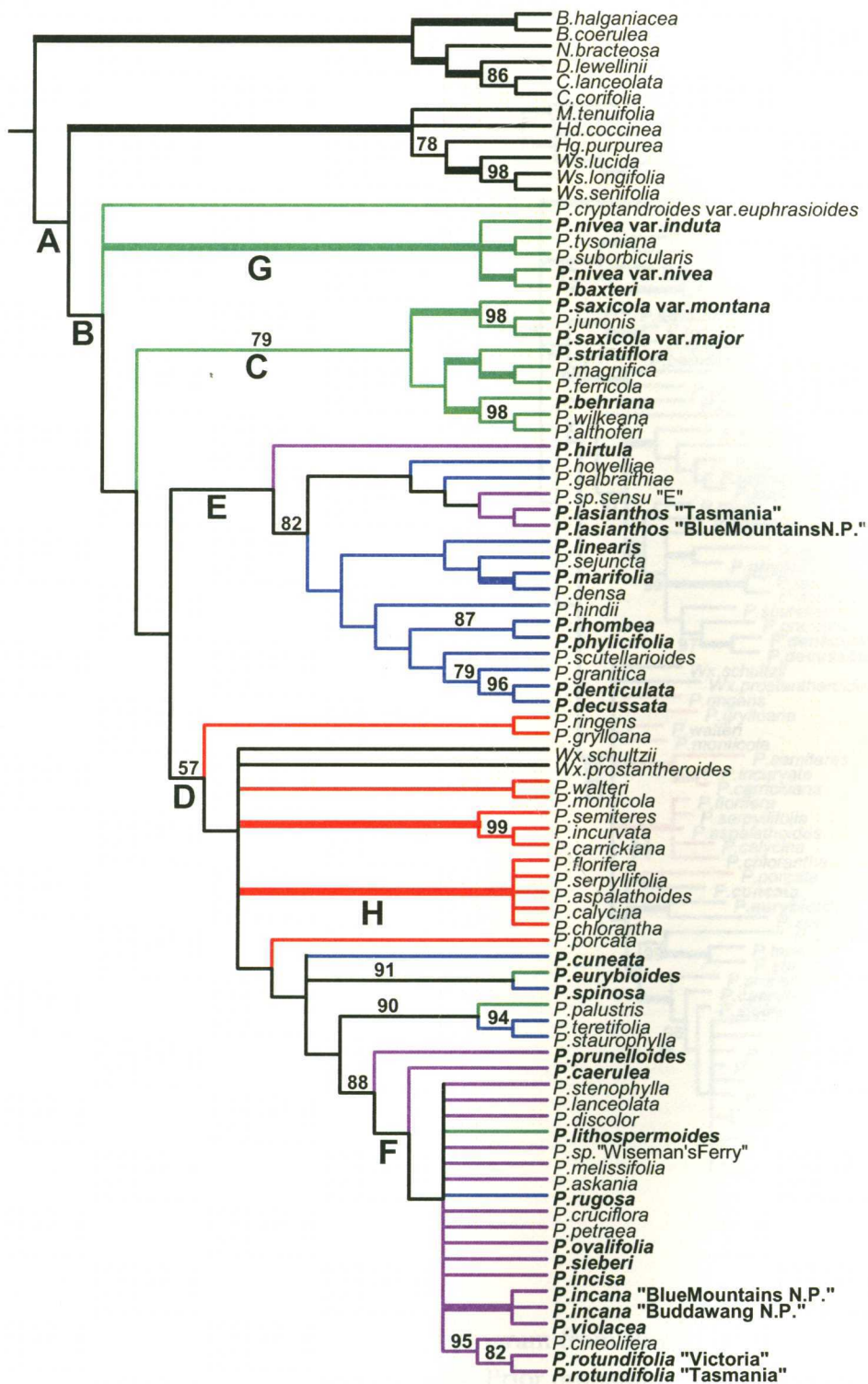


Figure 2.5. The strict consensus of 46142 trees from maximum parsimony (MP) analysis of the ETS sequence data for *Prostanthera* and its outgroups. Bootstrap values of 75% are reported above branches and thickened branches received 100%. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcaevae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

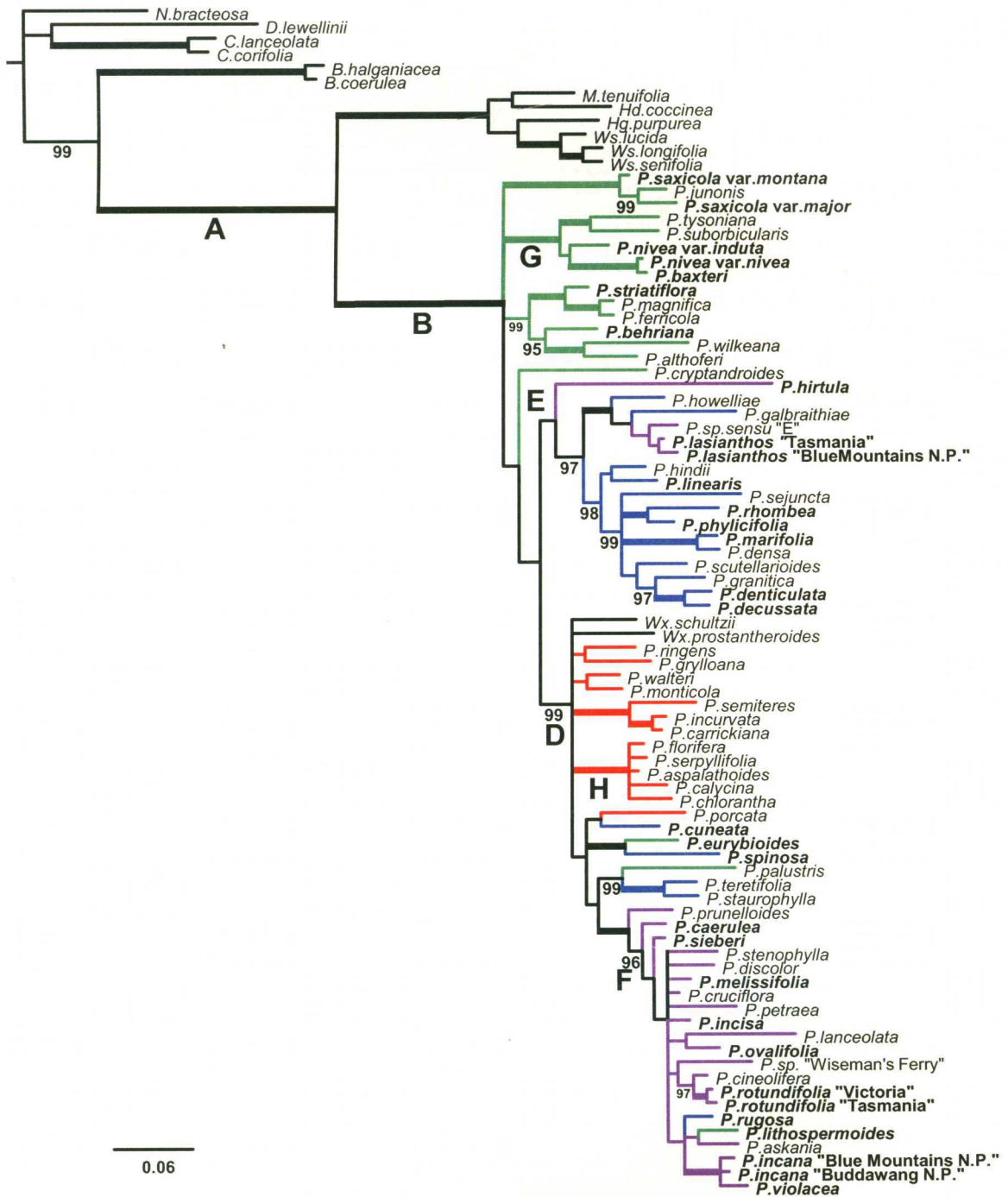


Figure 2.6. The 50% majority rule phylogram from Bayesian analysis of ETS sequence data for *Prostanthera* and its outgroups. Prior probabilities of greater than 0.95 are reported below branches and thickened branches receive 1.00. Values are formatted as a % rather than with a decimal place. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcaevae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

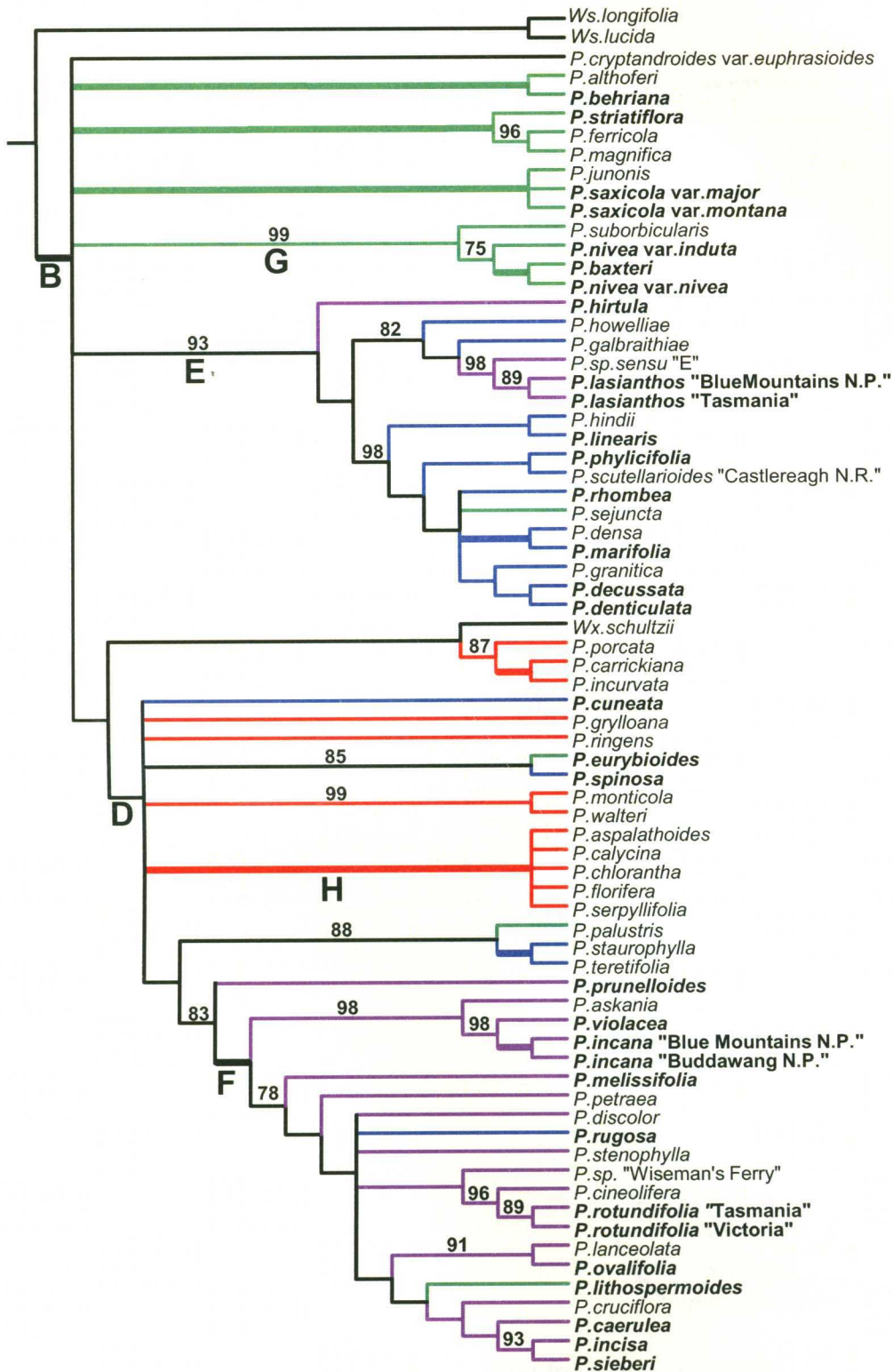


Figure 2.7. The strict consensus of 78 trees from maximum parsimony (MP) analysis of the combined *trnT-F*, *ndhF-rpl32*, and ETS sequence data for *Prostanthera* and *Westringia*. Bootstrap values of 75% are reported above branches and thickened branches received 100%. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcaevae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

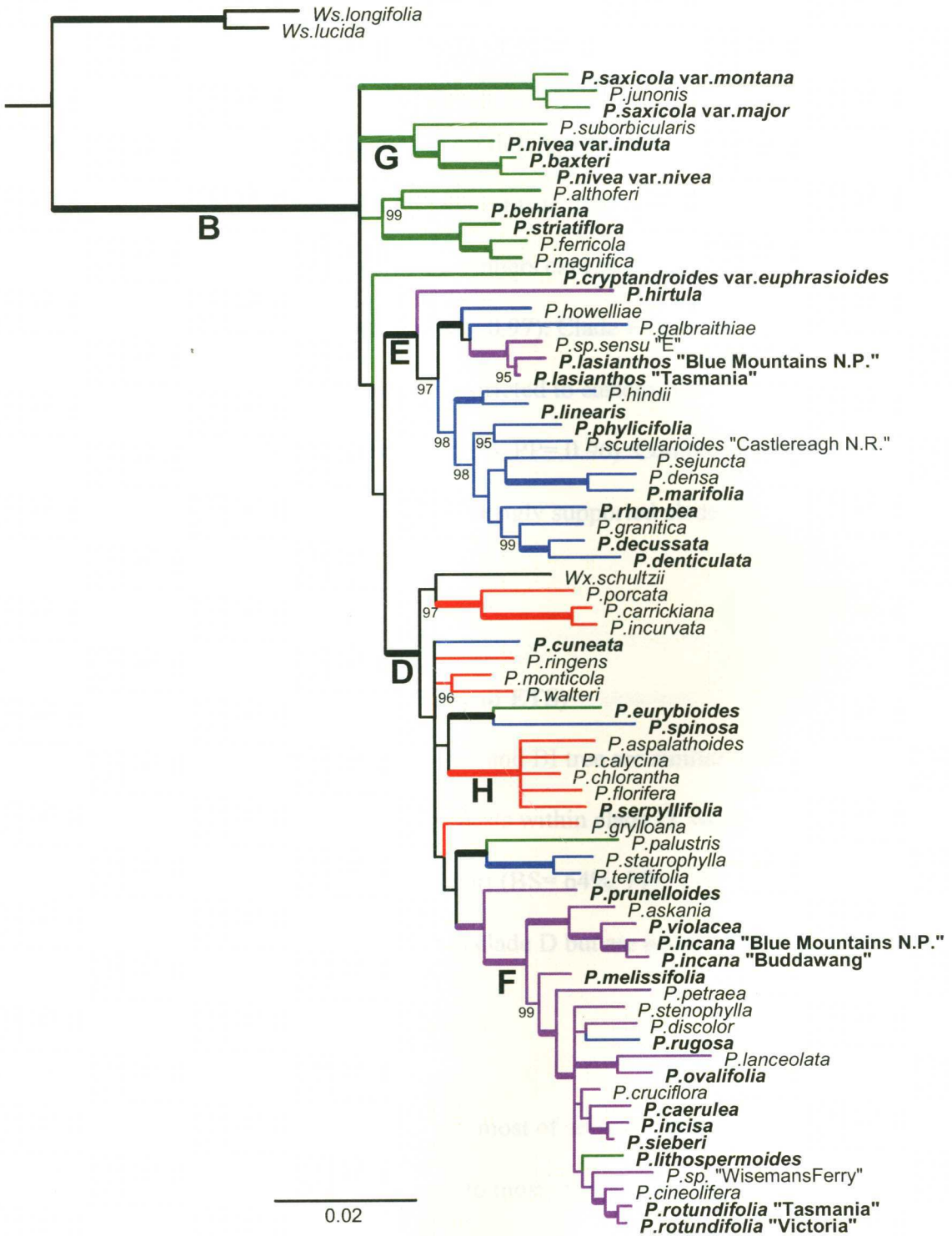


Figure 2.8. The 50% majority rule phylogram from Bayesian analysis of the combined *trnT-F*, *ndhF-rpl32*, and ETS sequence data for *Prostanthera* and *Westringia*. Prior probabilities of greater than 0.95 are reported below branches and thickened branches receive 1.00. Values are formatted as a % rather than with a decimal place. Generic abbreviations are listed in Fig. 2.1 and coloured branches refer to species grouped into Bentham's (1870) series of section *Prostanthera*: blue= *Convexae*; purple= *Racemosae*; green= *Subconcaevae*. Section *Klanderia* is coloured red. Species from the original treatment are in bold.

Most of section *Prostanthera* is resolved in three clades (E,F, and G). Clade G is well supported (BS= 100, PP= 1.0) and contains species strictly belonging to the *Subconcaevae*. Clade E contains species of section *Prostanthera* only found in eastern Australia that belong to the *Convexae* or *Racemosae*. It has weak bootstrap and no Bayesian support; within it, however, all members except *P. hirtula* are recovered in a clade with moderate support (BS= 82%, PP= 0.97). Clade F contains members of the *Convexae*, *Racemosae*, and *Subconcaevae* restricted to eastern Australia. It is supported in the BI tree but not in the MP tree (BS= 63%, PP= 0.96). Combined with the sister taxon *P. prunelloides*, however, it forms a strongly supported clade (BS= 88%, PP= 1.0).

Combined data set (*trnT-F*, *ndhF-rpl32*, and ETS)

The topology of the MP strict consensus tree and BI tree are similar to the results of the ETS data set (Figs. 2.7 and 2.8). *Wrixonia* nests within clade D, which has weak bootstrap support but strong Bayesian support (BS= 64%, PP= 1.0). All species of section *Klanderia* are also recovered within clade D but are poorly resolved. Clade H is a strongly supported (BS= 100%, PP= 1.0).

Similar to the results from the ETS data set, most of section *Prostanthera* is resolved in three clades (E,F, and G). Clade G is sister to most other groups of *Prostanthera* and has high support (BS= 100, PP= 1.0). All members of this clade belong to the *Subconcaevae*.

Clade E contains eastern distributed species of section *Prostanthera* that belong to the *Convexae* or *Racemosae*. Unlike the results from the ETS data set, this clade has

moderate bootstrap support and Bayesian support (BS= 93%, PP= 1.0). It is composed of two well resolved internal clades that have high support and each contain taxa of the *Convexae* and *Racemosae*.

Clade F has high support (BS= 100%, PP= 1.0) and mostly contains members of the *Racemosae* that are restricted to eastern Australia. *Prostanthera stenophylla* (*Convexae*) and *P. lithospermoides* (*Subconcaevae*), however, are also found within this clade.

Similar to the results from the ETS data set, clade E also forms a moderately supported clade (BS= 83%, PP= 1.0) with *P. prunelloides*.

Analyses were also run to include all species in a combined analysis, even if DNA regions were missing. However, this did not change the structure of the phylogeny or improve its resolution.

Discussion

The purpose of this study was to test the sectional classification of *Prostanthera*. Results are equivocal as to whether section *Klanderia* is monophyletic but the sectional classification is not supported by the phylogeny. Furthermore, it is found that *Wrixonia* is nested within *Prostanthera sens. lat.* and the classification of series within section *Prostanthera* does not correspond to the relationships recovered in the molecular phylogeny.

There is general consensus amongst all trees of each molecular region. However, the phylogeny reconstructed from the *ndhF-rpl32* region is poorly resolved. This low

resolution does not correspond to the high variability in closely related families such as the Scrophulariaceae or Plantaginaceae (Shaw *et al.* 2007; Dunbar-Co *et al.* 2008; Tay *et al.* 2010). It is likely that the well resolved tree of *Plantago* has better resolution because the *ndhF-rpl32* region is at least 300 base pairs longer. Other species, such as *Magnolia acuminata* L., *Trillium ovatum* Pursh, and *Hibiscus cannabinus* L. have regions similar to *Prostanthera* in size (~650BP) and also have a reportedly high variation, described as potentially informative characters (Shaw *et al.* 2007). The *ndhF-rpl32* region of *Prostanthera* has several sites of mononucleotide repeats (e.g. poly-A's) that vary in length amongst species. Since they are difficult to align they were not used in the phylogenetic analysis. If these sites greatly contribute to the number of potentially informative characters described by Shaw *et al.* (2007) then the *ndhF-rpl32* may not be useful to low-level molecular studies that require base pair alignments for phylogenetic analysis.

Classification in the Westringieae

The molecular phylogeny supports the position of *Prostanthera* and *Wrixonia* as sister to all other Westringieae (Cantino 1992; Conn 1992b). However, it places *Wrixonia* within *Prostanthera* and therefore contradicts previous morphological analyses that support *Wrixonia* as a sister clade to *Prostanthera*. Furthermore, none of the results recover both species of *Wrixonia* within a clade. Therefore this does not provide support that *Wrixonia* is a monophyletic clade, but its status remains unresolved since they are consistently recovered within a poorly resolved region of the phylogeny.

The placement of *Wrixonia* within *Prostanthera* suggests that the flower of *Wrixonia* has lost a functional pair of anthers as well as the appendages on its functional anthers.

This loss suggests that anther morphology has changed too rapidly to be a useful phylogenetic character at the generic level. The considerable morphological variation in anther appendages within each section of *Prostanthera* also indicates this as a rapidly evolving character. Furthermore, *Microcorys* is considered distinct from *Hemigenia* because it has two abaxial staminodes (Carrick 1976). Recent molecular evidence demonstrates these two genera are polyphyletic and hence a similar reduction in anther appendages may have occurred again in the Westringieae (Guerin 2008).

Classification in *Prostanthera*

Whilst the phylogeny is insufficiently resolved to reject the monophyly of section *Klanderia*, it does demonstrate the paraphyly of section *Prostanthera*. Although the *ndhF-rpl32* region recovers little resolution within *Prostanthera*, *trnT-F* and ETS regions consistently show that species of section *Klanderia* nest within *Prostanthera* in clade D (Figs. 2.2- 2.8). Clade F, which is composed of species from section *Prostanthera*, has strong support from the *trnT-F* and ETS regions and is more derived than section *Klanderia* (Figs. 2.2- 2.8). This therefore forms two separate clades composed of species of section *Prostanthera*. *Wrixonia*, *P. cuneata*, *P. eurybioides*, and *P. spinosa* are recovered with species of section *Klanderia* by better resolved trees (Figs. 2.5- 2.8). However, their placement is not consistent amongst these trees nor does their position receive any support. Therefore, the status of section *Klanderia* remains unresolved since there is a lack of evidence in support of the group and there is little evidence to reject it.

The paraphyly of section *Prostanthera* contradicts the previous assumption of monophyly derived from Bentham's (1870) classification. Unfortunately no subsequent

study of taxonomy in the Westringieae has included multiple representatives to test the monophyly of section *Prostanthera*. Two possible exceptions are morphological studies that support section *Prostanthera* and *P. tysoniana* (= *Eichlerago tysoniana*) as sister to section *Klanderia* (Cantino 1992; Conn 1992b). The ETS data set contradicts this since it recovers section *Klanderia* within section *Prostanthera*, which includes *P. tysoniana* (= *E. tysoniana*). In addition to some of the previously untreated species, the placement of many original members in each series does not correspond with the phylogeny.

Prostanthera lasianthos, for example, has racemose inflorescences but is recovered with members of the *Convexae*. This supports that inflorescence and leaf shape are not ideal for systematic treatment of *Prostanthera* (Hillson 1959). Despite these inconsistencies, however, there is some correlation between the phylogeny and the series. Most members of the *Racemosae* belong to clade F, which has high support from the *trnT-F* and ETS data sets and is also partially recovered in the BI analysis of the *ndhF-rpl32* region. Most members of the *Convexae* belong to clade E, which is resolved in all trees except the *ndhF-rpl32* MP tree. Most of the *Subconcavae* are recovered as sister to the rest of *Prostanthera*; this includes clade G which has high support in *trnT-F* and ETS trees. In addition to this general correlation, the reclassification of some of Bentham's decisions makes the correlation even stronger. For example, the current morphological descriptions of *P. spinosa* and *P. cuneata* fit the *Subconcavae* even though they are placed in the *Convexae* (Conn 1988; 1998). It appears that with detailed morphological and ontogenetic study across *Prostanthera* may resolve the inconsistencies between the phylogenetic and series classifications.

The trees of the *trnT-F* and ETS datasets recover the multiple accessions of a particular species into a well supported clade. *Prostanthera sejuncta* has been described as *P.*

spinosa until recently because they both share spinose branches (Williams *et al.* 2006). Their placement into separate clades is a surprising result that supports that the spinose morphology is homoplastic. *Prostanthera arapilensis* and *P. nudula* also have spinose morphology and their addition to the phylogeny is necessary to better understand the evolution of this morphology.

Conclusions

The results of molecular analyses demonstrate that *Prostanthera* is not monophyletic since *Wrixonia* nests within it. Since *Prostanthera* was described before *Wrixonia*, and both belong to a well supported clade, it is recommended that *Wrixonia* be transferred to *Prostanthera*. Poor resolution of the phylogeny prevents a conclusion about the monophyly of section *Klanderia*. The sectional classification should be rejected, however, since section *Prostanthera* is paraphyletic. The phylogeny does not correspond to the series classification proposed by Bentham (1870). It appears that with an improved morphological understanding of *Prostanthera*, however, these groups will be the foundation of a new internal classification of *Prostanthera*.

Chapter 3

Floral characters and shape support measureable pollination syndromes in *Prostanthera*

Introduction

Pollination syndromes have traditionally been characterised based on a collection of qualitative characteristics (Vogel 1954; Faegri and Van der Pijl 1979). For example, long red tubular corollas, a large volume of nectar, reflexed lower lobes, and exerted anthers are typical floral features that are used to estimate ornithophily (Faegri and Van der Pijl 1979; Cronk and Ojeda 2008). However, applying the concept of a pollination syndrome does not always allow correct prediction of pollinators and should not be considered failsafe (Faegri and Van der Pijl 1979; Proctor *et al.* 1996). For example, the flowers of the bird-pollinated *Banksia ericifolia* Smith (Proteaceae) do not have long red tubular corollas and the long red tubular corollas of *Peraxilla tetrapetala* (L.f.) Tiegh. (Loranthaceae) are not exclusively bird pollinated (Paton and Turner 1985; Robertson *et al.* 2005). Inferring pollinators based on floral characteristics might be improved if it is understood what measureable changes are necessary to distinguish them.

The few studies that have measured differences between pollination syndromes employ multivariate analysis of floral characteristics (Ollerton and Watts 2000; Wilson *et al.*

2004; Martén-Rodríguez *et al.* 2009). The choice of measurable characteristics for these studies was restricted to linear measurements such as petal reflexion, or corolla length. Flower shape, which has an influence on attracting pollinators and corresponds to their fit (Westerkamp and Classen-Bockhoff 2007), has so far been restricted to categorical description and has not been reliably measured objectively.

Relative warps analysis is a recent method developed to measure differences in shape (Rohlf and Marcus 1993; Mitteroecker and Gunz 2009). This analysis recognises shape as a set of landmarks and compares it with homologous landmarks on other shapes by using principal components analysis (Rohlf and Marcus 1993). The principal components, defined as relative warps, can be visualised by projecting them onto a thin-plate spline, which is a modification of Thompsons' (1942) cartesian grid (Bookstein 1989). Therefore patterns in the ordination can be associated with the deformations in shape caused by relative warp 1 (RW1) or relative warp 2 (RW2) on the thin-plate splines. The effect these deformations have on the entire shape can also be investigated by dissecting the total change in shape, which is known as the shape component, into two parts: the uniform shape component, which describes the effects of shearing and stretching, and the non-uniform shape component, which describes the effects of localised deformations in shape (Jensen *et al.* 2002). Relative warps analysis has had a wide variety of applications from taxonomic analysis of *Drosophila* to identifying trophic phenotypes amongst Cichlid fishes (Rohlf and Slice 1990; Rüber and Adams 2001). Its recent application for understanding zygomorphy in *Erysimum* flowers demonstrates that there is potential for this tool to investigate key changes of flower shape amongst pollination syndromes (Gómez *et al.* 2006)

Taxa are inferred to be either entomophilous or ornithophilous by qualitative observations of their flowers: a short, gullet-shaped, mauve or white corolla of section *Prostanthera* (Fig. 3.1a and Fig. 3.2b) implies entomophily whereas the long, red, tubular corolla of section *Klanderia* (Fig. 3.1c) implies ornithophily (Keighery 1980; Conn 1984). These pollination syndromes are speculative without quantitative analysis of morphology or visitors. In this chapter, multivariate analyses of linear measurement and shape are used to identify the difference in floral shape amongst groups identified in the phylogeny of the genus *Prostanthera*.



Figure 3.1 Three species of *Prostanthera* demonstrating the breadth of variation in flower morphology. a) *Prostanthera lasianthos*, female stage. b) *P. rotundifolia*, female stage. c) *P. aspalathoides*, male stage.

Materials and Methods

Specimen collection

Seventeen species of *Prostanthera* were selected for morphometric analysis and collected from across New South Wales, Victoria, and South Australia (Table 3.1).

Flowers were collected from more than one population of *P. aspalathoides*, *P. lasianthos*, *P. monticola*, and *P. nivea*. Two varieties of *P. nivea* (namely, var. *induta*

Table 3.1. Species of *Prostanthera* used for phenetic and geometric analysis. Section and series classification is based on Bentham (1870).

*=series classification adopted in Chapter 2. N.P.= National Park; N.S.W.= New South Wales; S.A.= South Australia; S.F.= State Forest; Vic.= Victoria.

Study Species	Section	Series	Location	Voucher #
<i>P. aspalathoides</i> Benth.	<i>Klanderia</i>	-	Gubbatta N.R., N.S.W	NSW803944
			Taleeban N.R., N.S.W	NSW803918
			Hiawatha S.F., N.S.W	NSW803950
<i>P. chlorantha</i> (F.Muell.) Benth.	<i>Klanderia</i>	-	Currency Creek, S.A.	NSW803952
<i>P. cruciflora</i> J.H.Willis	<i>Prostanthera</i>	<i>Racemosae</i> *	Mount Kaputar N.P., N.S.W.	NSW803951
<i>P. cuneata</i> Benth.	<i>Prostanthera</i>	<i>Convexae</i>	Mt. Buffalo N.P., Vic.	NSW844231
<i>P. lasianthos</i> Labill.	<i>Prostanthera</i>	<i>Racemosae</i>	Blue Mountains N.P., N.S.W.	NSW799707
<i>P. linearis</i> R.Br.	<i>Prostanthera</i>	<i>Convexae</i>	Brisbane Waters N.P., N.S.W.	NSW784600
<i>P. monticola</i> B.J.Conn	<i>Klanderia</i>	-	Mount Buffalo N.P., Vic.	NSW803943
<i>P. nivea</i> Benth. var. <i>induta</i>	<i>Prostanthera</i>	<i>Subconcaevae</i>	Warrumbungles N.P., N.S.W.	NSW803949
<i>P. nivea</i> Benth. var. <i>nivea</i>	<i>Prostanthera</i>	<i>Subconcaevae</i>	Mt. Kaputar N.P., N.S.W.	NSW803947
<i>P. ovalifolia</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Mendooran, N.S.W.	NSW803953
<i>P. porcata</i> B.J.Conn	<i>Klanderia</i>	-	Buddawang N.P., N.S.W.	NSW779742
<i>P. ringens</i> Benth.	<i>Klanderia</i>	-	Pilliga S.F., N.S.W.	NSW803945
<i>P. rotundifolia</i> R.Br.	<i>Prostanthera</i>	<i>Racemosae</i>	Mt. Buffalo N.P. Vic.	NSW779804
<i>P. saxicola</i> R.Br. var. <i>montana</i>	<i>Prostanthera</i>	<i>Subconcaevae</i>	Blue Mountains N.P., N.S.W.	NSW803916
<i>P. serpyllifolia</i> (R.Br.) Briq. subsp. <i>microphylla</i>	<i>Klanderia</i>	-	Pulletop N.R., N.S.W.	NSW813913
<i>P. sieberi</i> Benth.	<i>Prostanthera</i>	<i>Racemosae</i>	Royal N.P., N.S.W.	NSW844227
			Mt. Keira, N.S.W.	NSW803920
<i>P. staurophylla</i> F.Muell.	<i>Prostanthera</i>	-	Mt. Mckenzie, N.S.W.	NSW785873
<i>P. striatiflora</i> F.Muell.	<i>Prostanthera</i>	<i>Subconcaevae</i>	Mt. Yardi, S.A.	NSW449546

and var. *nivea*) were included to account for its full range of morphological variation. For other species, replicates were collected from different plants at one population.

The flowers of *Prostanthera* are bisexual and protandrous (Conn 1984). For most species of *Prostanthera*, shape change is continuous for the duration of the male flower stage whereas it ceases by the female stage (refer to Chapter 4). Therefore most phenetic data and all geometric data were collected from female stage flowers. For each species, one male stage flower and one female stage flower were collected for measurements of the phenetic data. Therefore the total number of flowers examined for these measurements was 34. Three female stage flowers were collected for each species to measure the geometric data. The total number of flowers used for the geometric analysis was 51.

Flowers were cut below the prophylls (refer to Chapter 1) and preserved in 70% ethanol. To keep the natural shape of the flower, care was taken to remove the flowers and place them in a vial large enough to suspend the flower in ethanol without deformation. Apart from anthers being slightly divergent, there was no change in morphology after preservation in ethanol.

Measurement of phenetic data

Bentham (1870) originally described flowers of *Prostanthera* as bilabiate with spreading lateral lobes of the labium, or bottom lip. However, it is often difficult to ascertain which lip these lateral petals associate with and so flower descriptions are here based on the morphology previously outlined by Conn (1984). In his terminology, the corolla is defined as consisting of five lobes: two adaxial, two lateral, and one abaxial

(Fig. 3.2). The adaxial lobes are often so completely fused that they are referred as the adaxial median lobe-pair (Conn 1984).

Twenty-three morphological characters (Table 3.2) were chosen on the basis that they are commonly used to distinguish insect and bird pollination syndromes (Wilson *et al.* 2004; Cronk and Ojeda 2008; Martén-Rodríguez *et al.* 2009; Ollerton *et al.* 2009b). Since flowers of *Prostanthera* are isobilateral (Conn 1984), measurements that were taken from one side of the flower were consistently made on the right (Figs. 3.2a and 3.2b). Most characters were measured with hand held calipers (to the nearest 0.05 mm) with the aid of a dissecting microscope. The profile for each specimen was photocopied so that characters of curvature and reflexion could be measured using a ruler and protractor.

Table 3.2. Twenty-three floral characteristics measured for the phenetic analysis of *Prostanthera*.

#	Floral character	#	Floral character
1	♀ stage flower length	13	Adaxial lobe reflexion
2	♂ stage flower length	14	Lateral lobe width
3	Abaxial tube length	15	Tube height
4	Adaxial tube length	16	Tube width
5	Abaxial lobe length	17	♀ stage female exertion
6	Adaxial median lobe-pair length	18	♂ stage female exertion
7	Abaxial curvature (length)	19	♂ stage male exertion
8	Adaxial curvature (length)	20	appendage length: abaxial anther
9	Abaxial curvature (angle)	21	appendage length: adaxial anther
10	Abaxial curvature (angle)	22	Spotting
11	Adaxial tip curvature (angle)	23	Corolla colour
12	Abaxial lobe reflexion		

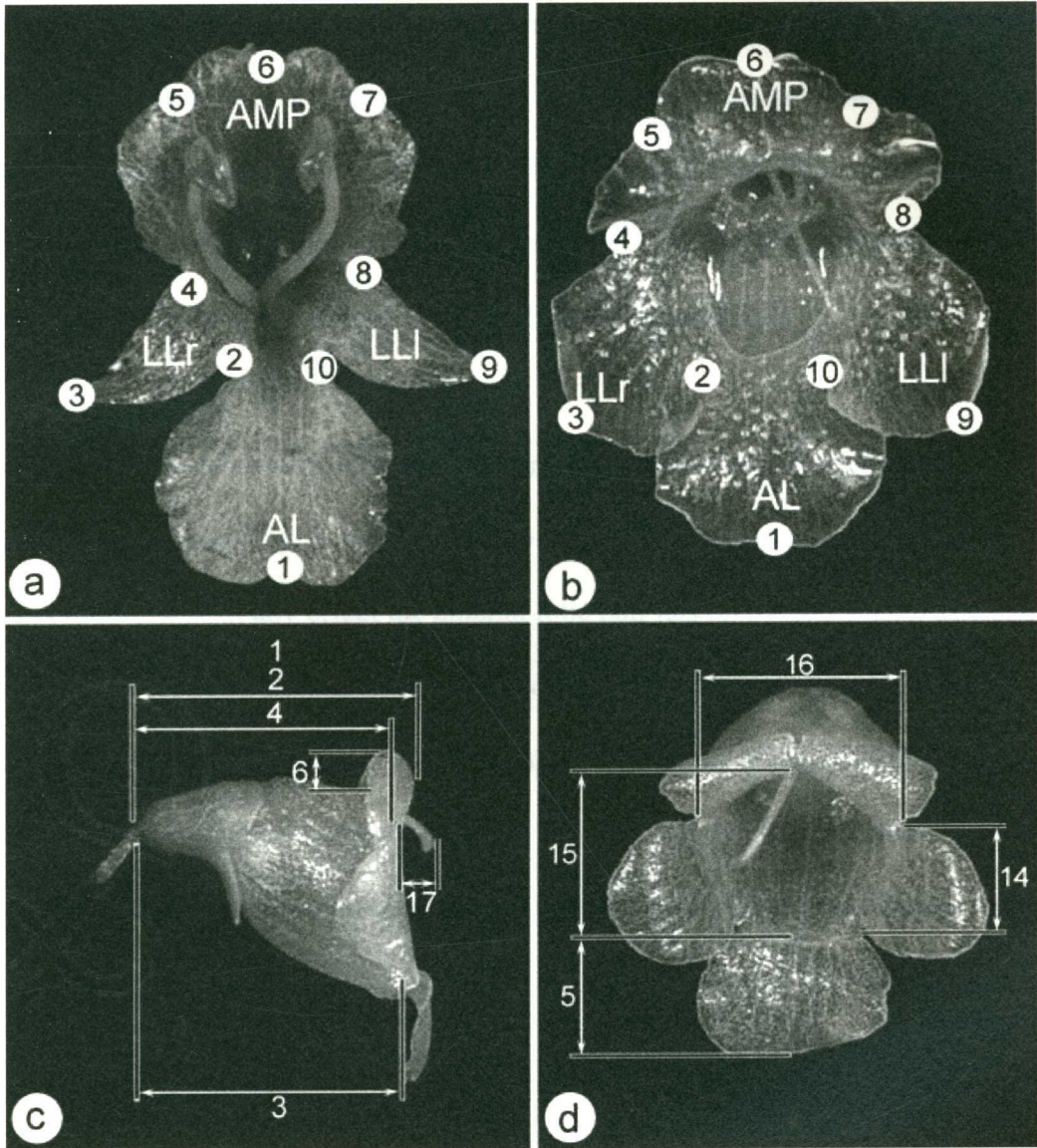


Figure 3.2. Image of flower illustrating the location of the 10 landmarks and linear morphometric characters applied to *Prostanthera* flowers. (a), landmarks on a *P. monticola* flower; (b), landmarks on a *P. sieberi* flower; (c), linear measurement of a *P. ovalifolia* flower in profile; (d), linear measurement of a *P. ovalifolia* flower in front view. AL= abaxial lobe; AMP= adaxial median pair; LLI= left lateral lobe; LLr= right lateral lobe; 1= female stage flower length; 2= male stage flower length; 3= abaxial tube length; 4= adaxial tube length; 5= abaxial lobe length; 6= adaxial median pair lobe length; 14= lateral lobe length; 15= tube height; 16= tube width.

Adaxial flower length (characters 1 and 2) was measured for male and female stage flowers whereas tube length (characters 3 and 4) was only measured in female flowers (Fig. 3.2c). Flower length (characters 1 and 2) was measured from the base of the calyx to tip of the adaxial median pair. Tube length (characters 3 and 4) was measured from the base of the corolla to the base of the lateral lobe. Lobe length (characters 5 and 6) was measured from the lobe base to its tip (Figs. 3.2c and 3.2d).

Length and angle of corolla curvature were measured by using abaxial tangent and adaxial tangent lines (Fig. 3.3a). Adaxial tip curvature and adaxial curvature were measured to establish any effect the adaxial lobe pair has on overall shape. The abaxial tangent is the line drawn tangentially to the curve of the abaxial side of the flower. The adaxial tangent is the line drawn as the tangent to the curve of the adaxial side of the flower. Corolla curvature length (characters 7 and 8) and angle (characters 9 and 10) were measured between the tangent and the junction of the abaxial and lateral lobe. Adaxial tip curvature (characters 11) was measured as the angle between the adaxial tangent and tip of the adaxial median pair.

The main flower axis was used to measure the angle of adaxial and abaxial lobe reflexion (Fig. 3.3b). The main flower axis is the line drawn from the base of the corolla through the junction between the lateral lobe and the abaxial lobe. Abaxial lobe reflexion (character 12) is the angle between the main flower axis and the distal tip of the abaxial lobe. Adaxial lobe (character 13) reflexion is angle between the main flower axis and the distal tip of the adaxial median pair.

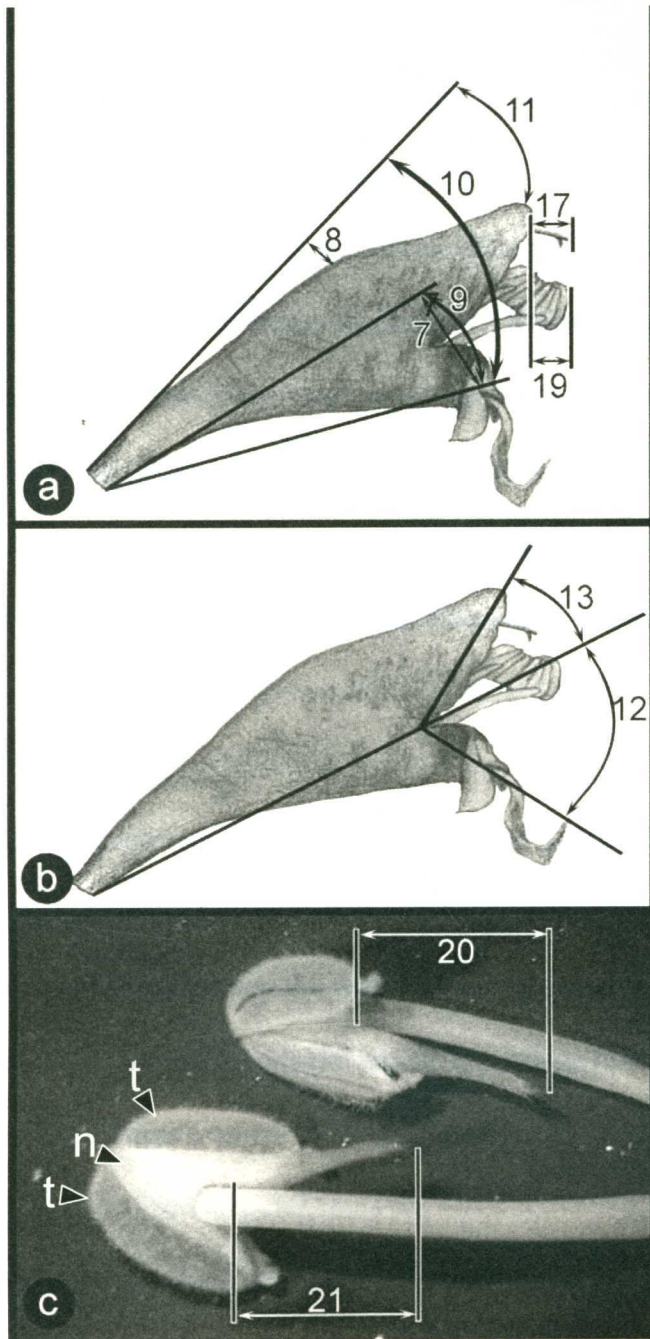


Figure 3.3. Images demonstrating measurements made for *Prostanthera* flowers. (a) and (b), Illustration of a flower representative of *Prostanthera* section *Klanderia*; (c), abaxial (top) and adaxial (bottom) anthers of *P. striatiflora*. The abaxial anther is positioned on its side whereas the adaxial anther is positioned ventral side down and shows how the appendage is an outgrowth of the enlarged connective tissue (n) between the two thecae (t). 7=abaxial curvature (length); 8= adaxial curvature (length); 9= abaxial curvature (angle); 10= adaxial curvature (angle); 11= adaxial tip curvature; 12= abaxial reflexion; 13= adaxial reflexion; 17= stigma exsertion; 19= stamen exsertion; 20= appendage length of abaxial anther; 21= appendage length of adaxial anther.

Measurements of height and width were taken from the point at which the lobes emerge from the floral tube (Fig. 3.2c). Lateral lobe width (character 14) was measured at the base of the lateral lobe. Tube height (character 15) was measured from the midpoint of the abaxial lobe to the midpoint of the adaxial median pair. Tube width (character 16) was measured at the base of the adaxial median pair. Stamen length and style length were measured from the base of the calyx to the tip of the anther and stigma, respectively (Fig. 3.2d). Female exertion (characters 17 and 18) and male exertion (character 19) were acquired by subtracting total flower length of the respective flower from this distance. At the female flower stage, stamens have senesced and so were not measured.

Some *Prostanthera* species have more than one type of androecial appendage (Conn 1984). This study is only concerned with the principal appendage, which originates from the connective tissue between the two thecae (Fig. 3.3c). The abaxial anther appendage (character 20) and the adaxial anther appendage (character 21) were measured from base to tip.

Corolla colour and the presence of spots were measured on fresh flowers in the field from approximately 200 female flowers for each species. The presence of spots on the corolla (character 21) was assessed as any markings on the abaxial lobe (Fig. 3.1a). Corolla colour (character 22) was coded from one to four in order from shortest wavelength to longest wavelength: 1= white (Fig. 3.1a), 2= blue-violet (Fig. 3.1b), 3= green-yellow, and 4= red (Fig. 3.1c).

Measurement of geometric data

The shape of the flower face (e.g. Fig. 3.2a) was used for relative warps analysis since it demonstrates many characteristics associated with pollination syndrome. Ten morphologically homologous points represent the apex of each petal lobe and each sinus between each of these lobes (Figs.3.2a and 3.2b). The apex of a petal was found at the terminus of the midvein and each sinus was chosen as the deepest point between petal lobes. These represent Type 1 landmarks (Slice *et al.* 1996). In the morphometric study of a leaf, sinuses are considered to be Type 2 landmarks since their exact positioning relies on geometric evidence rather than homology (Jensen 1990; Slice *et al.* 1996; Jensen *et al.* 2002). Sinuses in a corolla, however, can be considered Type 1 landmarks since they represent the division between petals.

Ethanol-preserved flowers were photographed using a stage mounted digital SLR camera (Canon EOS 30D) fixed with a 35mm Canon Lens EF (1:2). Four landmarks (1, 4, 6 and 8, Fig. 3.2a and Fig. 3.2b) were used to standardise flower position before digitisation by levelling them along a background horizontal line and the crosshairs of the camera viewfinder. The computer program Tps-Dig version 2.05 (Rohlf 2006) was used to digitise the location of homologous landmarks on each flower image and record the resulting data matrices.

Statistical analysis

Similarities of floral characters of *Prostanthera* were investigated using PATN version 3.03 (Belbin and Collins 2004). Cluster analysis and multidimensional scaling were conducted on the phenetic data in a two-part analysis. The first analysis was conducted using measurements of both sexual stages. A second analysis excluded the

measurements taken from male stage flowers. This was done in order to make comparisons with the geometric analyses that only analyse female stage flowers.

Cluster analysis was conducted using the Gower association measure and a flexible unweighted pair group method with arithmetic mean (UPGMA) sorting strategy with a beta value = -0.1. Gower's coefficient of similarity (Gower 1971) was used to prevent unequal character weighting since different quantitative measures are used. PATN subsequently changes these results to a dissimilarity matrix (Austin and Belbin 1982). The Kruskal-Wallis statistic was used to evaluate how well each character partitioned the groups found at a particular dissimilarity value in the cluster analysis. An analysis of similarities (ANOSIM) was used to evaluate the statistical significance between groups (Clarke and Green 1988).

Semi-strict hybrid multidimensional scaling (SSHMDS) and non-metric multidimensional scaling (NMMDS) ordinations were used to investigate spatial patterns of the floral characters after the data transformation described above. SSHMDS is recommended when there are a smaller number of operational taxonomic units (OTUs) than data variables because it is sensitive to differences between closely grouped OTUs (Rohlf 1972). Results of SSHMDS and NMMDS were congruent, therefore only the output of the former will be presented here. Principal component correlation (PCC) was used to generate a regression line for each variable in multivariate space. A coefficient of determination (r^2) measures the correlation of each variable from PCC. The reliability of the PCC was tested using Monte-Carlo attributes in ordination (MCAO) to determine how robust each linear regression was; a value less than 5% was considered as robust.

The landmark data were analysed using relative warps analysis (Rohlf 1993), cluster analysis and SSHMDS ordination. Relative warps was conducted with the program PAST (Hammer *et al.* 2001). A consensus shape for each species was generated from three replicates. For all species, the consensus shapes were then superimposed with each other using the Procrustes method to eliminate differences in size and rotation (Rohlf and Slice 1990). Relative warps analysis was conducted using principal components analysis (PCA) and then thin-plate splines were generated for relative warp one (RW1) and relative warp two (RW2).

The landmark data was also analysed with cluster analysis and SSHMDS to investigate the differential effect between the uniform and non-uniform shape component. The weight matrix was extracted using PAST and imported into PATN. Subsequent cluster analysis and SSHMDS ordination used the same settings as described for the analysis of phenetic data.

Results

Phenetic data

The data matrix for phenetic data is provided in Appendix 3.1. Two groups can be seen from the UPGMA dendrogram with a dissimilarity value of 0.527 (Fig. 3.4). Group 1 contains five members of section *Klanderia*. Group 2 contains *P. serpyllifolia* (section *Klanderia*) and all members of section *Prostanthera*. The flower of group 1 has a longer (characters 1, 2, 4, and 6), more curved (characters 7 and 8) corolla that reflects colour of a longer wavelength (character 23) (Fig. 3.5).

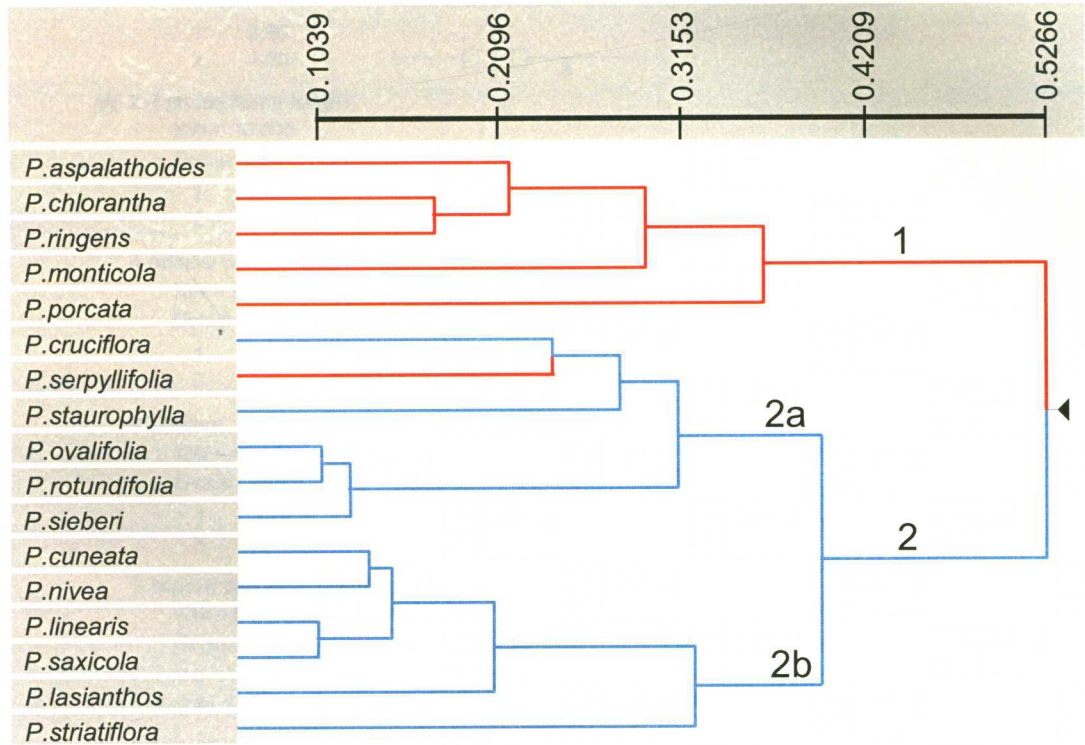


Figure 3.4. Dendrogram for the UPGMA analysis of the floral phenetic data from 17 species of *Prostanthera*. Three clusters can be identified from the dendrogram. Line colour represents sectional membership: red = section *Klanderia*; blue = section *Prostanthera*.

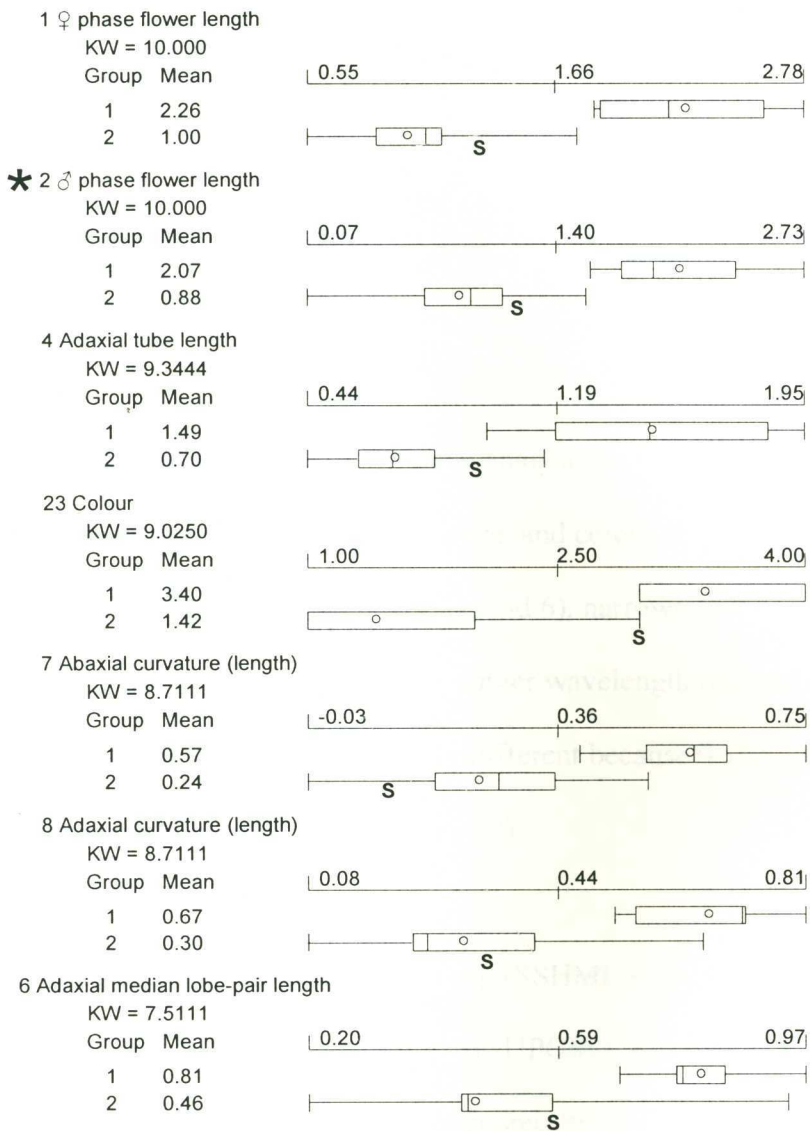


Figure 3.5. Box-whisker plots of UPGMA analysis of the floral phenetic data from 17 species of *Prostanthera*. Eight characters separate group 1 and group 2. Characters are ranked by Kruskal-Wallis values from highest (top) to lowest (bottom). Asterisk represents character retrieved only in the analysis using male and female stage flowers.

Two subgroups (group 2a and group 2b) are recovered in the UPGMA dendrogram at a dissimilarity value of 0.363 (Fig. 3.4). The flower of group 2a has a more exerted stigma (characters 17 and 18), shorter corolla (character 2), shorter abaxial lobe length (character 5), shorter anther appendages (characters 20 and 21), and a more narrow tube height (character 15) (Fig. 3.6).

The highest discriminatory characters from an analysis on taxa organised according to sectional classification are tube width, curvature, and colour (Fig. 3.7). The flower of section *Klanderia* is longer (characters 1, 2, 4, and 6), narrower (character 16), more curved (character 8) and reflects colour of a longer wavelength (character 23). The ANOSIM supports that they are significantly different because 0.10% scores (best fratio=1.48) are higher than the real f-ratio (1.42).

Semi-strict hybrid multiple dimensional scaling (SSHMDS) recovers three groups similar in composition to those recovered in the UPGMA dendrogram (Fig. 3.8). Most characters with high Kruskal-Wallis values scored from cluster analysis have high r^2 values that are significant (MCAO, $p < 0.05$) (Appendix 3.2). *Prostanthera serpyllifolia* (arrow) of group 2 is joined to group 1 by the minimum spanning tree. A group 1 flower is longer (characters 1, 2 and 4) and more curved (characters 7 and 8) (Fig. 3.6). If *P. serpyllifolia* is considered as group 1, then the flower of group 1 is also narrower (character 16) and reflects a colour with longer wavelength (character 23). Compared with only group 2b, the flowers of group 2a (circles) are wider (character 16), have more exerted stigmas (characters 17 and 18), and have longer anther appendages (characters 20 and 21).

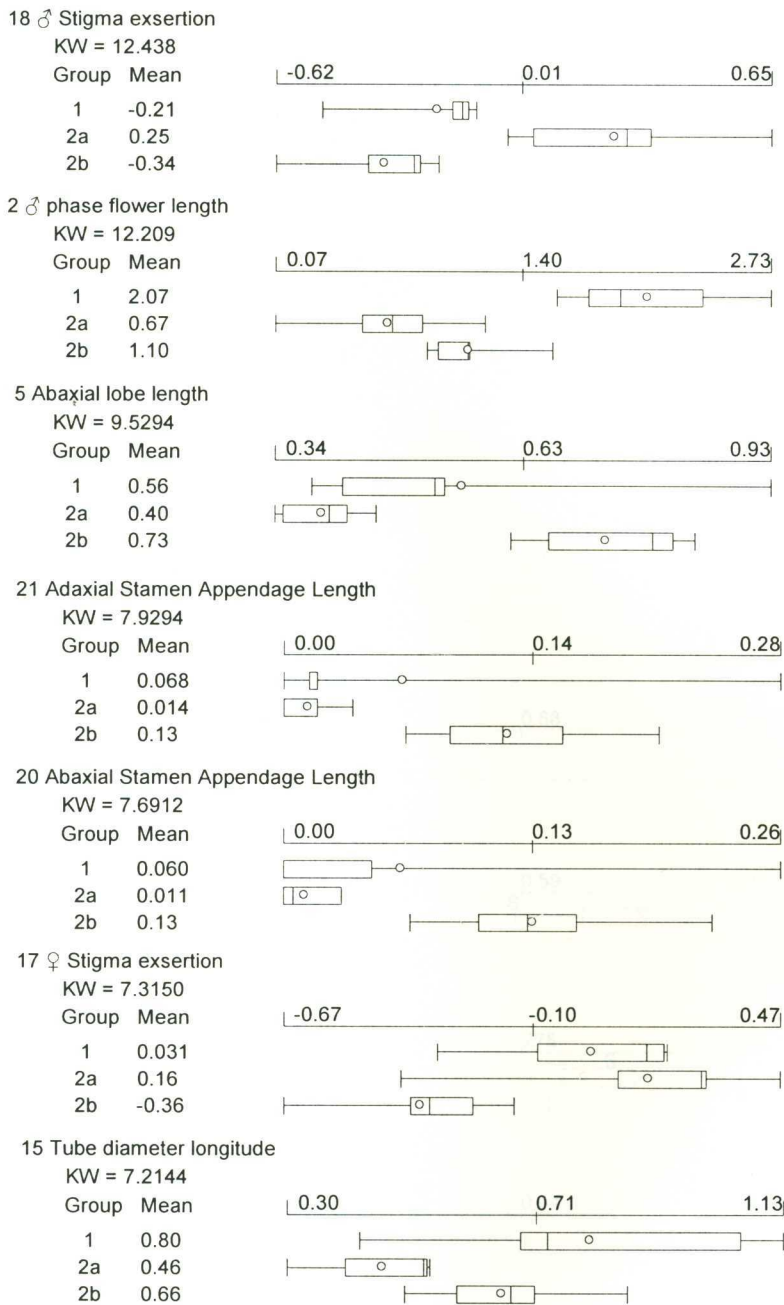


Figure 3.6. Box-whisker-plots from UPGMA analysis of floral phenetic data from 17 species of *Prostanthera*. Seven characters discriminate group 1, group 2a, and group 2b. Characters are ranked by Kruskal-Wallis values from highest (top) to lowest (bottom)

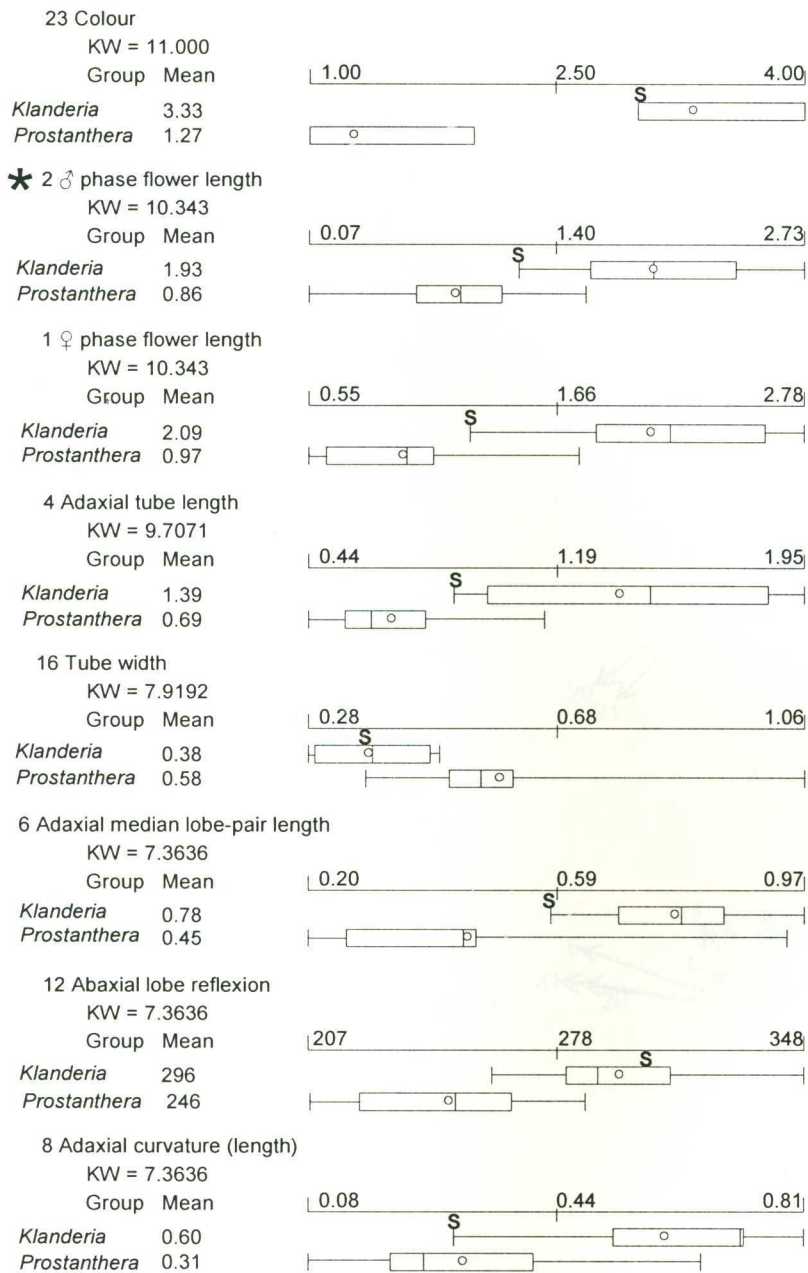


Figure 3.7. Box-whisker plots from the UPGMA analysis of floral phenetic data from 17 species of *Prostanthera*. The analysis uses the *a priori* groups section *Klanderia* and section *Prostanthera*. Eight characters separate section *Klanderia* and section *Prostanthera*. Characters are ranked by Kruskal-Wallis values from highest (top) to lowest (bottom). Asterisk represents character retrieved only in the analysis using male and female stage flowers. An S represents the approximate position of *P. serpyllifolia* in the box plots.

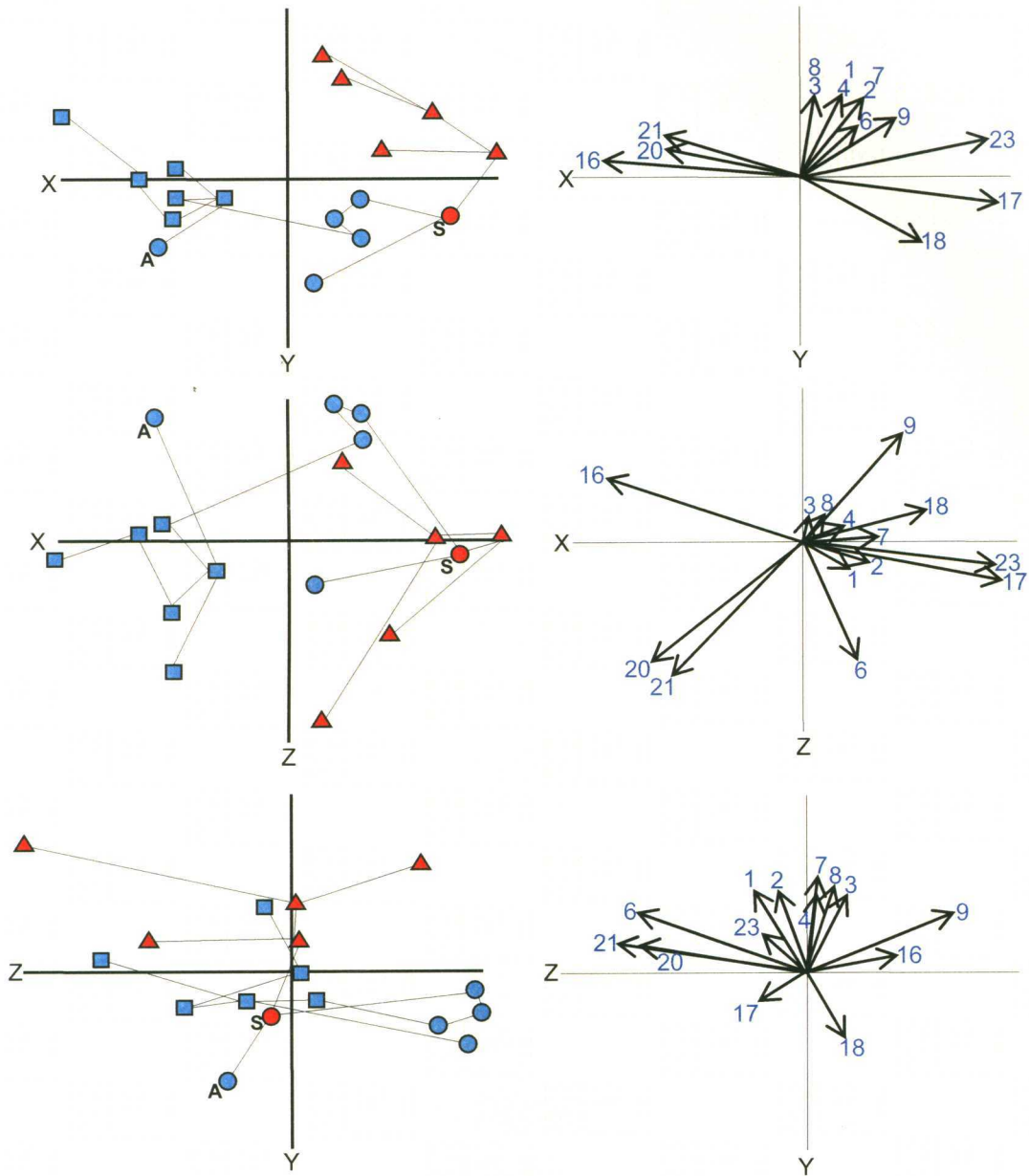


Figure 3.8. Pair-wise plots of the first three dimensions recovered from SSHMDS of the floral phenetic data from 17 species of *Prostanthera* (stress = 0.080). Character numbers from Table 3.2 are used in the vector diagrams. *Prostanthera serpyllifolia* is marked with an 's' and *P. staurophylla* is marked with an 'A'. Symbols represent cluster membership derived from flexible UPGMA (Fig. 3.4) and colours represent sectional classification. Triangle= group 1; circle= group 2a; squares= group 2b; red = *Prostanthera* section *Klanderia*; blue = *Prostanthera* section *Prostanthera*.

Cluster analysis recovers two groups at a dissimilarity value of 0.559 when male stage flower data are removed (Fig. 3.9). Group 1 contains five members of section *Klanderia*. group 2 contains *P. serpyllifolia* (section *Klanderia*) and all members of section *Prostanthera*. The flower of group 1 has a longer (characters 1, 4, and 6), more curved (characters 7 and 8) corolla that reflects colour of a longer wavelength (character 23) (Fig. 3.5).

Two subgroups (group 2a and group 2b) are recovered in the UPGMA dendrogram at a dissimilarity value of 0.402 (Fig. 3.4). The flower of group 2a has a more exerted stigma (characters 17), shorter length (character 1), more reflexed adaxial median pair (character 13), and shorter anther appendages (characters 20 and 21) (Fig. 3.10).

The SSHMDS recovers three groups similar in composition to those recovered in the UPGMA dendrogram (Fig. 3.11). Most characters with high Kruskal-Wallis values scored from cluster analysis have high r^2 values that are significant (MCAO, $p < 0.05$) (Appendix 3.2, Analysis 2). *Prostanthera serpyllifolia* of group 2 is joined to group 1 by the minimum spanning tree. A group 1 flower is longer (characters 1 and 4) and more curved (characters 7 and 8) (Fig. 3.6). If *P. serpyllifolia* is considered as group 1, then the flower of group 1 is additionally narrower (character 16), has a more reflexed abaxial lobe (character 12) and reflects a colour with longer wavelength (character 23). Compared to only group 2b, the flowers of group 2a (circles) are wider (character 16), have more exerted stigmas (character 18), and have longer anther appendages (characters 20 and 21).

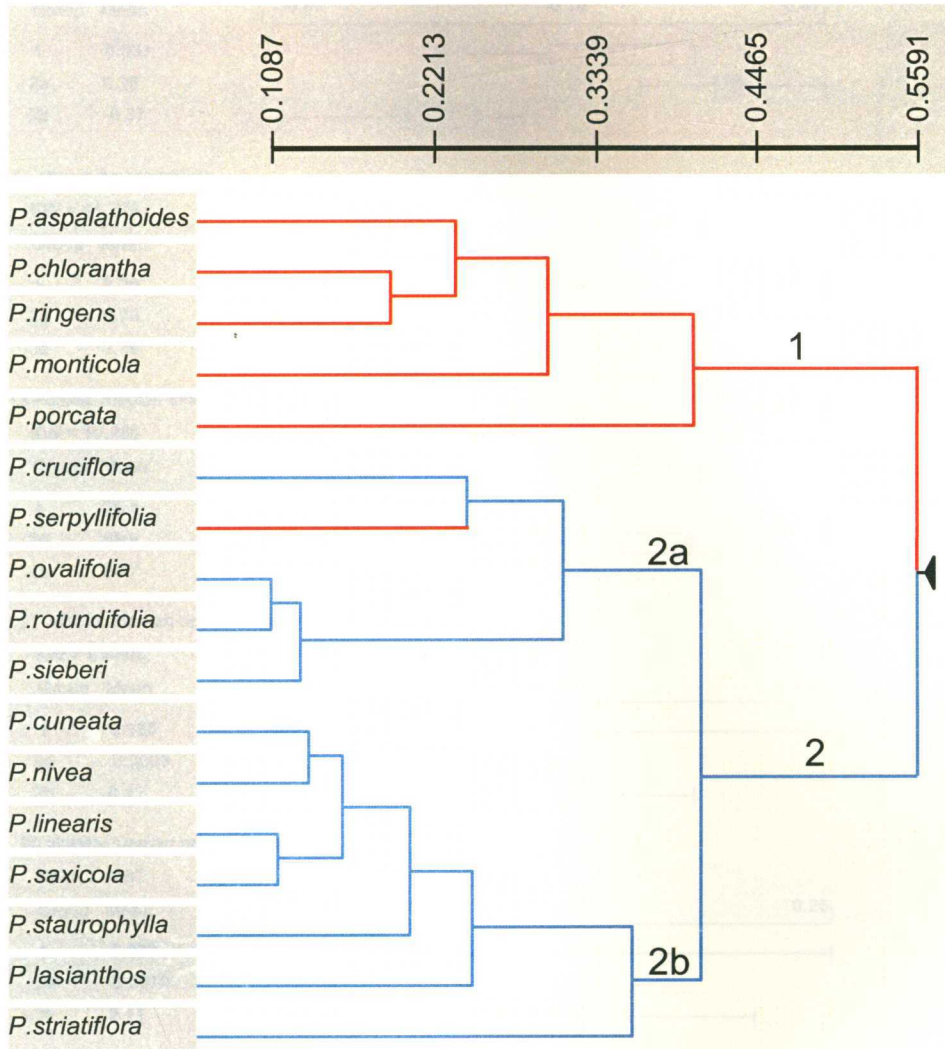


Figure 3.9. Dendrogram from the UPGMA analysis of the floral phenetic data from 17 species of *Prostanthera*. Data excludes characters from male stage flowers. Line colour represents sectional membership: red = section *Klanderia*; blue = section *Prostanthera*.

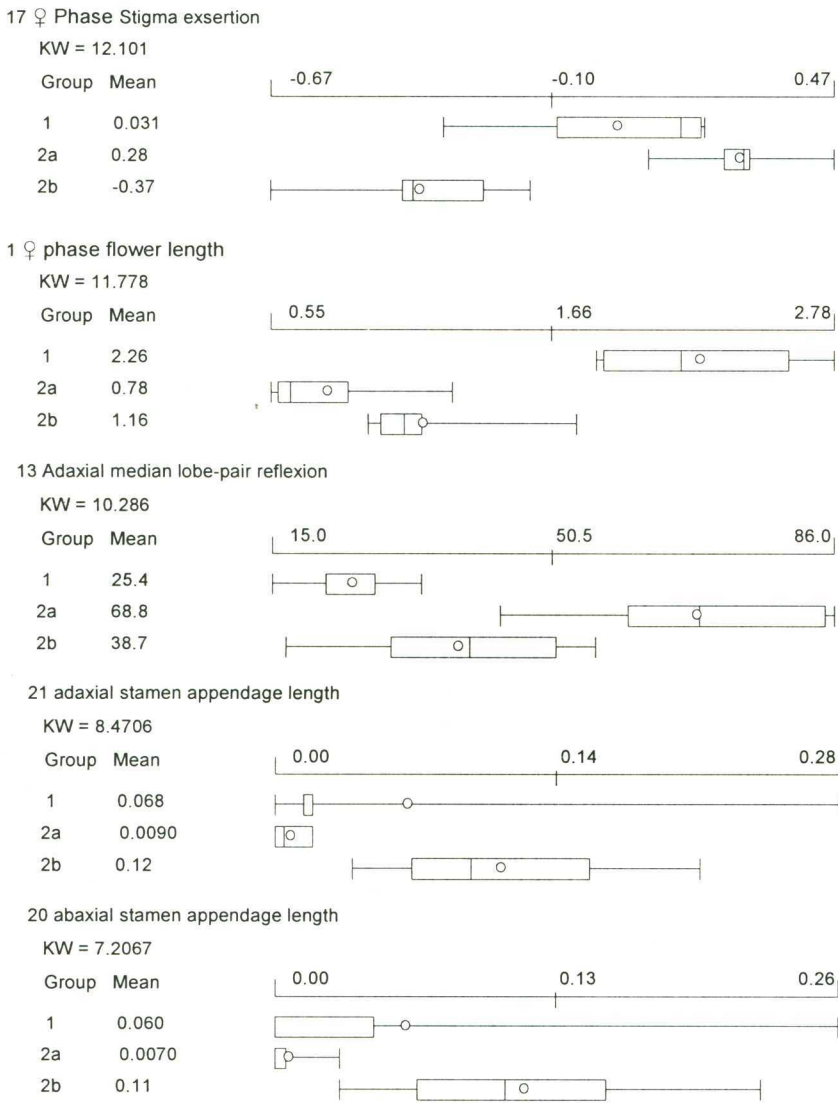


Figure 3.10. Box-whisker-plots from UPGMA analysis of the floral phenetic data from 17 species of *Prostanthera*. Data excludes characters from male stage flowers. Five characters discriminate group 1, group 2a, and group 2b. Characters are ranked by Kruskal-Wallis values from highest (top) to lowest (bottom)

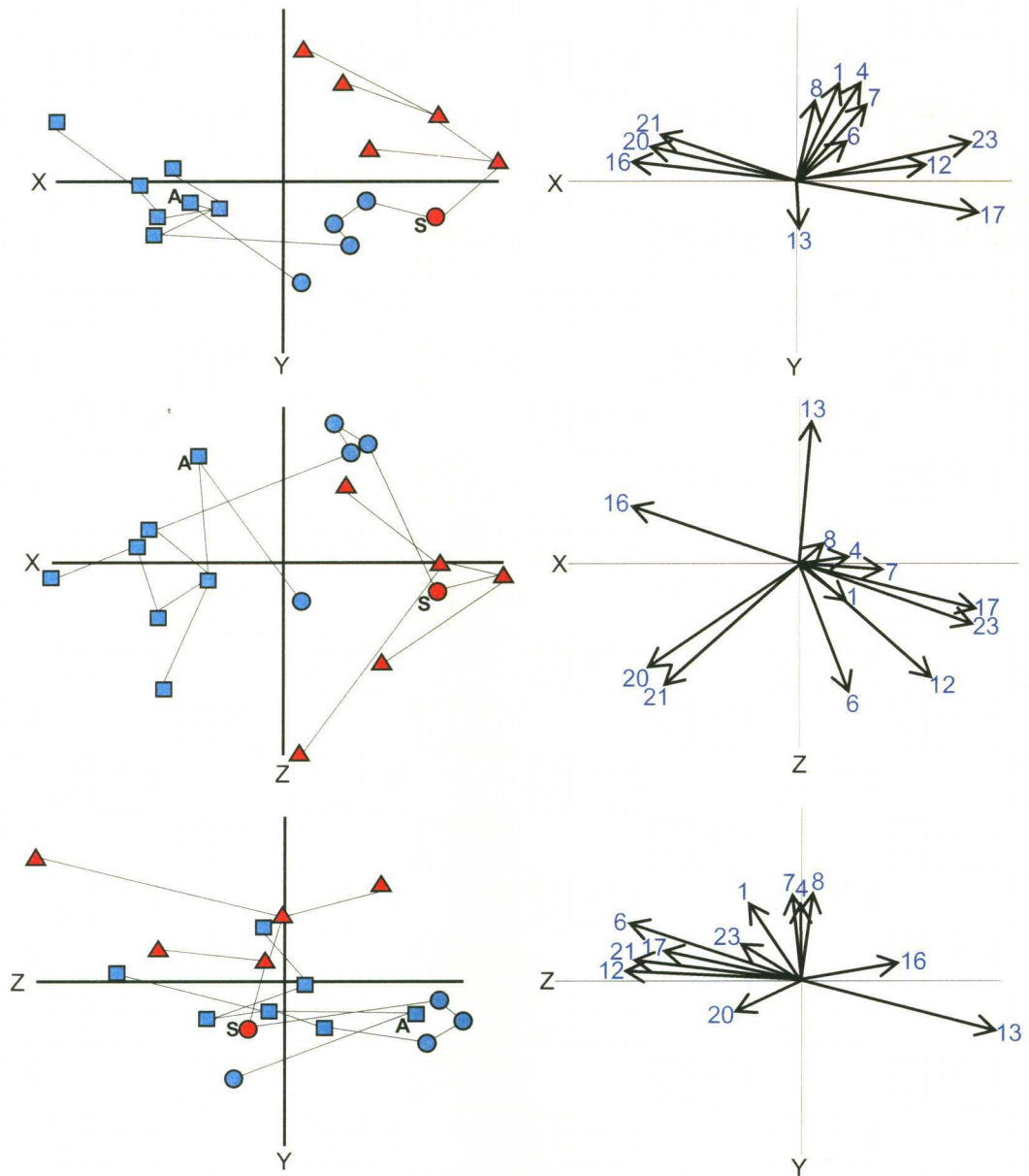


Figure 3.11. Pair-wise plots of the first three dimensions recovered from SSHMDS of the floral phenetic data from 17 species of *Prostanthera* (stress = 0.080). Data excludes male flower stage characteristics. Character numbers from Table 3.2 are used in the vector diagrams. *Prostanthera serpyllifolia* is marked with an ‘s’ and *P. staurophylla* is marked with an ‘A’. Symbols represent cluster membership derived from flexible UPGMA (Fig. 3.9) and colours represent sectional classification. Triangle= group 1; circle= group 2a; squares= group 2b; red = *Prostanthera* section *Klanderia*; blue = *Prostanthera* section *Prostanthera*.

Geometric data

The landmark data for all 17 species of *Prostanthera* is listed in Appendix 3.3. Two groups are recovered in relative warps analysis along relative warp one (RW1) (Fig. 3.12). The cohesiveness of these two groups is reinforced by the minimum spanning tree (MST) connections. *Prostanthera monticola* and *P. lasianthos* form the connection between the two groups. Group 2 is separated into two subgroups (2a and 2b) along relative warp 2 (RW2). The MST connects subgroups from *P. sieberi* to *P. lasianthos*. Both relative warps account for 80% (67.6 and 12.4, respectively) of the total shape variation. Both relative warps produce deformations of flower shape when projected onto a thin-plate spline (Fig. 3.13). In the ordination, RW1 defines a shift in size proportion between the adaxial median lobe-pair and the lower lobes (lateral lobes and abaxial lobe) of the flower. Its negative deformation increases the size of the adaxial median lobe-pair and decreases the size of the lower lobes. Its positive deformation decreases the size of the adaxial median lobe-pair and increases the size of the lower lobes. Negative deformation of RW2 causes the lateral lobes and the tips of the adaxial median lobe-pair to stretch upwards, increasing the radial symmetry of the flower shape. Positive deformation of RW2 causes the lateral lobes and tips of the adaxial median lobe-pair to bend downwards, increasing the bilateral symmetry of the flower shape. The consensus shape of group 1 is horizontally compressed in relation to the consensus shape of either subgroup of group 2 (Fig. 3.14).

Cluster analysis recovers two groups at a dissimilarity value of 0.426 based on the combination of uniform component (warp 2) and non-uniform component (warps 4, 10, and 12) (Figs. 3.15 and 3.16A). Group 1 has larger values of warp 4, 10 and 12 whereas Group 2 has a larger value for warp 2 (Fig. 3.16A). Group 1 contains members of

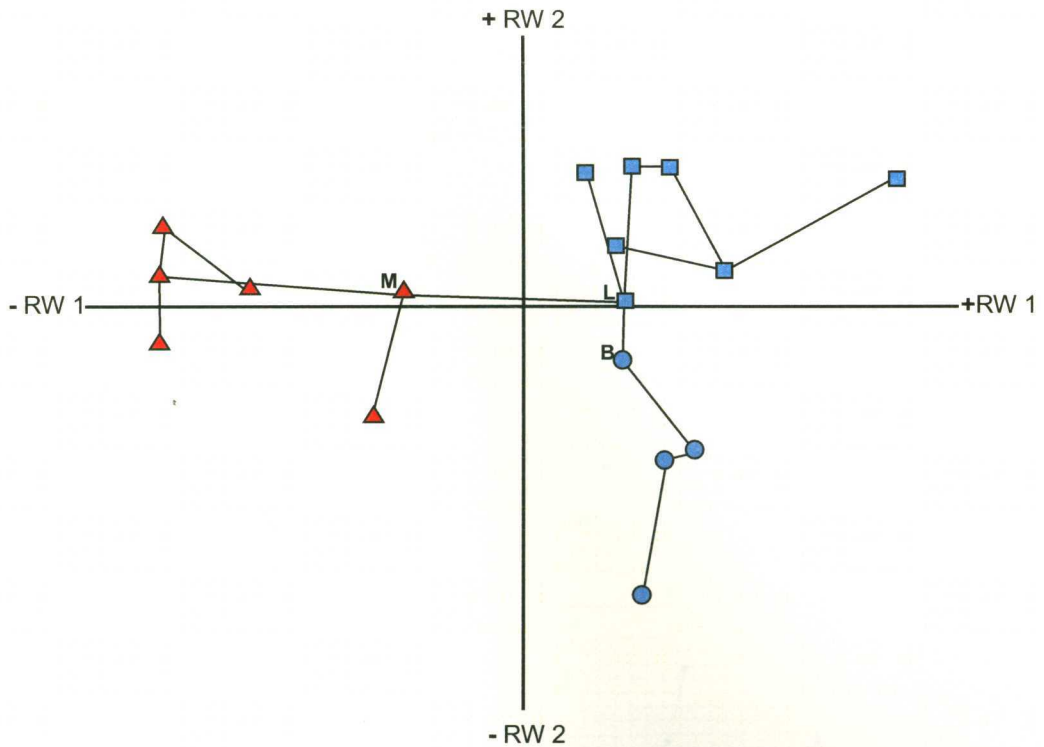


Figure 3.12. Ordination from relative warps analysis of the flower from 17 species of *Prostanthera*. Symbols represent cluster membership derived from flexible UPGMA of phenetic analysis (Figs. 3.4 and 3.9) and colours represent sectional classification. *Prostanthera lasianthos*, *P. monticola*, and *P. sieberi* are marked with an 'L', 'M', and 'B' respectively. See Figure 3.13 for the corresponding shape deformations represented by relative warp 1 (RW1) and relative warp 2 (RW2). Triangle= group 1; circle= group 2a; squares= group 2b; red = *Prostanthera* section *Klanderia*; blue = *Prostanthera* section *Prostanthera*.

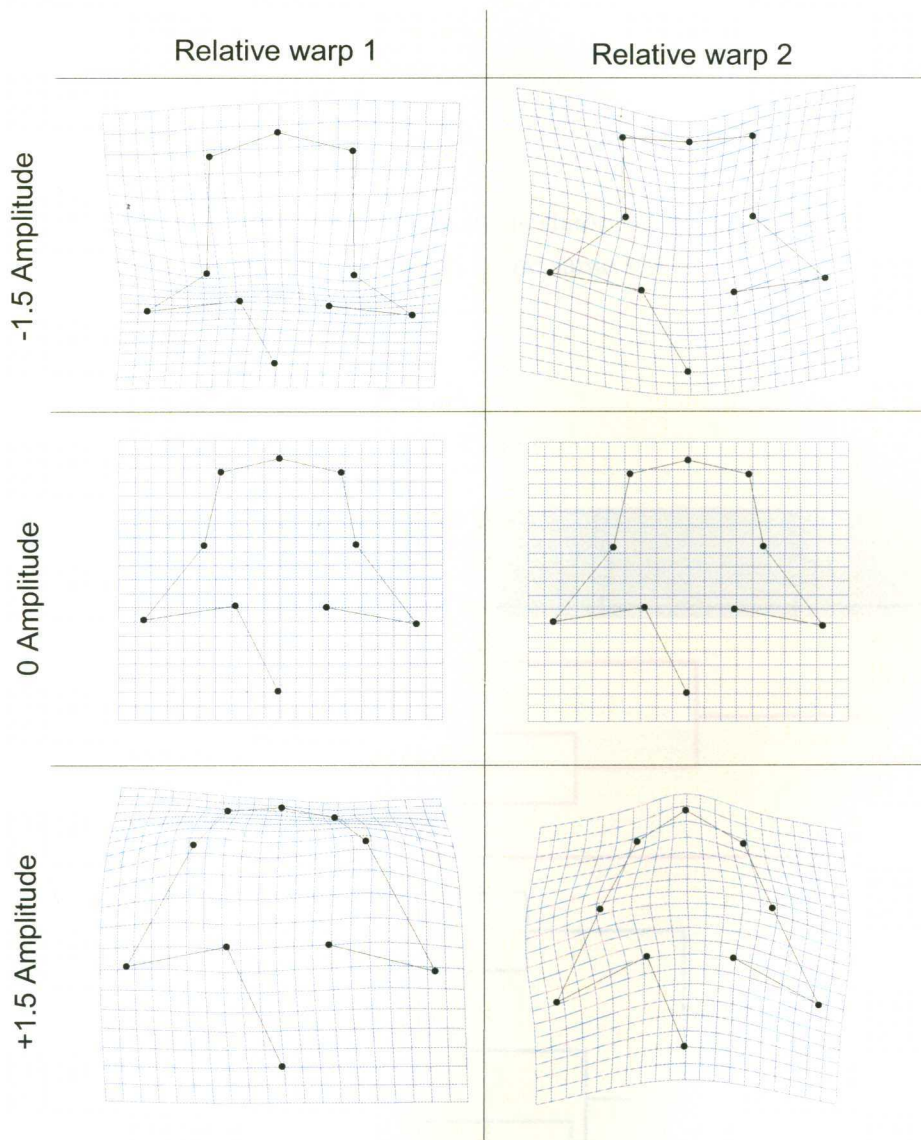


Figure 3.13. Thin-plate splines demonstrating the positive and negative amplitude deformations from relative warps analysis. Relative warp one stretches (-1.5 amplitude) or contracts (+1.5 amplitude) the shape of the flower constituting the adaxial median lobe-pair. Relative warp two bends the shape of the flower constituting the lateral lobes downwards (-1.5 amplitude) or upwards (+1.5 amplitude).

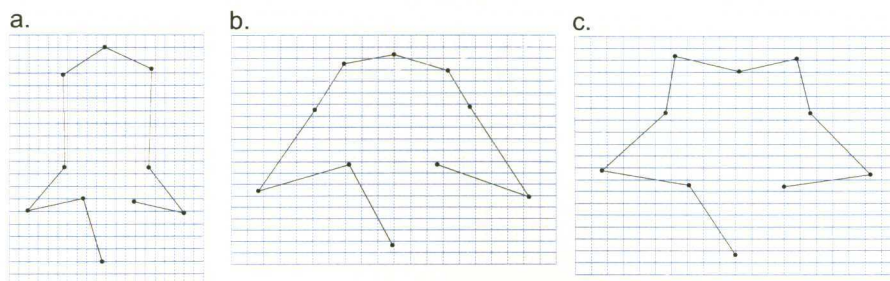


Figure 3.14. Thin-plate splines of the consensus shape for (a.) group 1, (b.) group 2a, and (c.) group 2b. All shapes are illustrated at the same scale.

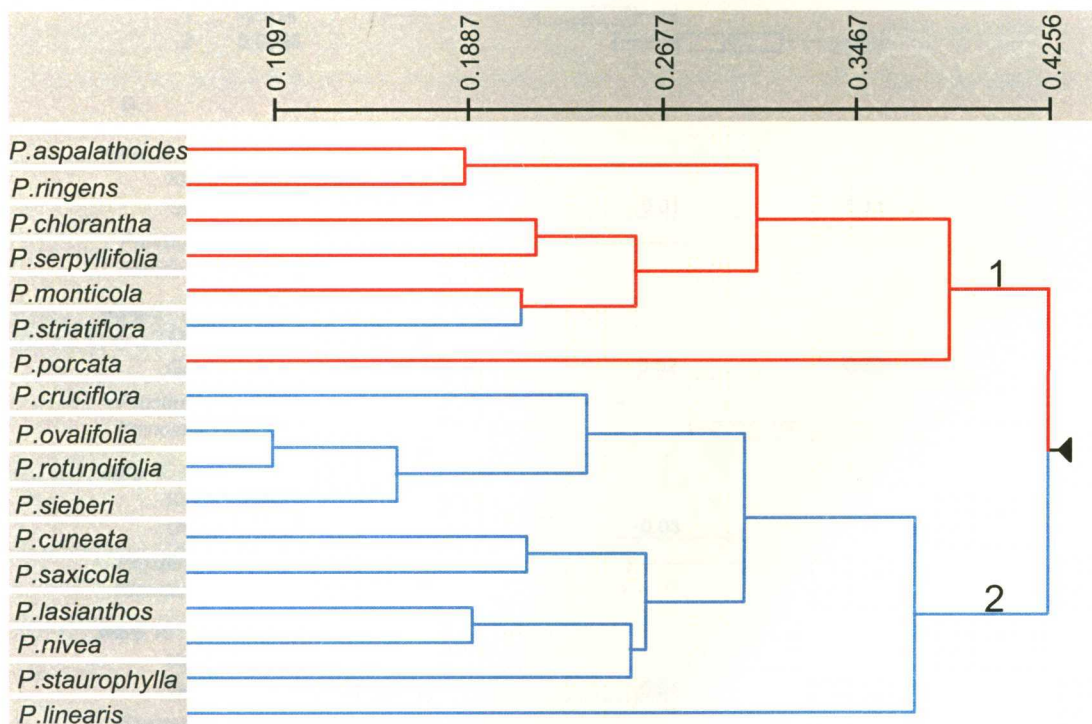


Figure 3.15. Dendrogram from the UPGMA analysis of the geometric data. Line colour represents sectional membership: red = section *Klanderia*; blue = section *Prostanthera*.

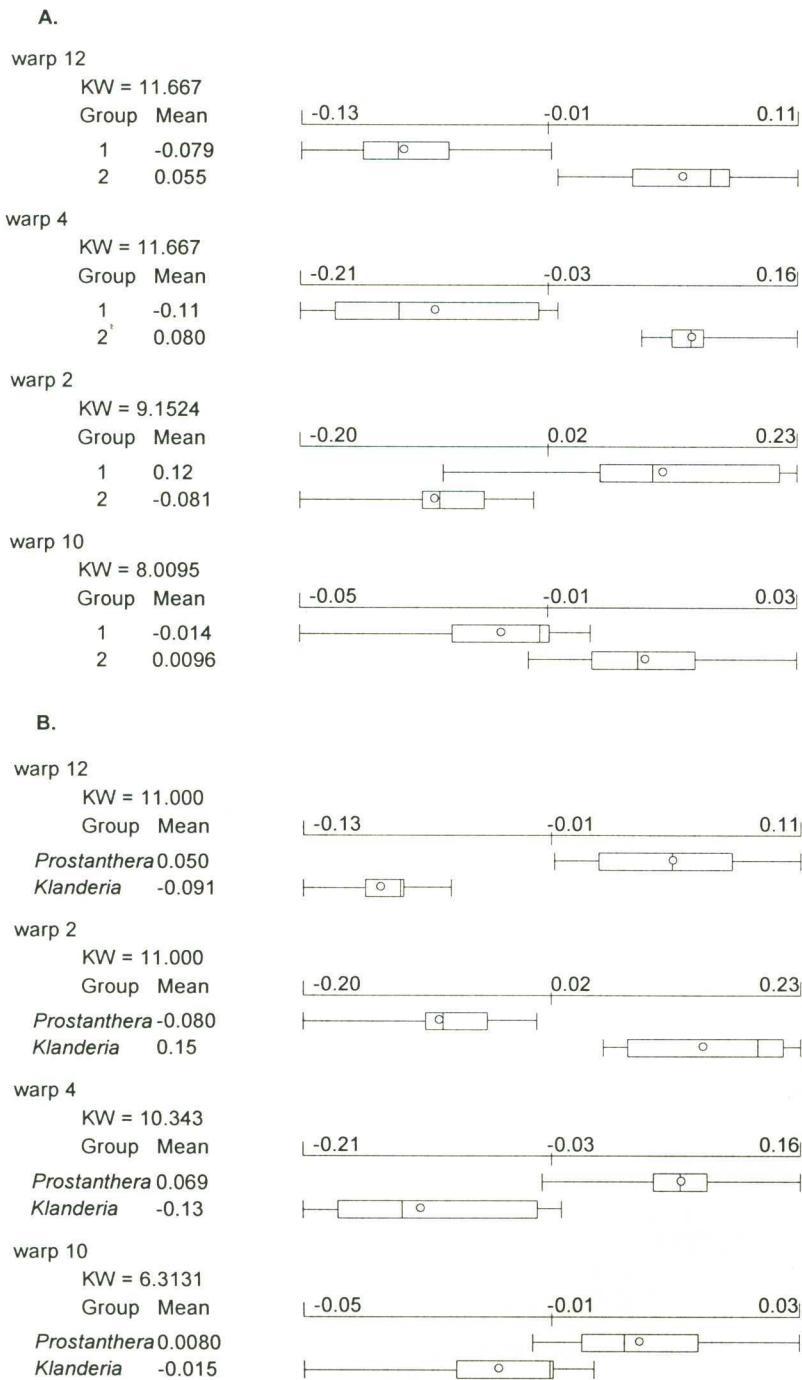


Figure 3.16. Box-whisker-plots from UPGMA analysis of geometric data. Four warps discriminate (A) group 1 and group 2, or (B) section *Klanderia* and section *Prostanthera*. Characters are ranked by Kruskal-Wallace values from highest (top) to lowest (bottom).

section *Klanderia* plus *P. striatiflora* and group 2 contains the remaining members of section *Prostanthera*. The groups remain significantly different if *P. striatiflora* is placed with section *Prostanthera* (ANOSIM: best-fratio= 1.28 < real f-ratio= 1.34) and there is no change in discriminatory characters (Fig. 3.16B).

SSHMDS recovers two groups similar in composition to those recovered in the UPGMA dendrogram (Fig. 3.15). The four characters (warps 2, 4, 10, and 12) have high r^2 values that are significant (MCAO, $p < 0.05$) (Appendix 3.2, Analysis 3). The two groups are linked by MST through *P. monticola* and *P. sieberi* (Fig. 3.17).

Discussion

The purpose of this study was to define measurable characteristics of pollination syndromes by identifying the differences between floral characteristics of groups within *Prostanthera*. Multivariate analysis and relative warps analysis confirm that ornithophilous floral characteristics separate section *Klanderia* from section *Prostanthera*. Two distinct flower morphologies within section *Prostanthera* are identified as having syndromes of melittophily or general entomophily.

Interpretation of phenetic analysis

Cluster analysis initially recovered two groups similar in membership to section *Prostanthera* and section *Klanderia*. An exception is that it recovered *P. serpyllifolia* with the section *Prostanthera* in group 2 rather than with section *Klanderia* in group 1. This is because the flower of *P. serpyllifolia* is smaller than any other species of section *Klanderia* (Conn 1984); characters such as shorter length and weaker curvature place it

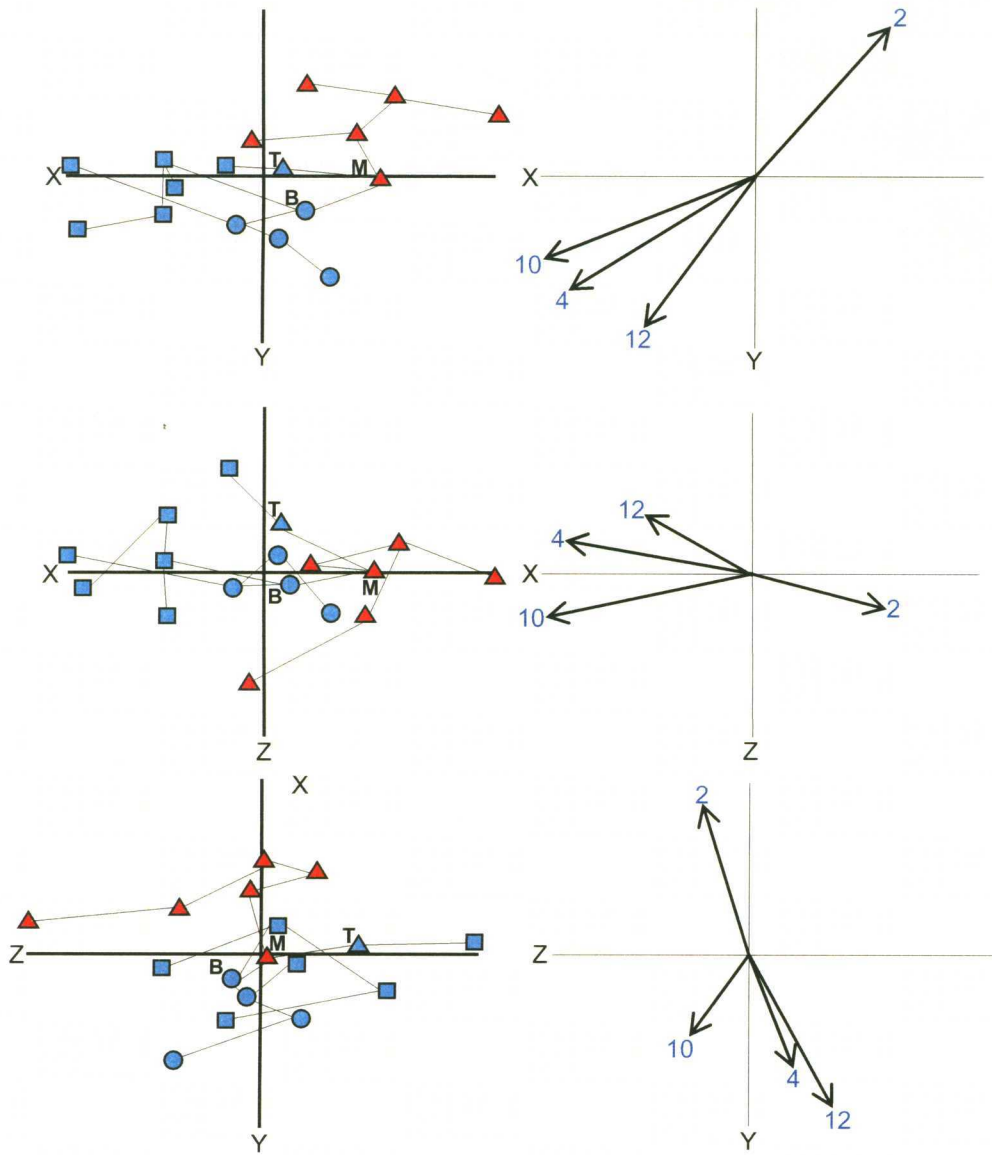


Figure 3.17. Pair-wise plots of the first three dimensions recovered from SSHMDS of the geometric data without male flower stage characteristics (stress= 0.1464). Character numbers from Table 3.2 are used in the vector diagrams. Symbols represent cluster membership derived from flexible UPGMA (Fig. 3.9) and colours represent sectional classification. *Prostanthera monticola* and *P. striatiflora* are marked with an ‘M’ and an ‘T’, respectively. Triangle= group 1; circle= group 2a; squares= group 2b; red = *Prostanthera* section *Klanderia*; blue = *Prostanthera* section *Prostanthera*.

amongst section *Prostanthera* (Fig. 3.5). In the SSHMDS plot, however, it is situated between the clusters of both sections and therefore its membership could also be part of section *Klanderia*. If analysis is conducted with it placed within section *Klanderia* then the significance between groups is retained. Compared to a flower of section *Prostanthera*, a flower of section *Klanderia* is longer, reflects longer wavelengths of light, and has a narrower corolla tube. The flowers of section *Prostanthera* have shorter corollas that reflect shorter wavelengths of light and the corolla tube is wider.

Cluster analysis also recovers two subgroups within section *Prostanthera*. Compared to a flower of group 2b, the flower of group 2a has a shorter corolla, abaxial lobe, and anther appendages, and has a more exerted stigma. *Prostanthera staurophylla* changes membership if the male flower characteristics are excluded from the analysis. In this case, abaxial lobe length does not distinguish flowers of either group. Instead, a group 2a flower has a more reflexed adaxial median lobe-pair. This group membership appears more accurate since *P. staurophylla* is situated closer to group 2b in both SSHMDS plots (Figs. 3.8 and 3.11). Between group 2a and group 2b there is a large difference in the adaxial median lobe-pair reflexion for the female stage since flowers in the early anthetic male stage are shown to be porrect. The degree of reflexion of *P. cruciflora*, *P. ovalifolia*, *P. rotundifolia*, and *P. sieberi* is much larger than the other species when in the female stage (compare Fig. 5.4a and Fig. 5.4i for an example).

Interpretation of geometric analysis

Relative warps ordination produces two groups corresponding to section *Klanderia* and section *Prostanthera*. OTUs of section *Klanderia* cluster in the negative scale of RW1. As demonstrated on the thin-plate splines of figure 3.13, negative amplitude of relative

warp one causes a deformation of the flower shape to increase the size of the adaxial median lobe-pair compared to the lower lobes. Conversely, the OTUs of section *Prostanthera* cluster in the positive scale of RW1. Therefore their flowers have larger lower lobes compared to their adaxial median lobe-pair.

A greater size of the adaxial median lobe-pair in the geometric analysis corresponds to the greater length of the adaxial median lobe-pair measured in the phenetic analysis. A greater size of the abaxial lobes in the geometric analysis, however, does not correspond to any change in length of the abaxial lobe in the phenetic analysis. Instead, a decrease in abaxial lobe size corresponds with an increase in abaxial lobe reflexion: reflexion can change the two-dimensional size of a shape when orientation is taken into consideration. Rather than a change in size, the appearance of small lower lobes is due to the reduction in size of the outline. This phenomenon has not been encountered before since previous shape analyses have interpreted shapes without great 3-D complexity (e.g. Rohlf 1993; Swiderski 1993; Marcus *et al.* 2000; Jensen *et al.* 2002; Neustupa and Němcová 2007). This presents a limitation to the choice of subjects relative warps analysis can handle until cost and speed of producing 3-D landmark files becomes sufficiently smaller.

The relative warps ordination also produces two clusters that match group 2a and group 2b recovered from phenetic analysis. The flower of group 2a has a more radially symmetric shape whereas the flower of group 2b has a more bilaterally symmetric shape. Part of the radial symmetry can be explained by the upward shifting of the adaxial median lobe-pair. Much like the change in size of the abaxial lobes between

each section of *Prostanthera*, the increased size of the adaxial median lobe-pair is explained by phenetic analysis as an increase in reflexion rather than length.

The results of UPGMA analysis and SSHMDS on the shape component are similar to the results using phenetic data as they confirm both sections of *Prostanthera* are significantly different and they form similar clusters in multivariate space. Since warp 2 is a component of the uniform shape it represents uniform stretching of the shape. In this analysis it describes that width and height ratio is significantly different between group 1 and group 2, which can be interpreted as similar to the decrease in width described by the phenetic analysis. A comparison of the consensus shape (Fig.3.14) of each group demonstrates this change in width.

A limitation of interpreting warps analysis is that the ordination compares the two highest ranking shape changes. Although the above changes represent a large portion of the shape variation amongst *Prostanthera*, 20% of this change cannot be compared. Important biological shape changes might not be correlated with large changes in shape. Therefore, it is possible that relative warps analysis might not be able to indicate the most important biological changes in shape.

Interpretation of pollination syndromes

The consensus of all analyses of floral characters describes that the difference between section *Klanderia* and section *Prostanthera* is based on the difference between ornithophilous and entomophilous characters, respectively. Red colouration, a long and narrow floral tube, and a reflexed lower lobe are typically associated with ornithophilous flowers and are thought to have evolved as preventative mechanisms for

insect visitors (Faegri and Van der Pijl 1979; Proctor *et al.* 1996; Schemske and Bradshaw 1999; Temeles and Rankin 2000; Castellanos *et al.* 2004; Cronk and Ojeda 2008). For example, red colours are less obvious to an insect, abaxial lobe reflexion prevents insects from landing while it improves the fit of a birds' head, a long and narrow tube exceeds the length of unspecialised insect mouthparts and prevents an insect from reaching the floral nectary (Raven 1972; Faegri and Van der Pijl 1979).

Blue or white colours, shorter and wider floral tubes, and the presence of a large lower abaxial lobe are flower characters that are identified as entomophilous. These are found in all the species of section *Prostanthera* of this study. Within this group, the floral diversity suggests there is a scale within the generalist pollination syndrome. The results from phenetic data demonstrate that group 2a has more exserted stigmas, a shorter flower length, a more reflexed adaxial median lobe-pair, and a shorter appendage length than group 2b. This seems to be described in part by geometric analysis that shows that group 2a is more radially symmetric and group 2b is strongly bilaterally symmetric. The stigma becomes exserted in group 2a due to the shortening of the flower length. Yet the anthers do not become exserted meaning that they are held closer to the gynoecium at the base of the flower. The consensus of all analyses of floral characters describes that the difference between group 2a and group 2b is based on the difference between general entomophilous characters and melittophilous characters, respectively (Faegri and Van der Pijl 1979; Proctor *et al.* 1996; Neal *et al.* 1998; Ollerton *et al.* 2007; Westerkamp and Classen-Bockhoff 2007). Bilaterally symmetric flowers are strong visual cues for hymenopterans and a porrect adaxial median lobe-pair hides anthers from pollen collecting bees. Furthermore, melittophilous plants often have complex mechanisms to deposit pollen in a place where a bee cannot remove it

(Claßen-Bockhoff *et al.* 2003; Reith *et al.* 2006; Claßen-Bockhoff 2007; Westerkamp and Classen-Bockhoff 2007). This mechanism in group 2b appears to consist of a long floral tube that channels a visitor to brush past elongated anther appendages. The appendages might then disrupt the hidden anthers to release pollen on the dorsal surface of the visitor. The flowers of group 2a have more generalised characteristics such as a shallow, open corolla tube which is not restrictive to various body shapes or unspecialised mouthparts of insects. Furthermore, anthers are held close to the gynoecium so that all floral rewards are held together. This is likely so that anthers can contact a visitor regardless of which way it accesses the flower. This places anthers in an accessible place for insects to collect pollen. However, if the flower is a generalist, then a wide range of pollinators other than bees are less inclined to collect pollen.

The species of group 2a and group 2b form separate clades in the phylogeny of *Prostanthera* (Figs. 2.3-2.8). Group 2b species are found at the base of the *Prostanthera* clade which suggests that their floral morphology is the ancestral state. The derived clade (clade F) composed of group 2a species indicates that their morphology has evolved more recently. Reduced anther appendages of group 2a imply that the pollen depositing mechanism has changed corresponding with the accessibility of the flower. The anther appendage has possibly become vestigial since a visitor can manage to avoid triggering it in an open flower. In *Salvia* this has happened because changes in the corolla force the pollinator into a specific position (Walker *et al.* 2004; Wester and Claßen-Bockhoff 2006a; Walker and Sytsma 2007; Wester and Claßen-Bockhoff 2007). The trend in section *Prostanthera* is opposite since the anther appendage is reduced in a more cup-shaped flower. The anthers of these flowers, such as *P. sieberi*, have many short barbs (triangular trichomes) found at the tips of the reduced appendages as well as

at the base of their lobes (Conn 1984; Conn 1992a; Conn 1997; 2006; Hunter *et al.* 2006). The anther appendage mechanism might still function like those of group 2b flowers but only requires the contact of the head or mouthparts of an insect rather than a thorax or abdomen.

Despite these characters, caution still must be used in determining pollinators because many generalist flowers have been shown to be an ecological specialist on one particular pollinator (Ollerton *et al.* 2007). Like the flowers of section *Klanderia*, these flower types need to be quantified for pollinators in the field to properly assess pollination syndromes in *Prostanthera*.

Conclusions

This study demonstrates measurable differences in groups of *Prostanthera* that correspond with the typical characteristics of either ornithophily and entomophily. The floral variation within *Prostanthera* suggests one group is potentially melittophilous and another is suited to more general entomophily. It would be incorrect to conclude that these results alone satisfy our understanding of the reproductive biology of *Prostanthera*. To build on this, further work must demonstrate pollinators of each flower type presented here in order to more confidently understand the pollination of *Prostanthera*.

Chapter 4

Investigation of pollination syndromes in *Prostanthera*

Introduction

Two sections are recognised in the genus *Prostanthera*: section *Prostanthera* and section *Klanderia*. *Prostanthera* section *Prostanthera* is regarded as entomophilous because the taxa have white or mauve gullet-shaped flowers. *Prostanthera* section *Klanderia* is regarded as ornithophilous because the taxa have red or green flowers that have longer corolla tubes. This floral diversity is very similar to the many other entomophilous or ornithophilous groups found in the Lamiaceae (Conn 1984; Vogel *et al.* 1984; Conn 1988; Huck 1992a; Guerin 2005; Wester and Claßen-Bockhoff 2007). *Prostanthera lasianthos* is a typical example of section *Prostanthera* because its flower has a broad corolla tube, is white, and has nectar guides (Conn 1992a; Conn 1994). Molecular and morphological analyses in this study (Chapters 2 and 3) have confirmed that the affinities of this species are with other species of section *Prostanthera*. Field observations, however, contradict the expected pollination syndrome for *P. lasianthos* because they report consistent visitation by birds (Loyn 1985). The extent of pollinator observations in the rest of *Prostanthera* is limited to four species of section *Klanderia* that have been observed being visited by birds of the Meliphagidae and five species of section *Prostanthera* (including *P. lasianthos*) that have been observed being visited by bees and wasps (Conn 1984; Brown *et al.* 1997; Hingston and McQuillan 2000).

The pollination syndromes of *Prostanthera* were first proposed using herbarium specimens (Keighery 1980). This was part of a large scale estimate of pollination of the southwestern flora of Australia aimed at using floral characteristics to determine the pollination syndromes of over 3000 species. However, it was recently found that the typical traits associated with generalised pollination syndromes in Australian flora are unreliable predictors of floral visitors (Hingston and McQuillan 2000). Very few flowers were found to be exclusively ornithophilous and no entomophilous flowers were exclusive to a specialised suite of insects. A recent test of pollination syndromes on flora of other continents also do not support pollination syndromes (Ollerton *et al.* 2009a). Recent descriptions of pollination biology in some species provide other examples of exceptions to pollination syndromes. For example, the flowers of *Tritoniopsis revoluta* appear specialised on long-proboscid fly pollinators because their long floral tubes exclude all other insects. However, in absence of flies the accumulation of nectar up the floral tube attracts the short-tongued bee *Amegilla fallax* which pollinates the flower (de Merxem *et al.* 2009). Fenster *et al.* (2004) argue, however, that the integrity of generalised pollination syndromes may still be maintained despite these exceptions by recognising ‘functional groups’ of pollinators. These groups consist of suites of pollinators, taxonomically related or not, that exert similar selection pressures.

In this chapter, I examine whether the different flower types observed in *Prostanthera* covary with observed pollinators using the recent method of classifying pollinators into ‘functional groups’ (Fenster *et al.* 2004). This is done by quantifying pollinator visits and organising them into ‘functional groups’ based on an assessment of their

morphology and behaviour. This follows methods that categorise species which are observed to interact with flowers in a similar manner, but distinguish similar species if they manipulate the flowers differently (Wilson *et al.* 2004; Martén-Rodríguez *et al.* 2009). Methods for defining pollinator effectiveness in plants include quantifying visitor duration, visitor abundance, pollen removal or deposition, as well as fruit set (Ivey *et al.* 2003). Tierney and Gross (2001) observed that individuals of *P. junonis* Conn produced higher fruit set when plants were not protected from pollinators; however, the pollinators responsible for this could not be recorded. No other quantitative measures of pollinator effectiveness have been described for *Prostanthera*. This study describes a pollinator based on observation of pollen transfer, however, this is only an estimation until more rigorous tests of pollinator effectiveness (Ivey *et al.* 2003) can be conducted on the observed visitors.

A barrier for understanding pollinators of *Prostanthera* is that the pollination mechanism in the flower and its diversity has not been examined. The most detailed description of pollination has been given for species of section *Klanderia*. Species of this group have protandrous flowers and have anthers that are held in pairs above the throat of the corolla (Conn 1984; Huck 1992b). If the corolla is distorted or anther appendages are contacted, the anthers separate laterally and are lowered onto the visitor for pollen deposition. It is speculated that stigmas are receptive after they become exerted beyond the corolla and after the anthers dehisce (Conn 1984).

In order to distinguish pollinators from visitors, the pollination mechanism and floral phenology are described across the floral variation observed in *Prostanthera*. Using the

pollinator data, the relationships for *Prostanthera* are then examined in multivariate space to test the integrity of pollination syndromes.

Materials and methods

Specimens and sites

Phenology and visitation were recorded in field observations for 12 species of *Prostanthera* and one species of *Westringia* during 2007, 2008, or 2009 (Table 4.1).

Table 4.1 Species used for the observations of floral phenology and floral visitation. Total refers to the total number of visitor censuses made at a particular population. Asterisks identify the four species used for detailed study of phenology. Abbreviations: N.P.= National Park; N.S.W.= New South Wales; S.F.= State Forest; Vic.= Victoria.

Study Species	Study Populations	Voucher #	Sites	Total
<i>Prostanthera aspalathoides</i> A.Cunn.ex Benth.	Gubbatta N.R., NSW	NSW803944	2	2
	Taleeban N.R., NSW	NSW803918	2	2
	Hiawatha S.F., NSW	NSW803950	2	2
<i>P. cruciflora</i> J.H.Willis	Mount Kaputar N.P., NSW	NSW803951	4	6
	Boggabri, NSW	NSW844233	2	2
* <i>P. lasianthos</i> Labill.	Blue Mountains N.P., NSW	NSW799707	5	14
	Mt. Buffalo N.P., Vic.	NSW844202	2	2
<i>P. linearis</i> R.Br.	Brisbane Waters N.P.	NSW784600	3	5
* <i>P. monticola</i> B.J.Conn	Mount Buffalo N.P.	NSW803943	5	13
<i>P. nivea</i> Benth. var. <i>nivea</i>	Mt. Kaputar N.P., NSW	NSW803947	2	2
<i>P. porcata</i> B.J.Conn	Buddawang N.P., NSW	NSW779742	2	4
<i>P. ringens</i> Benth.	Pilliga S.F., NSW	NSW803945	2	2
<i>P. rotundifolia</i> R.Br.	Mt. Buffalo N.P., Vic.	NSW779804	2	2
<i>P. saxicola</i> var. <i>major</i> Benth.	Mt. Tomah Botanic Gardens	NSW784611	-	-
<i>P. saxicola</i> var. <i>montana</i> A.A.Ham.	Blue Mountains NSW	NSW803916	1	1
<i>P. serpyllifolia</i> (R.Br.)Briq.subsp. <i>serpyllifolia</i>	Pulletop N.R., NSW	NSW813913	2	2
* <i>P. sieberi</i> Benth.	Illawarra Escarpment Conservation-Area, NSW	NSW884227	2	3
	Royal N.P., NSW	-	1	1
<i>P. staurophylla</i> F.Muell.	Mt. Annan Botanic Gardens	NSW785873	-	-
* <i>P. striatiflora</i> F.Muell.	Mt. Annan Botanic Garden	NSW449546	-	-
<i>Westringia senifolia</i> F.Muell.	Mt. Buffalo N.P., Vic.	NSW799706	-	-

Four additional species were observed in cultivation at botanic gardens. Where possible, observations were collected from multiple locations, such that populations (a group of interbreeding individuals, Barbour *et al.* 1987)- could be considered independent. Unfortunately, poor knowledge of pollinators made it difficult to assess whether pollinators connect these populations during visits. Furthermore, many species of *Prostanthera* have limited geographic distributions which prevented sampling additional populations. Separate locations that are possibly connected by pollinators were still sampled but are hereby called sites. Some sites were recorded during different periods of the flowering season and/or in two separate flowering seasons. Observations of flower phenology were made on at least three different plants from each population.

Observation of phenology

Phenology was studied on four species that have different flower morphologies and represent the four regions identified in the molecular phylogeny of *Prostanthera* (Chapter 2). The observations that were recorded include anther position relative to corolla, corolla exsertion, style position relative to corolla, separation of stigma lobes, and the abaxial calyx lobe position relative to the adaxial calyx lobe. Observations were made for *P. striatiflora* (13-17/October/2007, n=54 flowers), *P. lasianthos* (15-17/December/2007 and 8/January/2008, n=18 flowers on each date), *P. monticola* (7-10/December/2007, n=42 flowers; 13-18/January/2008, n=11 flowers) and *P. sieberi* (17/October/2007, n=36 flowers). Photographs were taken of all developmental stages using a digital SLR camera (Canon EOS 30D) with a 35mm Canon Lens EF (1:2).

Stigmatic receptivity

Receptive stigmas are characterised by high enzymatic activity of peroxidases or esterases. The presence of peroxidase was detected by immersing a freshly picked stigma in 3% hydrogen peroxide (Herrera 1987; Kearns and Inouye 1993). A positive result is indicated by rapid bubbling from the stigmatic lobes (Fig. 4.1a). The presence of esterase was detected by immersing a freshly picked stigma in Fast Blue stain (Kearns and Inouye 1993). A positive result is indicated by dark red staining after five minutes (Fig. 4.1b). Both tests were conducted on three states of the stigma: a) lobes closed b) lobes partially recurved, and c) fully recurved.

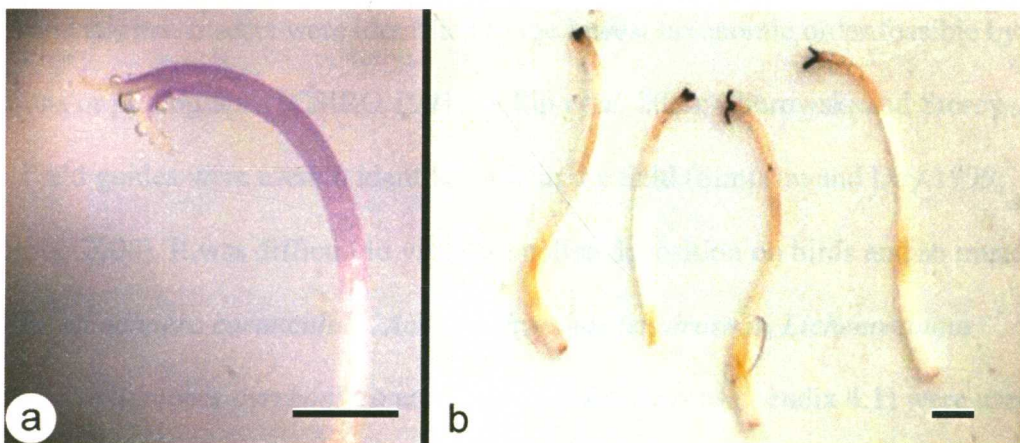


Figure 4.1. Images of stigmas tested for stigmatic receptivity (scale bar=500 μ m): (a) The stigma of *Prostanthera nivea* with fully recurved lobes immersed in peroxidase. (b) Stigmas of *P. linearis* treated with Fast Blue stain: left pair have partially recurved stigma lobes and the right pair have open stigma lobes.

Visitor censuses

A one-hour census nominated an observation patch that consisted of a target plant plus nearby individuals in line of sight. Insects were only recorded on the target plant in order to properly identify and monitor insect visitation. Birds were recorded over the entire patch since this method reduced the disturbance caused by the observer and it

increased the sampling for which bird visitation was quite often infrequent. Each site was observed for at least one full day period, which consisted of six evenly placed, one hour intervals. During summer day-lengths, a census consisted of 1) early morning (0600-0700 hours), 2) mid-morning (0900-1000 hours), 3) midday (1200-1300 hours), 4) mid-afternoon (1400-1500 hours), 5) late-afternoon (1700-1800 hours), and 6) night (2000-2100 hours).

A visit is here defined as any entry into the corolla tube or interaction with stamens or stigma. A visitor is estimated as a pollinator if it is able to visit male and female flower stages, if it has an appropriate surface to carry pollen, and if its surface can contact anthers and stigma. Insects were identified to the lowest taxonomic order feasible by collections or photographs (CSIRO 1991; Dollin *et al.* 2000; Zborowski and Storey 2003). Field guides were used to identify birds in the field (Simpson and Day 1999; Morcombe 2000). It was difficult to visualise pollen deposition on birds and so museum skins of *Anthochaera carunculata*, *Acanthorhynchus tenuirostris*, *Lichenostomus chrysopterus*, *Phylidonyris pyrrhoptera* and *Zosterops lateralis* (Appendix 4.1) were used to investigate pollen transfer in *P. monticola* and *P. lasianthos*. The head of each specimen was inserted into flowers at different stages and pollen deposition was observed.

A 4-point scale was implemented to categorise visitation numbers so that some species of *Prostanthera* with large numbers of flowers could be effectively monitored. The number of visits were pooled for all individuals of each species visiting a plant. No visitation is represented by 0, one visit is represented by 1, 2-10 visits are represented by 2, and 11+ visits are represented by 3. Observation data were transformed using a

value based on the number of observed plants so that the bird and insect visitation points were represented at a similar scale. This was done by dividing the bird visitation points by the number of plants observed in a patch. Afterwards, census data were pooled for each functional group and the data range was scaled to one. This was done by dividing the points scored by one functional group by the total points scored by all functional groups.

Environmental data were collected at 20 minute intervals and then averaged for a particular census. These data included percentage cloud-cover, percentage of direct sunlight cast on plant, temperature (°C), percentage of humidity, rain intensity (0, 1, or 2), and wind intensity (0, 1, or 2). Rain was assessed by no rain (0), light rain (1), or medium to heavy rain (2). Wind was assessed by the motion of the branches, and was scored as distal end of branches relatively still (0), shaking (1), or violently waving in the wind (2).

Statistical methods

Cluster analysis and semi-strict hybrid multidimensional scaling from PATN version 3.03 (Belbin and Collins 2004) were used to investigate patterns in visitation amongst species of *Prostanthera*. Visitor space was constructed using data from all visitors whereas pollinator space was constructed using data from visitors estimated to be capable of pollination. Two flower morphologies are identified within section *Prostanthera* (Chapter 3) and so additional analyses were conducted without section *Klanderia* in order to compare patterns of visitation with flower morphology.

Cluster analysis was conducted using the Bray-Curtis association measure and a flexible unweighted pair group method with arithmetic mean (UPGMA) sorting strategy with a beta value = -0.1. Bray-Curtis was chosen since many data entries contain at least one zero and it is a reputable method for use with ecological data (Beals 1984). Principal component correlation (PCC) was used to generate a regression line for each variable in multivariate space. The Kruskal-Wallis statistic, analysis of similarity (ANOSIM), and Monte-Carlo attributes in ordination (MCAO) were used to test the significance of *a priori* groups in the same fashion as described in chapter 3.

Fisher's exact test (SPSS version 9.2, SPSS Inc., Chicago, Illinois) was used to investigate if positive stigmatic receptivity tests were significantly different amongst the three states recognised for the stigma lobes. Kendall's Tau-b statistic was calculated to investigate whether there was association amongst these states and its significance test was calculated using 1000 replicates.

The remaining statistical analyses were conducted in PAST (Hammer *et al.* 2001). Bird observations were unexpected for *P. lasianthos*. Since different pollinators operate at different times or environmental conditions then a particular habitat might explain a shift in pollinator suites. Therefore the relationship amongst environment, time, and visitors were investigated using canonical correspondence analysis (Legendre and Legendre 1998). *Prostanthera lasianthos* was compared with *P. cruciflora*, *P. linearis*, and *P. sieberi* that all receive expected visitation rates for section *Prostanthera*. Night censuses were excluded from the analysis since most species did not receive visits at this time. Rain intensity was removed from the analysis of *P. sieberi* since it did not rain in any census. Functional groups that were not observed for a particular species

were excluded from the analysis. Permutation tests used 10000 iterations to test the significance of the relationship between time and visitor.

Flower number

Flowers were quantified for a comparison amongst species of *Prostanthera*. The total number of flowers for an individual was estimated by multiplying the flower count of a branch by a multiplication factor. The flower count of a branch was acquired by counting the number of flowers in anthesis on a branch that represented a similar state of flowering of the entire individual. The multiplication factor is the estimated number of these branches that are needed to compose the flower portion of the individual.

Nectar collection

The amount of nectar resources were quantified for a comparison amongst species of *Prostanthera*. The ‘standing crop’ of a flower was used to measure the nectar encountered for a particular pollinator at a particular time (Possingham 1989; Kearns and Inouye 1993). Nectar was collected using micro-capillary tubes (Microcaps[®], Drummond Scientific Co.). At least three flowers were sampled from at least two plants of each population. Where possible, nectar was collected between 0700 and 0900 hours from an equal number of flower stages that are described in the results.

Results

Floral development

i) *Prostanthera striatiflora*

The duration of anthesis measured for 19 flowers was a mean of 3 days (min= 2 days, max= 6 days). The unopened flower bud is demonstrated in Figure 4.2a. Inside the unopened corolla, the abaxial anthers are tightly appressed to each other, and are distal to the appressed adaxial pair of anthers (Fig. 4.2b). A shallow 'groove' is formed between the anthers of each pair. The decurved style rests on top of the anthers within this groove and it ends in an unopened (non-receptive), bilobed stigma. The epidermal surface of the stigma lobes appears smooth under 10X magnification.

At early anthesis, the corolla is white and the throat has yellow spots that cover the lower throat and burgundy striations that surround the rest of the throat (Fig. 4.2c). Anther pairs are located adjacent to the adaxial median lobe-pair and their appendages point downwards into the throat of the flower (Figs. 4.2c and 4.2d). Each anther is dehiscent and is capable of shedding pollen if it is separated from its partner. The style presses against the adaxial median-lobe pair and nectar can be found at the base of the ovary.

During mid-anthesis, anthers senesce by first changing from purple-blue to faded brown (compare abaxial and adaxial anther pairs in Figs. 4.2d and h). The abaxial anthers senesce first and then separate and are translocated horizontally to the side of the corolla tube. The adaxial anthers undergo the same process while the style begins to decurve (Fig. 4.2d).

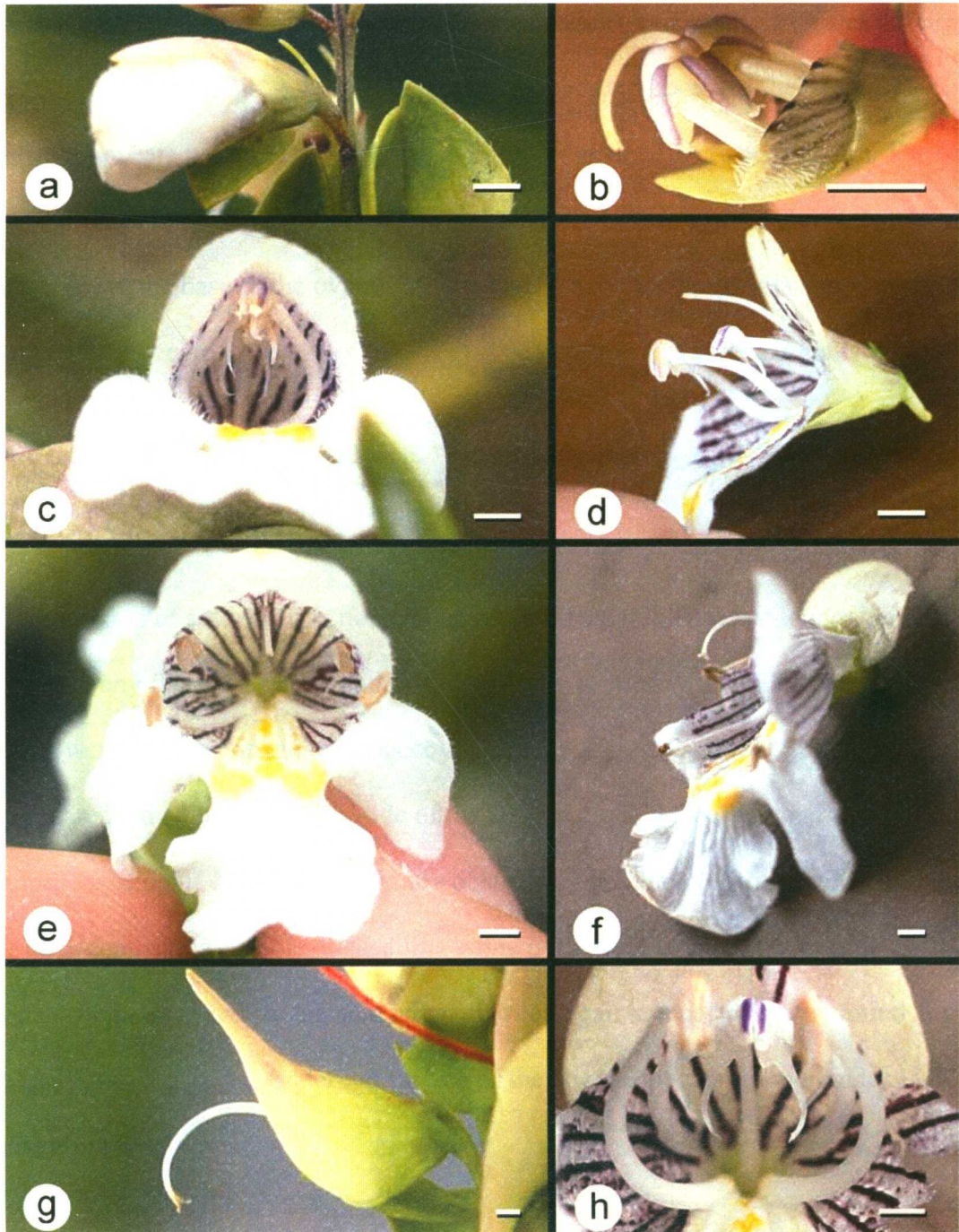


Figure 4.2. *In vivo* (left column) and dissected (right column) photographs of the development of a *Prostanthera striatiflora* flower. Scale bar=2mm. (a), flower bud in profile; (b), flower bud in profile with corolla removed; (c), apical view of a flower in male stage; (d), profile view of a flower in male stage with corolla removed; (e), apical view of a flower in female stage; (f), profile view of a flower in female stage with corolla partially removed; (g), profile view of a flower after having abscised its corolla; (h), apical view of a flower in transition between male and female stage. The two distal anthers have begun to separate.

In late anthesis the anthers are relocated, the corolla is maximally open and the stigma is fully decurved (Figs. 4.2e and 4.2f). Stigma lobes are open and their epidermal surface appears wrinkled or warty when observed under 10X magnification. The stigma is positioned approximately where the anthers were held in early anthesis. Nectar is observed at the base of the ovaries. At the end of anthesis the corolla abscises and the stigma lobes begin to senesce (Fig. 4.2g). The abaxial calyx lobe gradually bends to press against the adaxial calyx lobe.

ii) *P. lasianthos*

The duration of anthesis was not quantified, however it did not last longer than seven days for any flower. At early anthesis, the corolla is white with yellow spots covering the lower throat and magenta spots covering the surrounding throat (Fig. 4.3a). Anther pairs are held adjacent to the inside of the adaxial median lobe-pair and anther appendages point downwards partially obstructing the throat of the corolla. Each anther is dehiscent and is capable of shedding white pollen if it is separated from its partner. The style presses against the adaxial median-lobe pair and nectar can be found at the base of the ovary. The epidermal surface of the stigma appears smooth under 10X magnification.

During mid-anthesis, anthers of *P. lasianthos* begin senescing by changing from purple-blue to faded brown. The abaxial anthers senesce first and separate horizontally until each one is located laterally on a side of the corolla throat (Fig. 4.3b). The adaxial anthers undergo the same process and the style begins to decurve after the abaxial anthers have translocated.

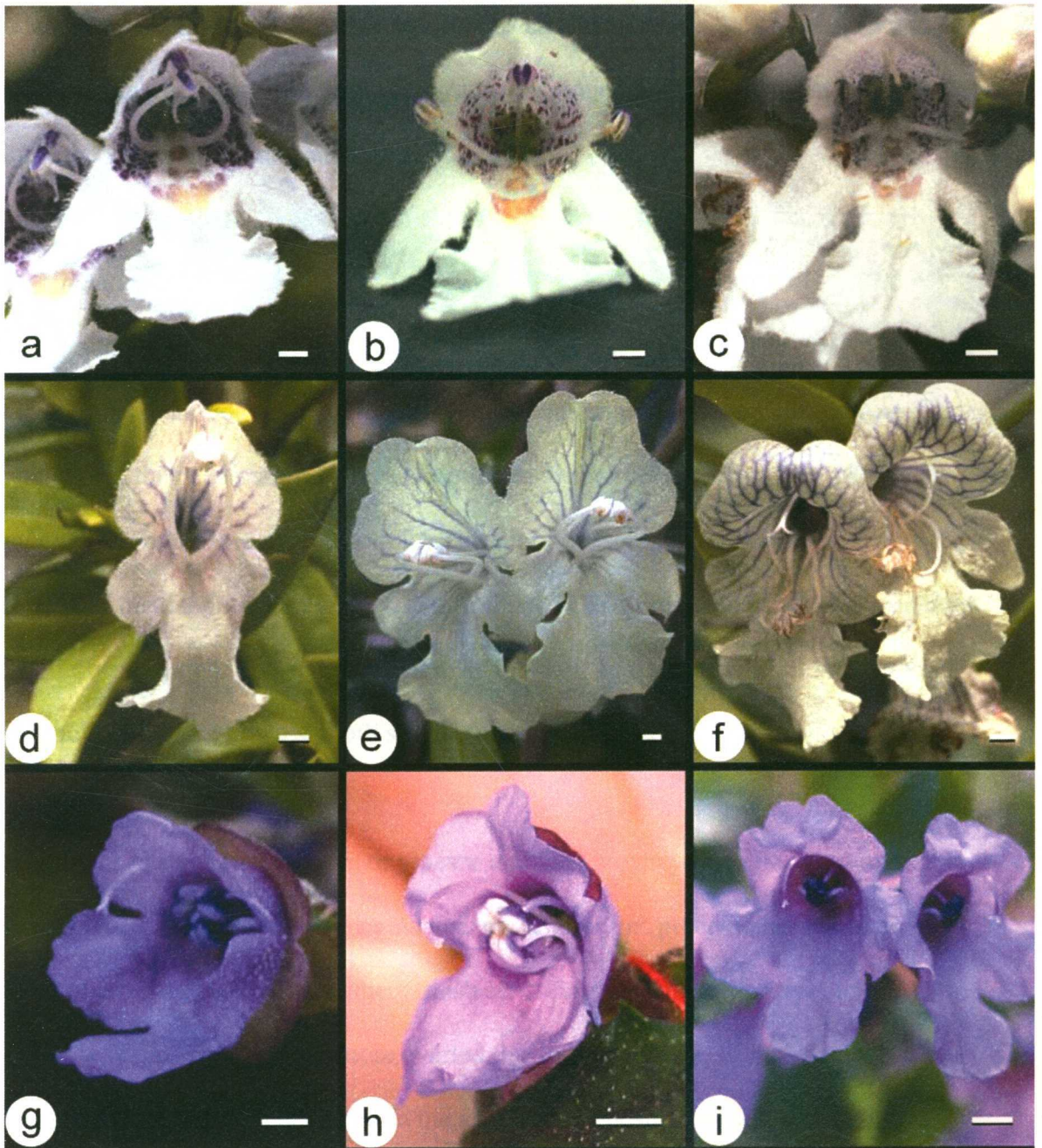


Figure 4.3. Photographs of male, hermaphroditic, and female developmental stages of *P. lasianthos*, *P. monticola*, and *P. sieberi* flowers. Scale bar=2mm. (a), *P. lasianthos* in male stages; (b), *P. lasianthos* in hermaphroditic stage; (c), *P. lasianthos* in female stage; (d), *P. monticola* in male stage; (e), *P. monticola* in hermaphroditic stage; (f), *P. monticola* in female stage; (g), *P. sieberi* in male stage; (h), *P. sieberi* in hermaphroditic stage; (i), *P. sieberi* in female stage.

At late anthesis, the straightened style is decurved and its stigma lobes are open and their epidermis appears wrinkled. The style is positioned in the approximate position to where the anthers were originally held (Fig. 4.3c).

iii) *P. monticola*

The duration of anthesis for any flower did not exceed seven days. The corolla varies in colour from cream-coloured to greenish yellow and has purple-blue veins that are less distinct near the petal margins. In early anthesis, the corolla is narrow and tubular due to the porrect adaxial median lobe-pair. The adpressed anther pairs are held adjacent to the adaxial median lobe-pair (Fig. 4.3d). Anthers are dehiscent and capable of shedding if partially separated. The style is straight and presses against the adaxial median lobe-pair and is terminated by a smooth stigma (under 10X magnification).

At mid-anthesis all anthers begin to senesce and fade brown while relocating to the lower lip, (Fig. 4.3e). The style begins to decurve after this process and by late anthesis the corolla stops opening, the anthers finish senescing, and the style stops curving (Fig. 4.3f). The stigma now appears more exerted due to the reflexion of the adaxial median lobe-pair. The stigma is positioned in the approximate position to where the anthers were held and its lobes have a wrinkled epidermis.

iv) *P. sieberi*

The duration of anthesis for any flower did not exceed seven days. At early anthesis the corolla and gynoecium are mauve and the anthers are usually dark purple (Fig. 4.3g). Anthers are held in pairs and their appendages point towards the ovary. They are

dehiscent and shed pollen if they are dislocated from their partner. The style is decurved and extends past the anthers and terminates in an unopened stigma.

During mid-anthesis anthers begin to senesce and fade to brown. The stigma becomes more exerted as the lobes of the corolla gradually reflex.

At late anthesis, the corolla is fully reflexed and the anthers have senesced but their position remains unchanged (Fig. 4.3i). The two lobes of the stigma are opened and their epidermal surface appears wrinkled (under 10X magnification).

v) *Prostanthera* spp. and *Westringia senifolia*

Flowers of *P. cuneata*, *P. linearis*, *P. nivea* var. *nivea*, *P. saxicola* var. *major*, *P. saxicola* var. *montana*, and *P. staurophylla* demonstrate similar early and late anthetic stages as reported in *P. striatiflora* or *P. lasianthos* (Fig. 4.4a, 4.4c, and 4.4e). Flowers of *P. aspalathoides*, *P. porcata*, *P. ringens*, and *P. serpyllifolia* have similar early and late anthetic stages as reported in *P. monticola* (Fig. 4.4b, 4.4d, and 4.4f). All species except *P. porcata* have extremely reduced anther appendages. The female stage of *Prostanthera aspalathoides* has a stigma that is exerted much further than the adaxial median lobe-pair (Fig. 4.4f). Flowers of *P. rotundifolia* demonstrate early and late anthetic stages as reported in *P. sieberi*. (Fig. 4.4i).

The flowers of *P. cruciflora* and *W. senifolia* have white corollas with yellow and orange markings on their lower lip (Fig. 4.4g, 4.4h, and 4.4j). The inner surface of the corolla throat and all anthers of *P. cruciflora* are pink whereas *W. senifolia* has an adaxial pair of blue anthers and an abaxial pair of white (sterile) staminodes. In early

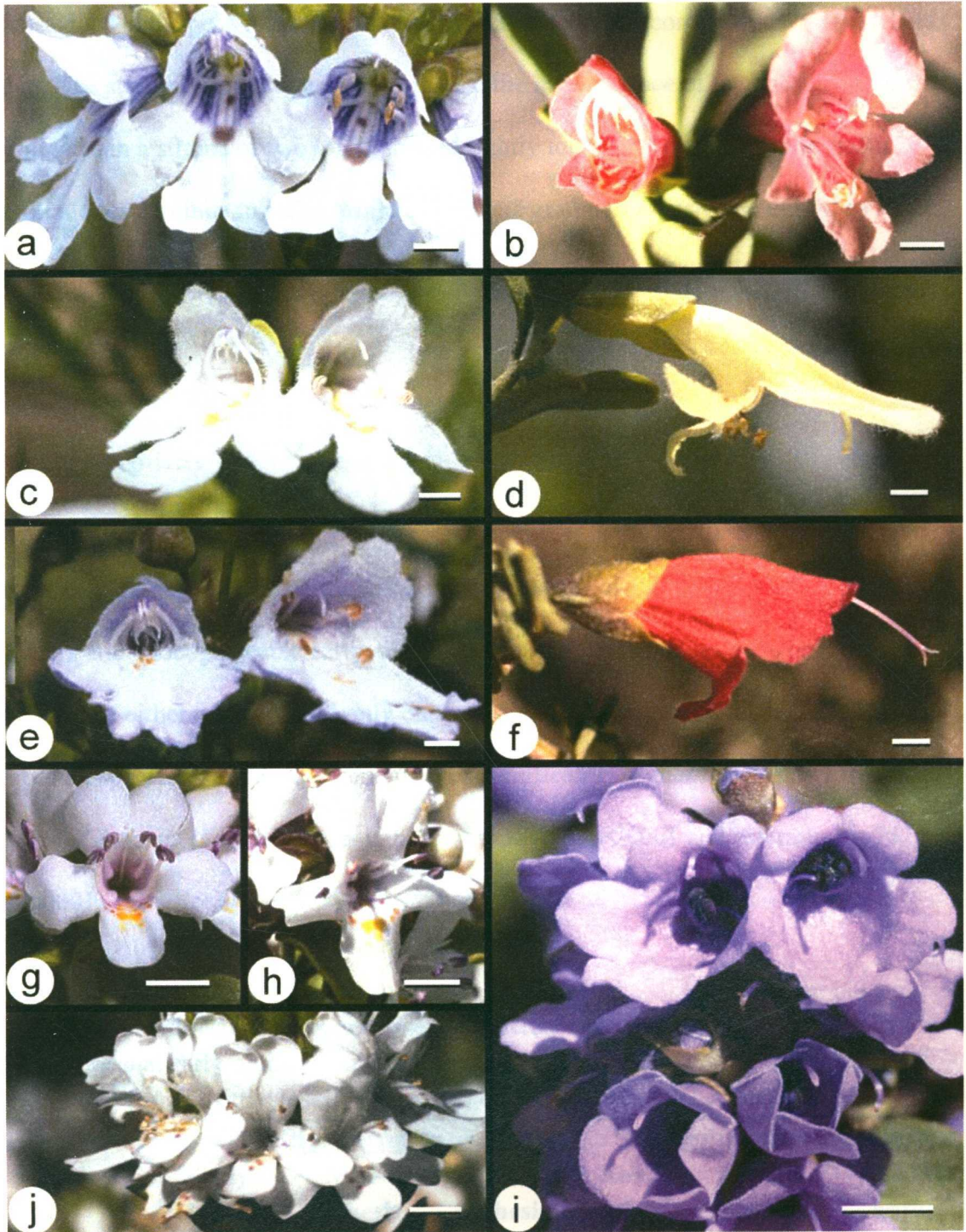


Figure 4.4. Photographs of *Prostanthera* flowers. Scale bar= 4mm. (a), male (left) and female (right) flower of *P. saxicola*; (b), male (left) and female (right) flower of *P. porcata*; (c), male (left) and female (right) flower of *P. nivea*; (d), female flower of *P. ringens*; (e), male (left) and female (right) flower of *P. linearis*; (f), female flower of *P. aspalathoides*; (g), male flower of *P. cruciflora*; (h), female flower of *P. cruciflora*; (i), male (below) and female (above) flowers of *P. rotundifolia*; (j), female flowers of *W. senifolia*.

anthesis for both species, anthers are held exerted from the corolla throat and are held apart. The straight white style presses against the inner surface of the corolla and it terminates in a stigma of two lobes pressed tightly together. Until late anthesis, the corolla lobes continue to reflex while the anthers gradually translocate until they lie next to the corolla lobes. The style also becomes decurved and the two stigma lobes separate from each other.

Female receptivity

A total of 219 flowers were tested for peroxidase and 62 flowers were tested for esterase on 14 species of *Prostanthera* and one species of *Westringia* (Appendix 4.2). Flowers at the recognisable early, middle, and late anthetic stages were tested. Positive reactions of either test appeared only on the inside of the stigmatic lobes. Fisher's exact test of all species in either test demonstrates significantly higher receptivity for later stages of anthesis (Table 4.2, peroxidase: $\chi^2=136.473$, $p<0.001$ and esterase: $\chi^2=56$, $P<0.0001$).

Table 4.2 Pooled results for peroxidase and esterase tests on stigmas of 14 species of *Prostanthera* at early-anthesis, mid-anthesis, or late-anthesis. Sample size (n) and percent (%) for a positive result is given for each developmental stage.

Species	<u>Positive peroxide test</u>						<u>Positive esterase test</u>					
	Early anthesis		Mid-anthesis		Late anthesis		Early anthesis		Mid-anthesis		Late anthesis	
	n	%	n	%	n	%	n	%	n	%	n	%
Total	91	13	19	53	106	92	26	0	8	50	34	97

Visitor observations

Of the total 365 1-hour visitor censuses, 231 censuses were conducted in which at least one visit was recorded. Visitor species are allocated to functional groups below (Fig. 4.5). Their interactions with *Prostanthera* flowers are described and are categorised as either visitors or pollinators.

'*Apis mellifera*' collects nectar or pollen from most species of *Prostanthera* (Fig. 4.5a). It is a likely pollen vector since its dorsal surfaces contact the anthers/stigma if it is foraging for nectar. It is excluded from the multivariate analyses, however, since it was only introduced to Australia in 1816 (Weatherhead 1986), which would make it an unlikely candidate for the selection pressures that have created the floral diversity of *Prostanthera*.

'Bird' species from the Meliphagidae (*Anthochaera carunculata*, *Acanthorhynchus tenuirostris*, *Lichenostomus chrysops*, *L. leucotis*, *L. ornatus*, *Manorina flavigula*, *Meliphaga lewinii*, *Phylidonyris novae-hollandiae*, *Phylidonyris pyrrhoptera*, *Philemon corniculatus*, *Plectorhyncha lanceolata*) and Zosteropsidae (*Zosterops lateralis*) were observed visiting *Prostanthera*. Their mode of interacting with flowers involved inserting their bills into corollas (Fig. 4.5b). Using museum skins to imitate a visit, the upper portion of the bill (all skins of Meliphagidae) or frons (*Z. lateralis* only) contacted the anthers in a male stage flower and was able to collect pollen. The deposited pollen could be successfully transferred to the stigma when skins were inserted into flowers of a female stage flower. Birds are therefore considered potential pollinators since their heads are large enough to contact stamens and stigmas and they have appropriate surfaces (feathers) for carrying pollen.

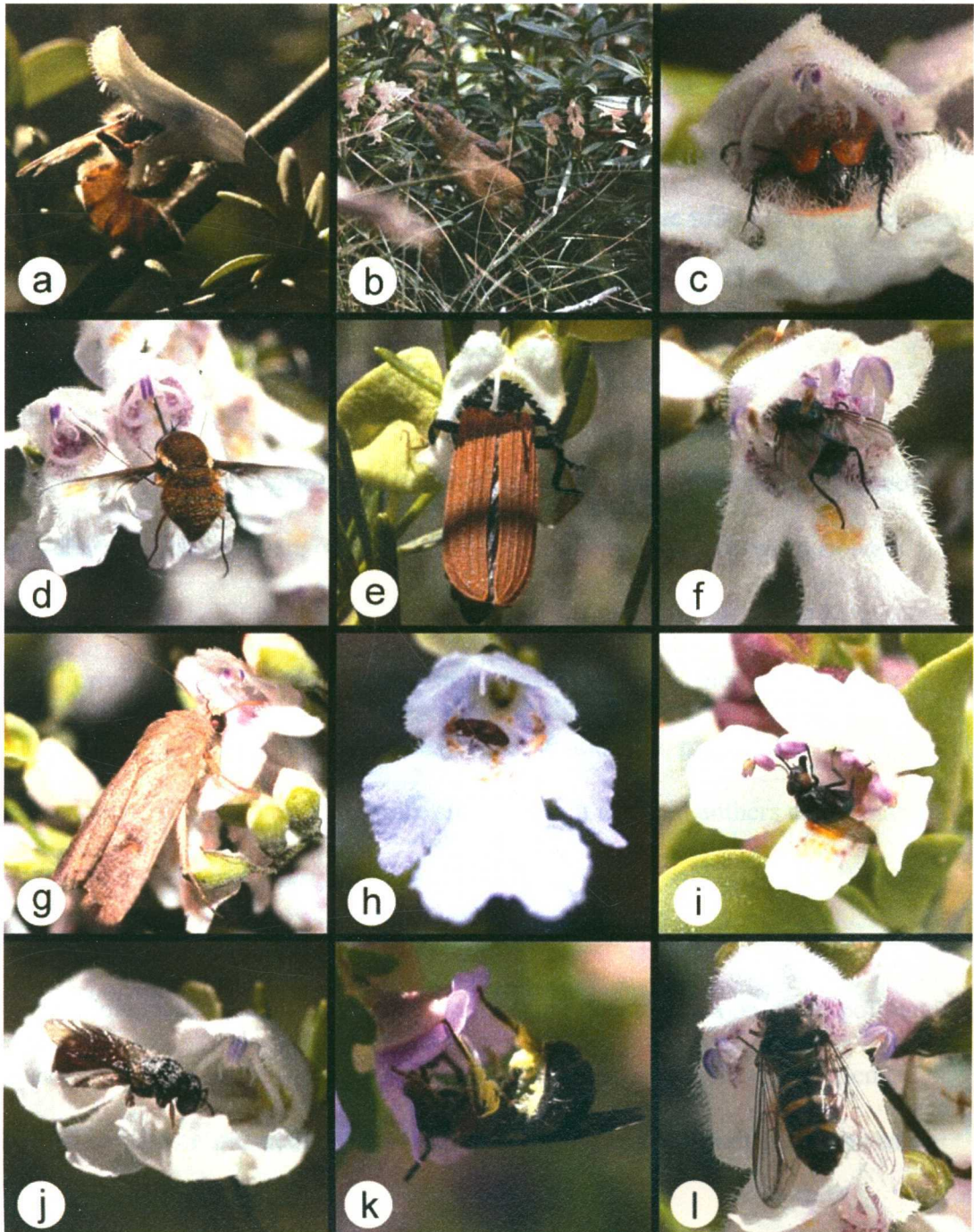


Figure 4.5. Photographs of visitors to *Prostanthera* flowers. (a), *Apis mellifera* visiting *P. ringens*; (b), juvenile *Acanthorhynchus tenuirostris* visiting *P. monticola*; (c), *Phylotocus* sp. (Cetoniinae) visiting *P. lasianthos*; (d) *Staurostichus* sp. (Bombyliidae) visiting *P. lasianthos*; (e), *Porrostoma* sp. (Lycidae) visiting *P. nivea*; (f), unidentified fly (Calliphoridae) visiting *P. lasianthos*; (g), moth (Lepidoptera) visiting *P. lasianthos*; (h), small beetle visiting *P. linearis*; (i), unidentified small fly visiting *P. cruciflora*; (j), *Exoneura* sp. visiting *P. nivea*; (k), *Lasioglossum* subgenus *Parasphecodes* sp. visiting *P. sieberi*; (l) unidentified Syrphidae visiting *P. lasianthos*.

Staurostichus sp. ('Bombylidae') are not considered pollinators since they use their long mouthparts to reach into the base of the corolla (Fig. 4.5c). It is unlikely that the narrow mouthparts contact anthers and their narrow surface offers little surface area to carry pollen.

Suborder 'Cetoniinae' (Scarabaeidae) are a group of beetles that visit flowers for nectar (Fig. 4.5d). They are considered a potential pollinator since their dorsal surface contacts anthers or stigma and were observed carrying pollen in transit between flowers.

The 'Formicidae' represents all ant visitors and 'Hemiptera' includes a variety of unidentified bugs that forage at the base of the gynoecium, presumably for nectar. They are unlikely pollinators since they are small enough to avoid anthers or stigmas and have not been observed carrying pollen.

'Large Coleoptera' (Cantharidae and Lycidae) are considered a potential pollinator since they contact anthers and stigmas and were observed dusted with pollen (Fig. 4.5e). They are considered distinct from the Cetoniinae considering they are more active even at lower temperatures.

'Large Diptera' (Calliphoridae, Muscidae, Sarcophagidae, Tabanidae, and Tachinidae) are considered potential pollinators since their bodies brush against anthers or stigmas and they were observed carrying pollen on their dorsal surfaces (Fig. 4.5f).

Amegilla sp. (Apidae) are classified as ‘large Hymenoptera’ since it is much larger than other bee visitors. They are approximately twice the body width in comparison to *Exoneura* sp., *Leioproctus* sp., and *Lasioglossum* sp. (Dollin *et al.* 2000). It is considered a pollinator since its body is large enough to contact anthers or stigmas of *P. monticola*. Furthermore its size and strength enable it to distort the corolla which is necessary to cause the anthers of *P. monticola* to release pollen.

‘Lepidoptera’ (all observed moths and butterflies) are unlikely pollinators because their long mouthparts only enter the corolla and do not appear to contact anthers and stigmas and provide a poor surface for pollen adhesion (Fig. 4.5g). Some of the butterflies identified include *Graphium macleayanum*, *Belenois* sp., *Pieris* sp., *Papilio* sp., and *Delias* sp.

‘Small Coleoptera’ (including Curculionidae, Meloidae, Mordellidae) and ‘small Diptera’ (Culicidae and Drosophilidae) are not considered pollinators since they mostly visit nectaries and are too small to accidentally contact anthers and stigmas and were never observed dusted with pollen (Fig. 4.5h and 4.5i). Some Diptera visited anthers but did not collect pollen on their bodies.

‘Small Hymenoptera’ include *Exoneura* sp. (Apidae), *Leioproctus* sp. (Colletidae), *Megachile* sp. (Megachilidae) and *Lasioglossum* sp. (Halictidae). These bees are potential pollinators because they were observed dusted with pollen and they brush their dorsal surfaces against anthers or stigma as they enter a corolla to forage for nectar (Fig. 4.5j). Less often they will clasp the adaxial median lobe-pair while they gather pollen from the anthers (Fig. 4.5k).

Members of the ‘Syrphidae’ mostly enter a corolla to forage for nectar and rarely observed feeding on pollen from anthers (Fig. 4.51). Pollen could not be observed on their dorsal surfaces after visitation but since their bodies are large enough to contact stamens and stigmas they are still considered potential pollinators.

Insect and bird visitations were recorded from both sections of *Prostanthera* (Fig. 4.6a). Bird visitation in section *Klanderia* is highest at all times when *Apis mellifera* is excluded from the results. *Apis mellifera* and ‘Lepidoptera’ were observed visiting *P. ringens* and all functional groups of bees (‘*Apis mellifera*’, ‘large hymenoptera’ and ‘small hymenoptera’) were observed visiting *P. monticola*. *Acanthorhynchus tenuirostris* was observed once visiting *P. cruciflora* (Fig. 4.6b). *Acanthorhynchus tenuirostris* and *Z. lateralis* were observed visiting *P. lasianthos*.

Multivariate analysis of visitors

Multivariate analyses were conducted on the visitation data for *Prostanthera* (Appendix 4.3). Statistics for r^2 , MCAO and KW are presented in Appendix 4.4 for all analyses. The UPGMA dendrogram finds two significant groups at a dissimilarity value of 1.361 (Fig. 4.7A) that are separated by ‘bird’ visitation (Fig. 4.7B). ANOSIM produces no scores (best f-ratio= 1.56) higher than the real f-ratio (1.62) demonstrating that the difference between these groups is statistically significant. The two groups are observed in the MDS plot if the ‘bird’ visitation vector is oriented along axis 1 (Fig. 4.8). The fidelity of *P. lasianthos* to group 2 was tested by including it as a member of group 1 in an analysis of *a priori* groups. ANOSIM produces 0.2% scores (best f-ratio= 1.64) higher than the real f-ratio (1.64). A separate analysis of Group 2 species (section

Prostanthera) finds two groups at a dissimilarity value of 0.82 based on visitation of ‘small Diptera’ and ‘small Hymenoptera’ (Fig. 4.7A and 4.7C). These two groups are not significantly different because ANOSIM produces 43% of scores (best f-ratio=1.31) higher than the real f-ratio (1.01). The MDS plot shows no strong clustering of taxa based on these groups (Fig. 4.9A). The regression line drawn for any vector in this analysis is not significant since they have high (>5%) MCAO scores.

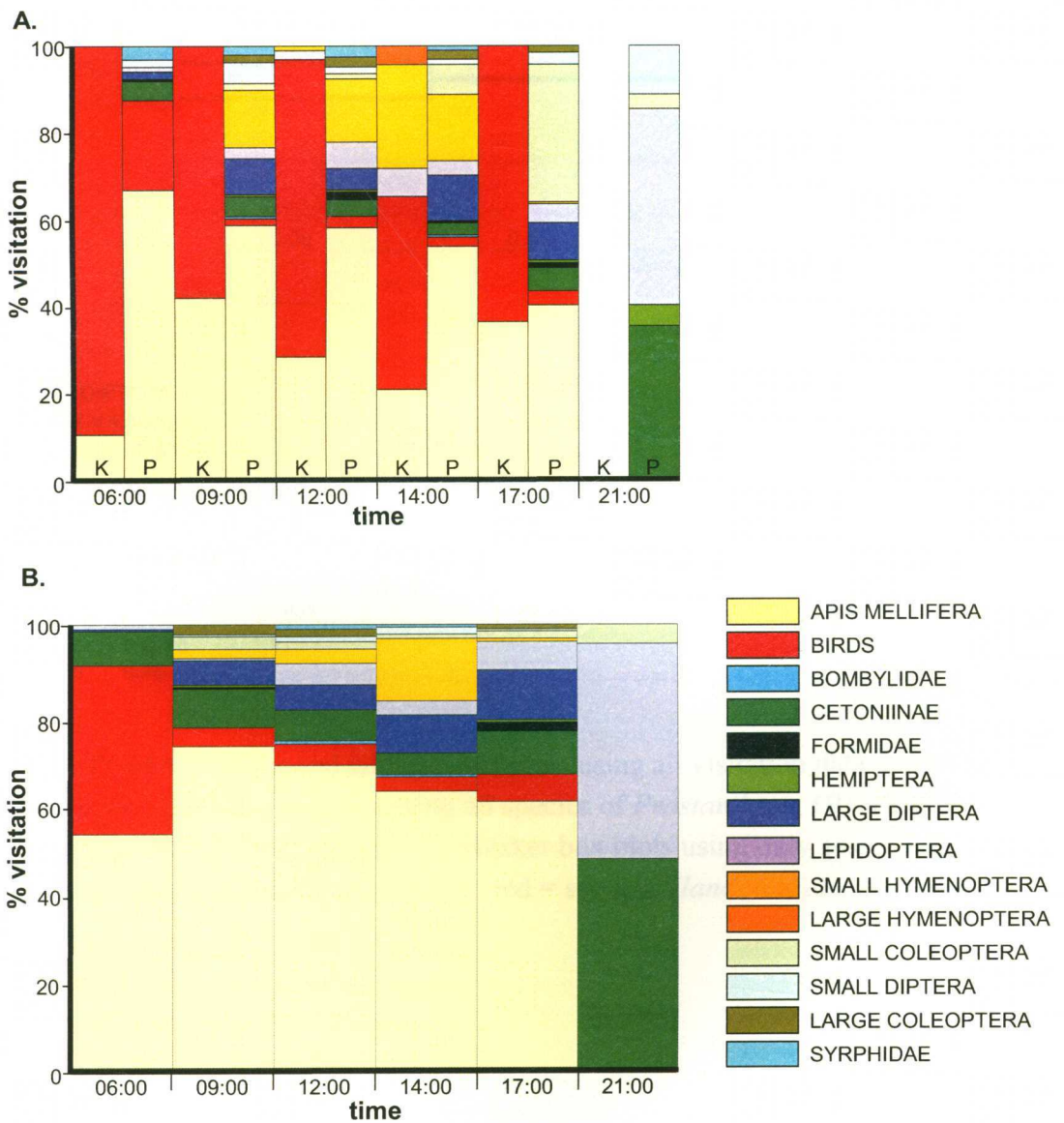


Figure 4.6. Proportion of visitation by functional groups during six different census times representing a day. (A)=visitation of (K) section *Klanderia* and (P) section *Prostanthera*; (B)=visitation to *P. lasianthos*.

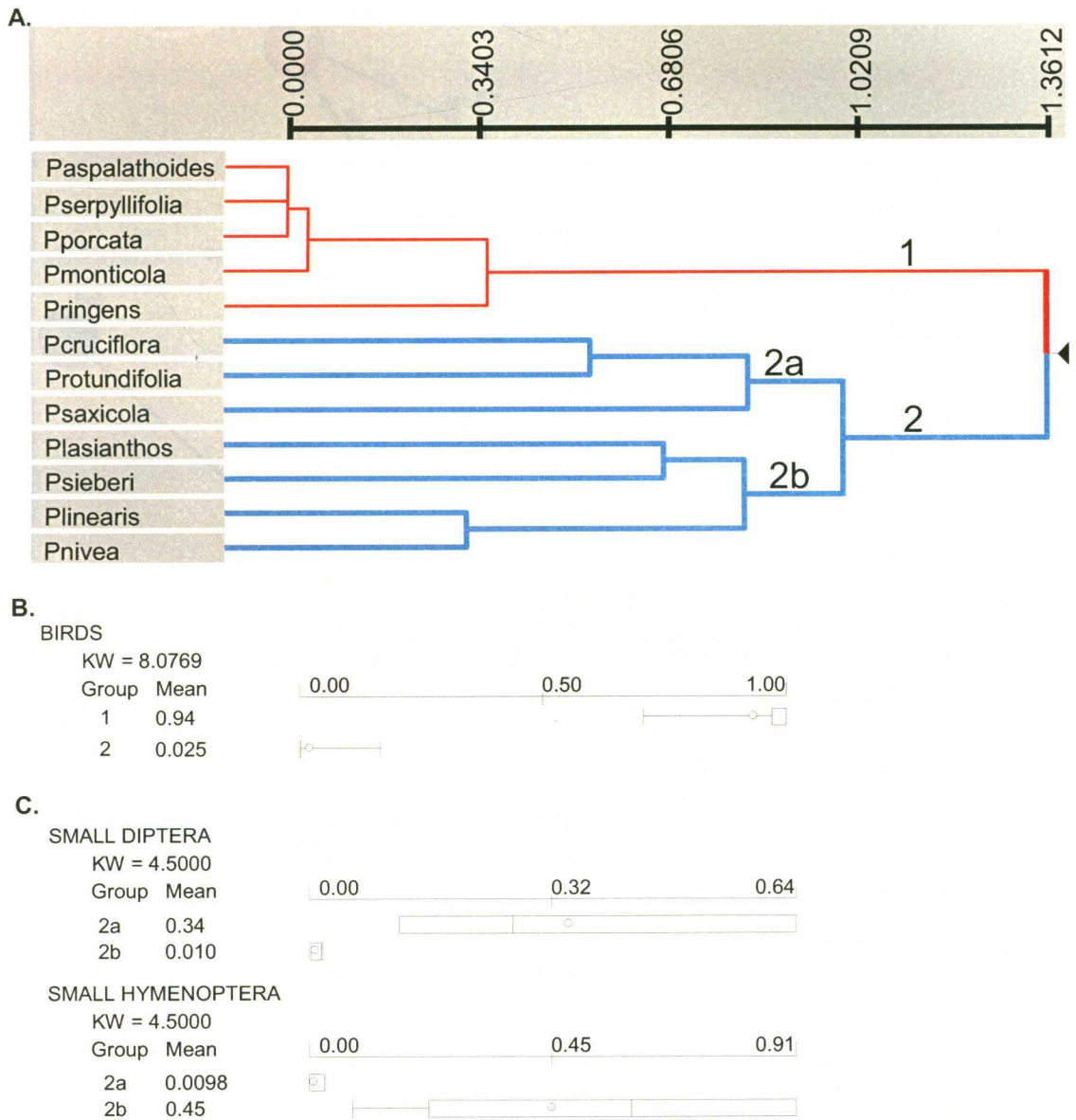


Figure 4.7. UPGMA analysis and whisker box plots using all visitation data except for *Apis mellifera*. (A) UPGMA analysis using all species of *Prostanthera*; (B) whisker box plots using all species of *Prostanthera* (C) whisker box plots using only group 2 data. Line colour represents sectional membership: red = section *Klanderia*; blue = section *Prostanthera*.

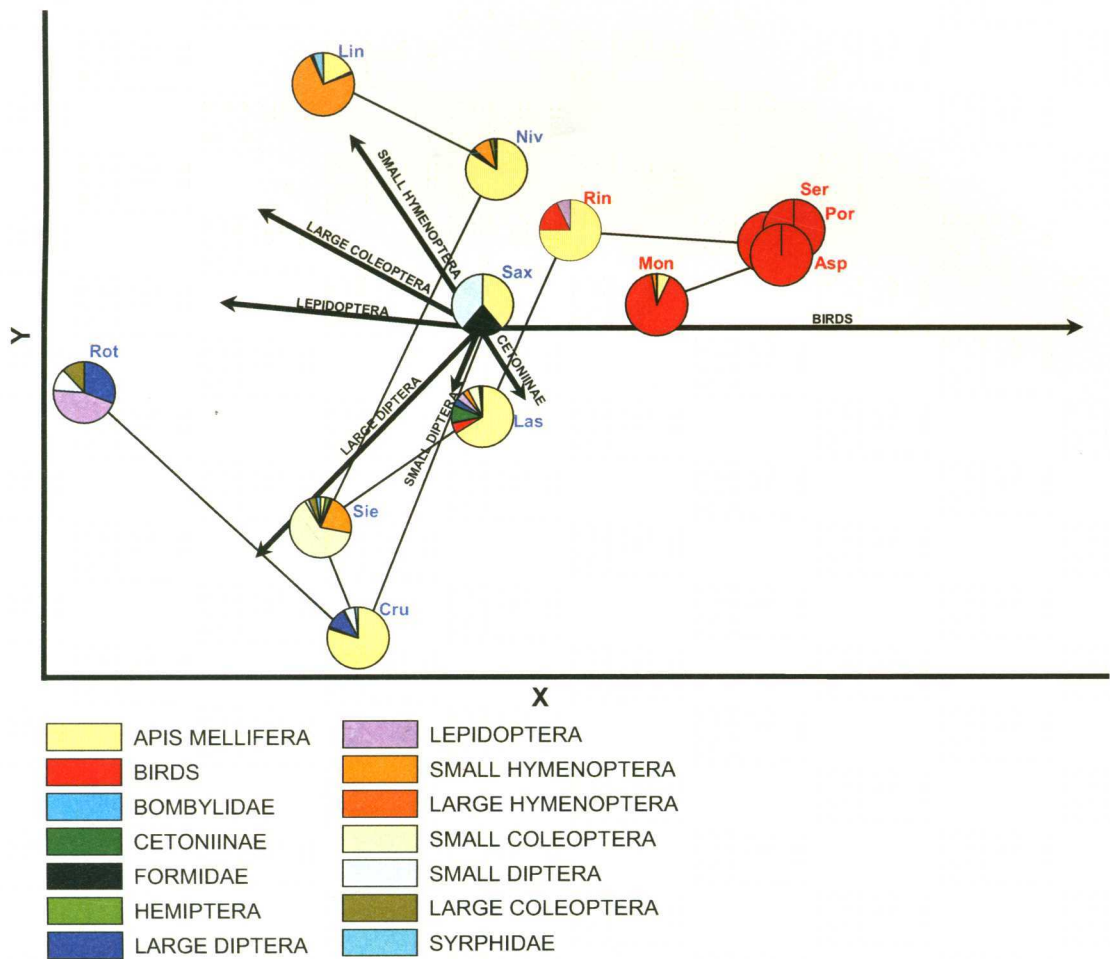


Figure 4.8. SSHMDS plot of *Prostanthera* using all visitation data except for *Apis mellifera*. Each operational taxonomic unit represents a species of *Prostanthera* and is accompanied by a pie chart demonstrating the proportion of all observed visitors, including *A. mellifera*. Stress = 0.110. Species abbreviations are as follow: Asp=*P. aspalathoides*; Cru=*P. cruciflora*; Las=*P. lasianthos*; Lin=*P. linearis*; Mon=*P. monticola*; Niv=*P. nivea*; Por=*P. porcata*; Rin=*P. ringens*; Rot=*P. rotundifolia*; Sax=*P. saxicola*; Ser=*P. serpyllifolia*; Sie=*P. sieberi*.

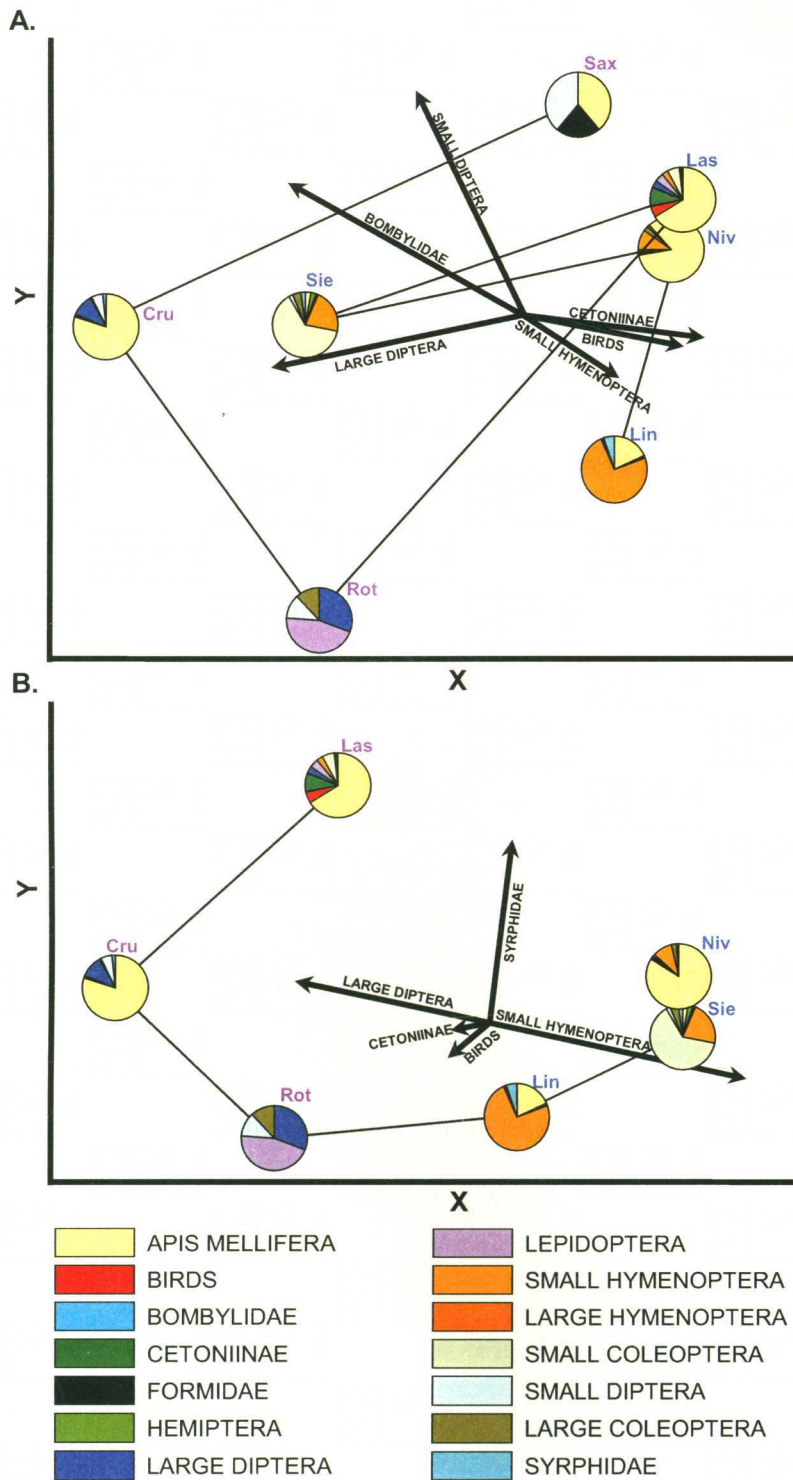


Figure 4.9. SSHMDS plot of *Prostanthera* section *Prostanthera* using (A) all visitation data except for *Apis mellifera* and (B) pollinator data. Each operational taxonomic unit represents a species of *Prostanthera* and is accompanied by a pie chart demonstrating the proportion of all observed visitors, including *A. mellifera*. Stress = 0.063. Species abbreviations correspond to those listed in Figure 4.8.

Multivariate analysis of pollinators

Non-native species and non-pollinators were removed from the data set (Appendix 4.5) and analyses were conducted again (Appendix 4.4). *Prostanthera saxicola* was not included since pollinators were not observed visiting it. The UPGMA dendrogram finds two significant groups (1 and 2, dissimilarity value= 1.439) that are separated by visitation data of birds and ‘large Coleoptera’ (Fig. 4.10A and 4.10B). The MDS plot demonstrates two groups if the ‘Bird’ visitation vector is oriented along axis 1 (Fig. 4.11). These groups are significantly different because 3% of ANOSIM scores (best f-ratio= 2.45) are higher than the real f-ratio (2.35). If *P. lasianthos* is included as a member of group 1, ANOSIM produces no scores (best f-ratio= 2.35) higher than the real f-ratio (2.45).

With section *Klanderia* species removed the UPGMA dendrogram finds the best two groups at a dissimilarity value of 0.84 that are separated by ‘large Diptera’ and ‘small Hymenoptera’ visitation (Fig. 4.10 A and 4.10C). ANOSIM demonstrates that the groups are not significantly different because 28% of scores (best fratio=2.16) are higher than the real f-ratio (1.10). The MDS plot demonstrates that group 2b are positively correlated with ‘small Hymenoptera’ visits whereas group 2a are positively correlated with ‘large Diptera’ visits (Fig. 4.9B). These vectors both have high r^2 values but the regression line drawn for either is not significant since they have high (>5%) MCAO scores.

Analysis of pollinators and environmental variables

Canonical correspondence analysis was compared amongst *P. lasianthos*, *P. cruciflora*, *P. linearis*, and *P. sieberi* to investigate correspondence with visitation, time and

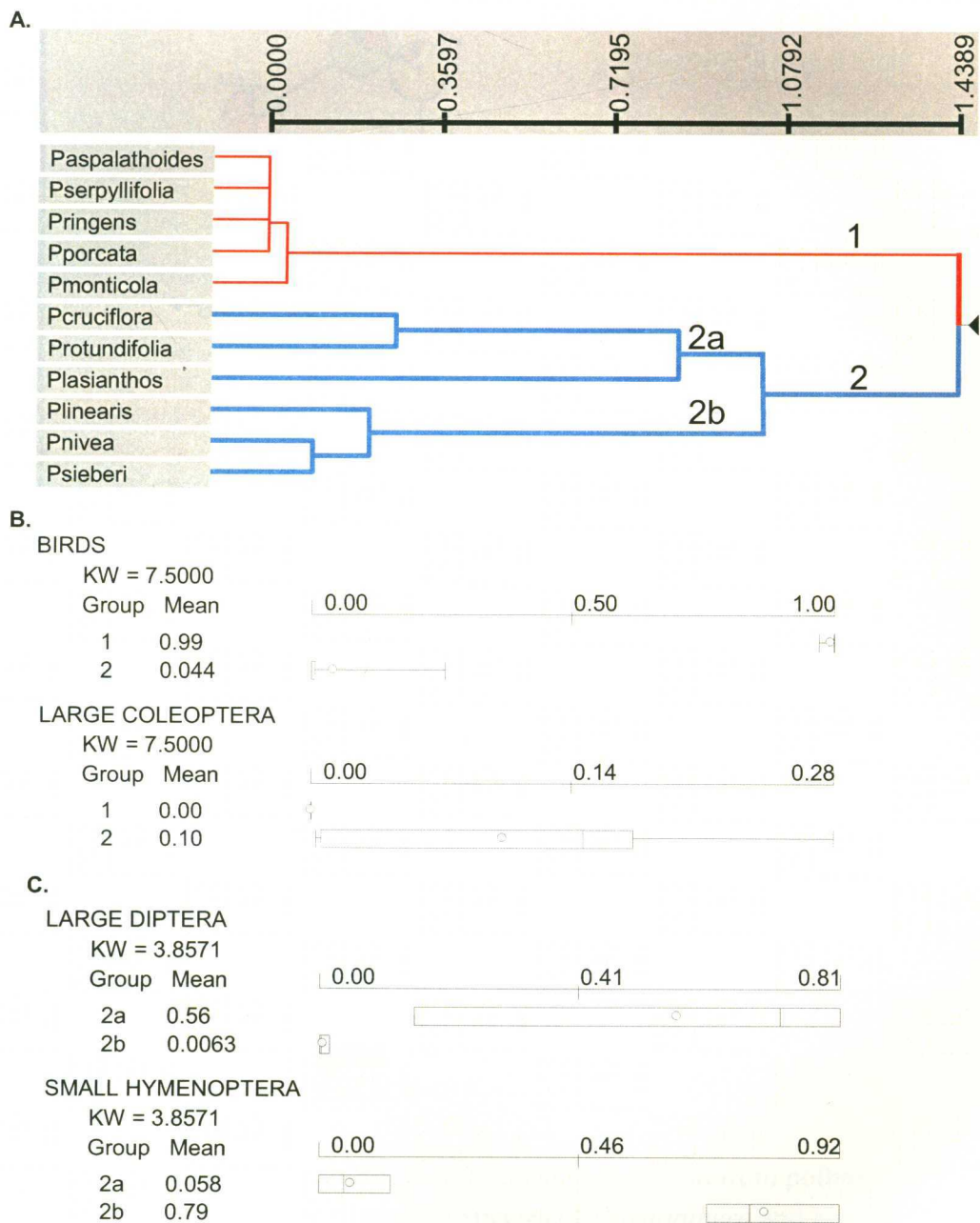


Figure 4.10. UPGMA and whisker box plots using only pollinator data except for *Apis mellifera*. (A) UPGMA analysis using all species of *Prostanthera*; (B) whisker box plots using all species of *Prostanthera* (C) whisker box plots including only group 2 species. Line colour represents sectional membership: red = section *Klanderia*; blue = section *Prostanthera*.

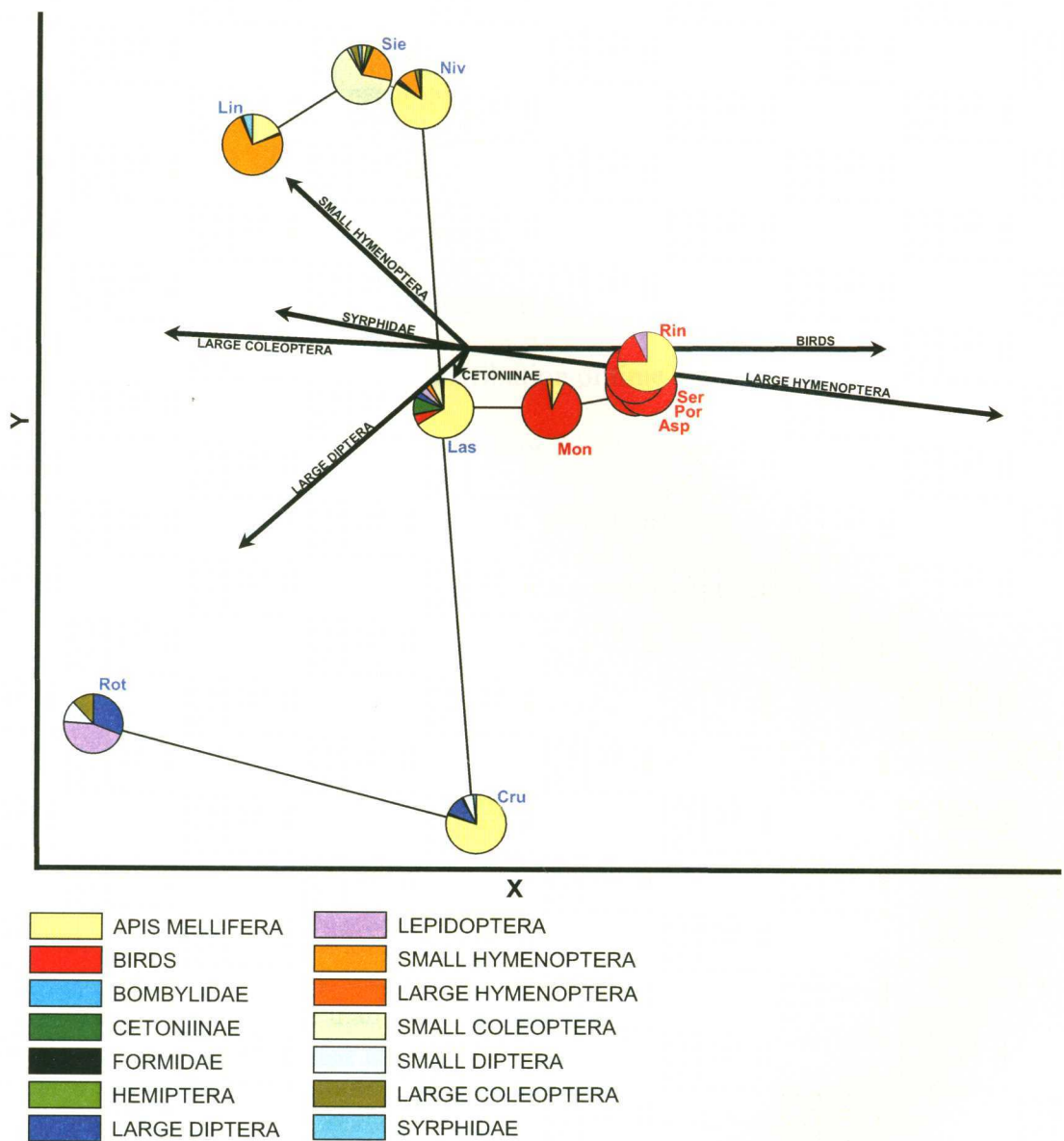


Figure 4.11. SSHMDS plot of *Prostanthera* using visitation from pollinators. Each operational taxonomic unit represents a species of *Prostanthera* and is accompanied by a pie chart demonstrating the proportion of all observed visitors, including *A. mellifera*. Stress = 0.128. Species abbreviations correspond to those listed in Figure 4.8.

visitors (Appendix 4.6). Most insect visitations are positively correlated with temperature and direct sunlight (Fig. 4.12). ‘Bird’ visits are positively correlated with rain and/or wind. ‘*Apis mellifera*’ has no association with any vectors for *P. cruciflora* and *P. lasianthos* and is either associated with higher temperatures for *P. linearis* or lower temperatures for *P. nivea*. The distribution of time 3 (1200 hours) and 4 (1400 hours) is associated with higher temperature or direct sunlight in most ordinations. Results of permutation tests for each analysis retrieve p-values all outside the 95% confidence limit ($p > 0.05$) indicating that the relationships between the species and environmental variables are independent (Appendix 4.7).

Flower number and nectar

Box plots show that *P. lasianthos* has the largest estimated mean number of flowers (Fig. 4.13, Appendix 4.8). *Prostanthera saxicola*, *P. nivea* and species of section *Klanderia* did not have more than 1000 flowers per shrub. Most species of section *Klanderia* have consistently more than three times the standing crop per flower than any species of section *Prostanthera* (Fig. 4.14, Appendix 4.9). *Prostanthera lasianthos* has the highest nectar volume per shrub when the estimated mean number of flowers is multiplied with the mean nectar volume of standing crop (Fig. 4.15).

Discussion

The goal of this study was to determine the pollination biology of *Prostanthera* and then quantify the pollinators of the recently described flower morphologies in Chapter 3. It was found that some species received unexpected visitation according to their

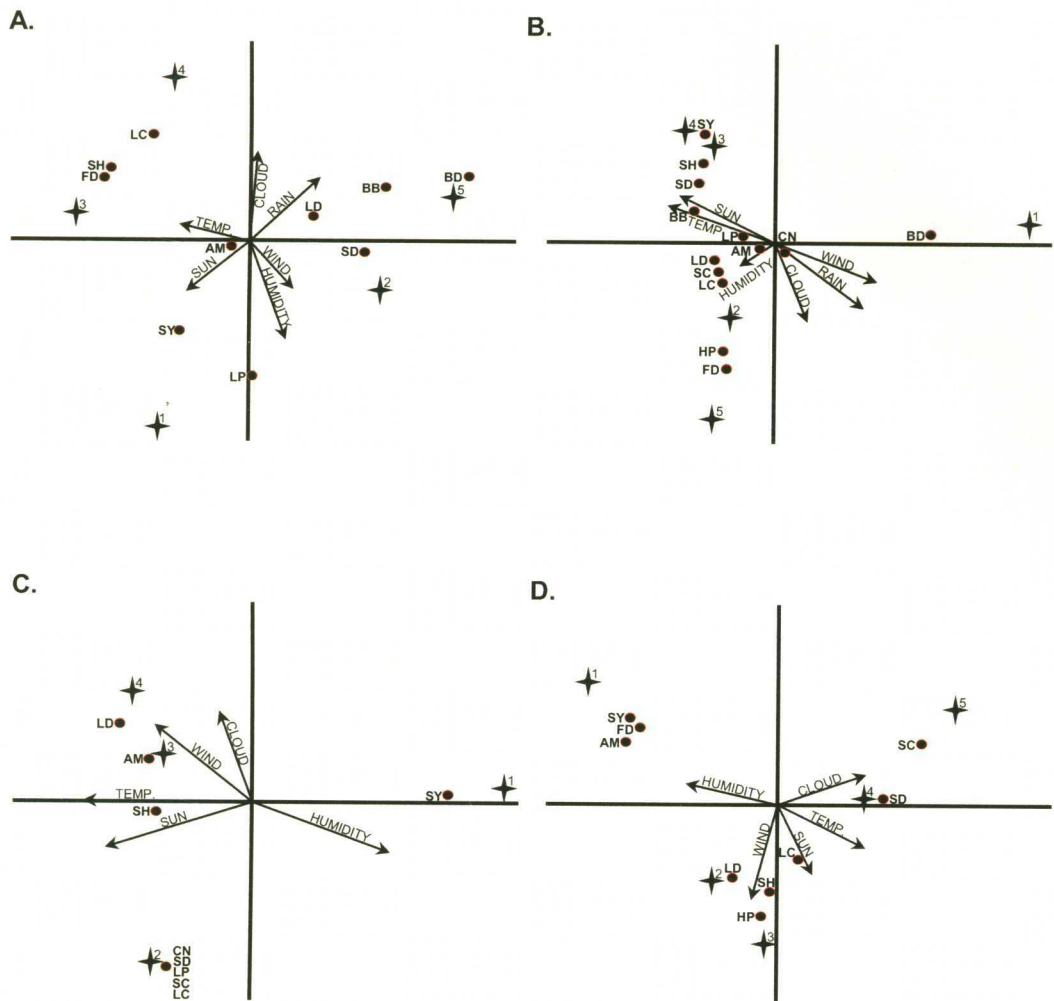


Figure 4.12. Canonical correspondence analysis using environmental variables, time and visitor data for 4 species of *Prostanthera*. Functional groups and census times are ordinated with respect to environmental variables and ordinations are drawn using type 2 scaling. Plots= (A) *Prostanthera cruciflora*; (B) *P. lasianthos*; (C) *P. linearis*; (D) *P. sieberi*. Functional group abbreviations = Birds (BD); Cetoniinae (CN); Syrphidae (SY); small Hymenoptera (SH); small Diptera (SD); Bombylidae (BB); Lepidoptera (LP); *Apis mellifera* (AM); large Diptera (LD); large Coleoptera (LC); Formicidae (FD); Hemiptera (HP); small Coleoptera (SC). Census time = (1) 0600 hours; (2) 0900 hours; (3) 1200 hours; (4) 1400 hours; (5) 1700 hours.

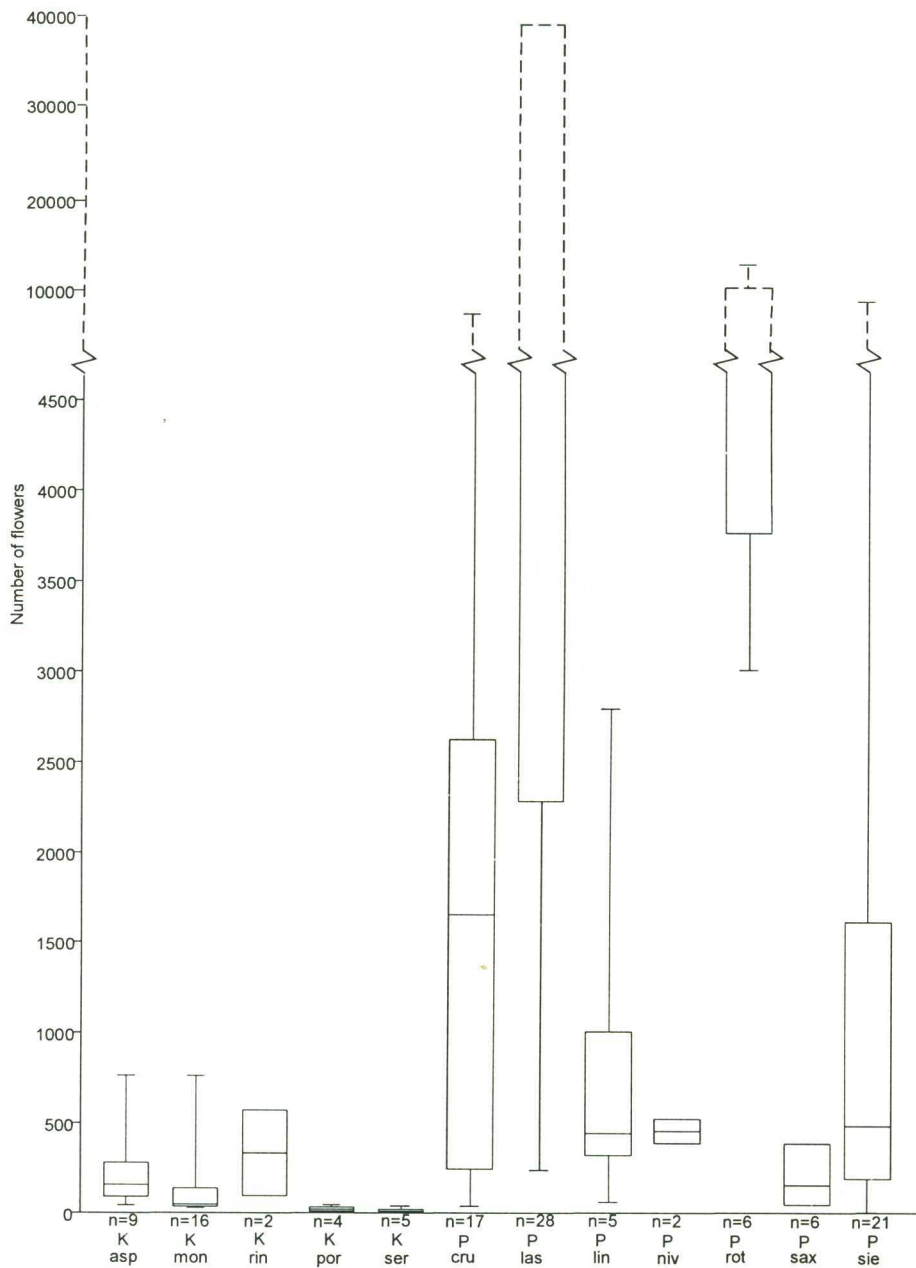


Figure 4.13. Estimated number of flowers per plant for 12 species of *Prostanthera*. K= section *Klanderia*; P= section *Prostanthera*, asp= *P. aspalathoides*; cru= *P. cruciflora*; las= *P. lasianthos*; lin= *P. linearis*; mon= *P. monticola*; niv= *P. nivea*; por= *P. porcata*; rin= *P. ringens*; rot= *P. rotundifolia*; sax= *P. saxicola*; ser= *P. serpyllifolia*; sie= *P. sieberi*.

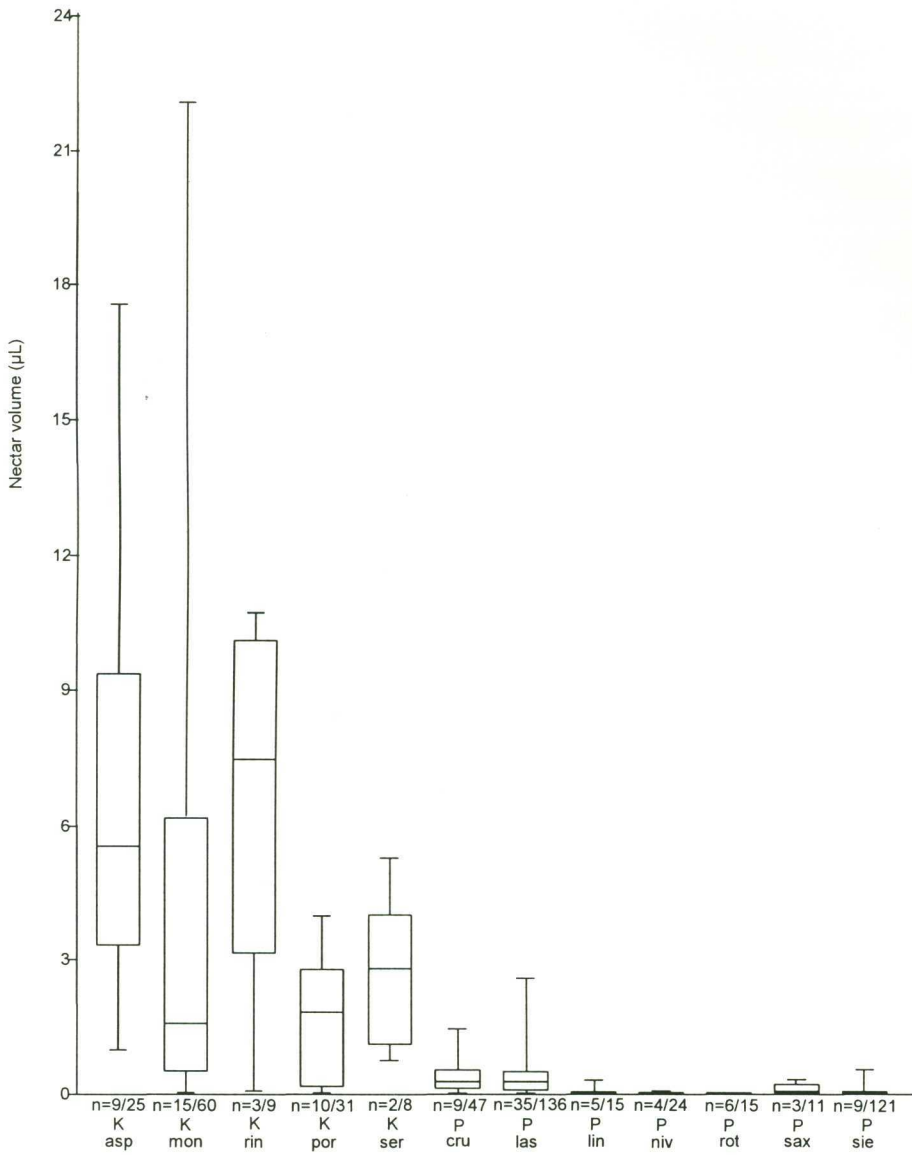


Figure 4.14. Measured nectar volume of standing crop per flower ($\mu\text{L}/\text{flower}$) for 12 species of *Prostanthera*. n= number of plants/number of flowers. Abbreviations correspond to those of Figure 4.14.

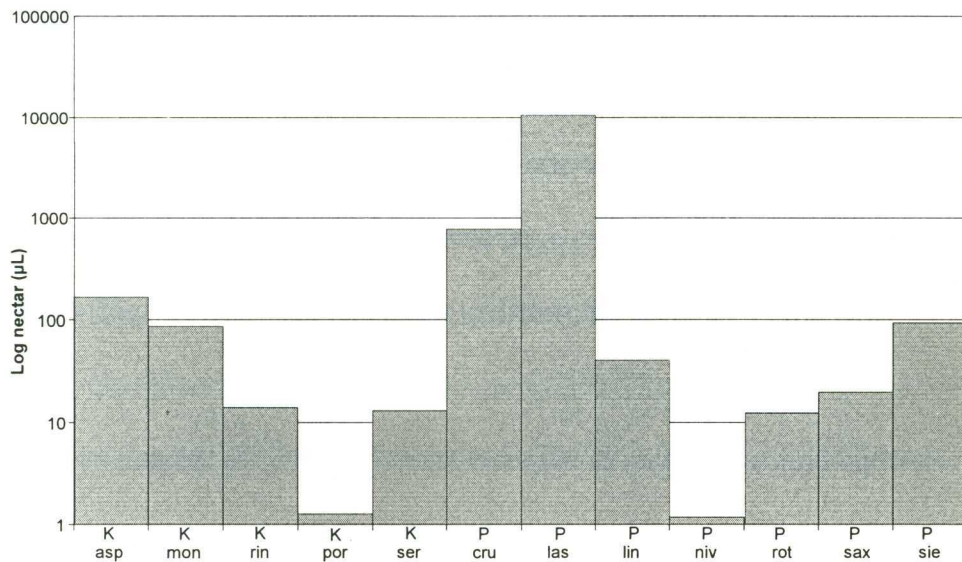


Figure 4.15. Estimated nectar volume of standing crop per individual ($\mu\text{L}/\text{plant}$) for 12 species of *Prostanthera*. Abbreviations correspond to those of Figure 4.14.

floral morphology. Flower phenology, visitors and an investigation to exceptions are discussed in this section.

Floral biology of *Prostanthera*

Observations of flower phenology support the conclusion that flowers of *Prostanthera* are weakly protandrous (Conn 1984). The first stage of anthesis is the male stage because pollen is shed and most stigmas tested were not receptive. The low number of positive peroxide results (13%) are likely due to damaged stigmas that will produce bubbles in hydrogen peroxide even if they are not receptive (Kearns and Inouye 1993). The floral characteristics that distinguish the male stage are dehiscent anthers and a closed stigma with a smooth epidermal surface. The second stage is the female stage because pollen has been shed and most stigmas that were tested were receptive (Table 4.2). It is characterised by a fully open corolla, senescent anthers, and a bifid stigma. The design of protandrous flowers minimizes autogamy on a flower (Faegri and Van

der Pijl 1979) which underlines the importance for floral visitors in *Prostanthera*. For most species, the placement of the stigma in a similar location after the anthers senesce suggests that precise pollen deposition onto biotic vectors is required for pollination. Pollen release was only aided by organisms large enough to agitate the anther appendages (Fig. 4.5c) or strong enough to distort the corolla.

The increased number of flowers that are recorded as receptive at mid-anthesis demonstrates a gradual transition to the female stage. The pale stain for the presence of esterase on stigma lobes at this point also demonstrates a gradual transition to the female stage (Fig. 4.1b). Since anthers have not senesced during this process there is an overlap of male and female stages and therefore *Prostanthera* demonstrates incomplete dichogamy. Although its duration was not measured, it usually lasted approximately a day in any observed species of *Prostanthera*. This hermaphroditic stage resembles the rapid 'intermediate stage' described for *Lavandula latifolia* (Herrera 1987). The hermaphroditic stage can be determined in all *Prostanthera* by observing partially open stigmatic lobes. It is more obvious in species with flowers represented by *P. lasianthos* and *P. monticola* because it corresponds with translocating anthers and style curvature (Fig. 4.3b and 4.3e). In this state, it is likely that there may be contact between stigma and anthers since low levels of autogamy have been measured in *P. junonis* (Tierney and Gross 2001). *Prostanthera* is therefore another example of a protandrous labiate designed for outcrossing that demonstrates a small level of autogamy (Ubera-Jimenez and Valdés 1983; Owens and Ubera-Jimenez 1992; Navarro 1997).

A herkogamous function is implemented in *Prostanthera* for the likely reason to maintain the separation of sexes while the flower is hermaphroditic. The two flower

types represented by *P. lasianthos* and *P. monticola* reposition their anthers away from the stigma as it becomes receptive (Fig. 4.3a-h). The way in which anthers are repositioned is vertical in a flower of section *Klanderia* whereas it is horizontal in the flower type represented by *P. lasianthos* (Compare Figs. 4.3g-i with 4.3d-f). This process has been described in other genera of the Lamiaceae (Whitten 1981; Raju and Reddi 1989; Da Silva *et al.* 2005; Potgieter *et al.* 2009) but never for the Westringieae since the relocation of anthers in *Hemigenia* and *Microcorys* flowers is the result of withering (Guerin 2005).

Flowers represented by *P. sieberi* demonstrate a different type of herkogamy than described above. The same position of anthers is able to be maintained throughout anthesis by having an exerted stigma (Fig. 4.3g-i). Stigma exertion has already been shown as significantly greater in flowers of *P. sieberi* (and those of Group 2a described in Chapter 3) than in flowers with anthers that translocate through anthesis (described as group 2 in Chapter 3). The phylogenetic position of group 2a in Chapter 2 suggests that this morphology is recently evolved corresponding to a loss in movement of the anthers and stigma. *Prostanthera cruciflora* is related to this group and also shares many similar floral characteristics (see Chapter 2 and Chapter 3). It is unique to *Prostanthera*, however, since its anthers are separated at early anthesis and continue to separately translocate towards the side of its symmetrical corolla throughout anthesis. These features are very similar to *Westringia* which demonstrates a possible convergence to a distinct pollination syndrome.

Despite the separation of sexes on a flower through protandry and herkogamy, during the flowering period, flowers of all sexual stages can be observed on a plant as well as

on the same branch. Geitonogamy is likely possible, especially since low levels of autogamy were measured in *P. junonis*. In this study, most visitors were commonly observed visiting several flowers on one plant.

Assessment of pollination syndromes in *Prostanthera*

Hummingbird and hymenoptera groups have been distinguished amongst “penstemons”, which refers to the Cheloneae of the Scrophulariaceae (Wilson *et al.* 2004). Similar to these results, two distinct groups of *Prostanthera* are distinguished based on bird and insect visitation. Section *Klanderia* is significantly different from section *Prostanthera* due to a higher number of bird visits. These groups are the same as those produced in Chapter 3 based on floral morphology. Therefore, this relationship supports a correlation between ornithophilous traits and bird visitation. Most of the insect visitation to section *Klanderia* does not correspond to pollination with the possible exception the bee *Amegilla sp.* The bee visited flowers of *P. monticola* for nectar and was large enough to encounter anthers or stigmas. This reflects a similar bimodal pollination system in *Tritoniopsis revoluta* (Burm.f.) G.J. Lewis (Iridaceae) where *Amegilla* bees are capable pollinators of its apparently specialist long-proboscid fly pollination syndrome (de Merxem *et al.* 2009).

Earlier reports demonstrate that spinebills (*Acanthorhynchus tenuirostris*) feed on nectar of *P. lasianthos* but do not describe it as a potential pollinator (Loyn 1985). The observations of this study provide the first support that birds are pollinators of *P. lasianthos*. *Prostanthera lasianthos* was observed to have a large number of visits by silvereyes (*Zosterops lateralis*) as well as *A. tenuirostris*. Both birds are nectar feeders and their geographic ranges overlap with that of *P. lasianthos* from Tasmania to

southern Queensland (Ford *et al.* 1979; Conn 1992a; Carthew 1993; Simpson and Day 1999; Hackett and Goldingay 2001). *Zosterops lateralis* is likely to be a good pollinator since pollen carrying feathers (Knox *et al.* 1985) of its frons can contact anthers and stigma. The long, slender bill of *A. tenuirostris* can come in contact with anthers and stigma of *P. lasianthos*. Although it seems unsuitable for carrying pollen, it has been shown to carry equal pollen loads when compared with feathered heads of other nectarivorous birds (Paton and Turner 1985).

Based on floral differences quantified in Chapter 3 it is hypothesised that *P. cruciflora*, *P. sieberi*, and *P. rotundifolia* attract a more generalist suite of insects since their flowers have shallow corolla tubes and reflexed petals (McCall and Primack 1992; Proctor *et al.* 1996). The other entomophilous species are hypothesised to be Hymenopteran specialists since their flowers are bilabiate and have longer tubes (Westerkamp and Classen-Bockhoff 2007). The above hypotheses are unsupported because the groups formed by visitation data are not significantly different and they do not match the groups retrieved by floral characters. Furthermore, a distinction between a specialist and generalist is not supported either since some putatively melittophilous species received a more diverse group of pollinators than the suspected generalist species (e.g. see *P. lasianthos*, Appendix 4.5). Similar results amongst melittophilous “penstemons” suggest that specific types of entomophily are more subtle than compared with ornithophily (Wilson *et al.* 2004). This supports the trend that plants receive a wider diversity of pollinators across orders of insects than across classes of animals (Ollerton 1996). It may be possible that both flower types are different ways of using the same suite of pollinators. The putatively melittophilous group could have originally

evolved under a strong selection pressure by bees, but in a current ecological context, these pressures are different (Ollerton 1996)

Environmental influence on pollination

Ordinations for *P. cruciflora*, *P. lasianthos*, *P. linearis* and *P. sieberi* show that insect visitation is positively correlated with increased temperature and negatively correlated with the presence of rain, wind and increased cloudiness (Kevan and Baker 1983; Inouye and Pyke 1988; Totland 1994). Also, the ordinations of *P. lasianthos* and *P. cruciflora* show that bird visitation is positively correlated with rain and wind. These relationships match expectations that insects are more influenced by environmental extremes based on their smaller size and ectothermic physiology (Cruden 1972; McFarland 1986). However, it appears counter intuitive that bird visitation should be positively influenced by the presence of these extreme conditions. There is no support for any trend since no analysis demonstrates significant correlation between environmental variables and visitation. This result is more likely due to the small data sets for each species. The largest data set (*P. lasianthos*) consisted of 12 observed days spanning two years. Although a more extensive data set is required to investigate the correlation of environment and time of day on pollination, these preliminary results can be explained. When insects are not active during certain times, this should reduce competition with birds, thereby increasing bird visitation. The presence of *Apis mellifera* has been shown to reduce the visits made by honeyeaters (Paton 2000), although this correlation has never been tested with native Australian insects. Furthermore, a lack of insects might also result in an accumulation of nectar, improving the value of a flower as a resource and thereby increasing the number visits. A change in pollination syndrome based on environmental factors has already been described in

other flora (de Merxem *et al.* 2009). This underlines the importance of improving this data set to investigate how environmental factors might affect pollination syndromes.

Influence of rewards on pollination

The comparison of standing crop nectar shows a significant difference between most volumes of bird-visited and insect-visited species. Higher nectar volumes are found in species of section *Klanderia* and lower nectar volumes are found in species of section *Prostanthera*, which includes *P. lasianthos*. This pattern of relative nectar volumes in *Prostanthera* is consistent with nectar volumes found in other ornithophilous and entomophilous species of Lamiaceae (Whitten 1981; Raju 1989; Raju and Reddi 1989; Vos *et al.* 1994; Da Silva *et al.* 2005; Petanidou 2005; Wester and Claßen-Bockhoff 2007; Ford and Johnson 2008). Therefore nectar volume per flower alone does not demonstrate why birds visit *P. lasianthos*. If a large enough number of flowers is produced, however, the available amount of nectar might match the volume offered by a typically ornithophilous plant. *Prostanthera lasianthos* is the largest species of *Prostanthera* (Conn 1992a) and is here recorded as having the highest number flowers per individual (Fig. 4.14). This demonstrates that the nectar offered by one plant of *P. lasianthos* could be as much as 100 times greater than any other ornithophilous *Prostanthera* (Fig. 4.16). It has been suggested that nectivorous birds the size of *A. tenuirostris* are efficient foragers that are able to exploit smaller amounts of nectar (McFarland 1986). These data suggest that a plant size/flower number threshold for bird visitation may be indicative of ornithophily.

Visitation by *Apis mellifera*

Apis mellifera makes a large contribution of visits to species from section *Prostanthera* and section *Klanderia* throughout a day. Deleterious effects on pollinator assemblages have been measured when *A. mellifera* visits Australian native plants. It has been shown to remove significant amounts of pollen (Paton 1993; Vaughton 1996; Horskins and Turner 1999; Paton 2000), outcompete native bees (Paton 1993; Gross and Mackay 1998; Horskins and Turner 1999; Gross 2001), and reduce visitation rates of Meliphagids (Paton 1993). It is also known to make visits to specific flowers until their resources have been depleted (Westerkamp 1991). Since solitary bees constantly forage on new resources, they have a better chance at depositing pollen on different plants of the same species, which makes *A. mellifera* a poorer pollinator in comparison. Pollen removal was not measured in this study, however, *A. mellifera* was observed collecting pollen from all species of section *Prostanthera*. The relatively high morning visits observed by *A. mellifera* for all *Prostanthera* (Fig. 4.6) suggests that nectar and pollen are reduced before native insects are active. These observations suggest that *A. mellifera* has changed the pollinator assemblage and so it must be acknowledged that this might cause some misinterpretation between morphology and relative visitation rates by native pollinators. It may, however, be very difficult to conduct investigations in the absence of *A. mellifera* since they have become feral in many environments of Australia (Paton 1993).

Conclusions

This study supports that the inferred pollination syndromes of *Prostanthera* are typical of entomophily and ornithophily. The ornithophilous characteristics of section *Klanderia* correspond with an almost exclusive number of visits by birds whereas the

entomophilous characteristics of section *Prostanthera* correspond with an almost exclusive number of insect visits. It is found, however, that floral characters are not predictive of all pollinators since insects were found visiting *P. monticola* and birds were found visiting *P. lasianthos*. Furthermore, the discrete floral morphologies within section *Prostanthera* do not receive significantly different suites of visitors. These exceptions support that pollination syndromes are more general and dynamic than a rigid concept (Waser *et al.* 1996) and that syndromes are typical and not exclusive.

The visitation by *A. tenuirostris* and *Z. lateralis* to the putatively entomophilous *P. lasianthos* was the most regularly observed exception of a pollination syndrome. *Prostanthera lasianthos* provides rewards in terms of nectar volume per individual similar to other ornithophilous species. A plant size/flower number threshold might therefore be an important characteristic of ornithophily. Other features of nectar such as concentration, sugar type, and amino acid quality have already been shown to be correlated with ornithophily (Baker and Baker 1986; Stiles and Freeman 1993; Nicolson 2002; Dupont *et al.* 2004; Amela García and Gottsberger 2009) and may therefore provide an explanation for bird visitation at *P. lasianthos*. Further investigation of these features plus the flower number threshold for bird visitation on different plant sizes are needed to provide a better explanation of the pollination syndrome for *P. lasianthos*.

Chapter 5

The evolution of pollination in *Prostanthera* inferred from morphological and phylogenetic analyses

Introduction

A high proportion of ornithophily is one of the distinctive features of the Australian flora (Paton 1986). It makes up approximately 1% of pollination in angiosperms, yet in some parts of Australia it is assumed to be as high as 15% (Proctor and Yeo 1973; Ford 1985b; a; Lloyd 1985). The chief bird pollinators of Australia are the honeyeaters (Meliphagidae) and are the second most speciose group of nectarivorous birds (compared with the hummingbirds) in the world. Their centre of diversity is in Australia (Ford 1985a; b; Christidis and Schodde 1993; Driskell and Christidis 2004) and it is presumed that this may be correlated with the paucity of social bees (Apidae) on the continent (Ford 1985b; a).

Ornithophilous characteristics readily distinguish *Prostanthera* section *Klanderia* from *Prostanthera* section *Prostanthera*. Compared with section *Prostanthera*, taxa of section *Klanderia* have an increased abaxial lobe reflexion and a narrow corolla tube (Chapter 3). The recent pollinator observations for representatives of each group demonstrated that these characteristics are borne by species that are visited by higher numbers of bird pollinators than insect pollinators (Chapter 4). In the absence of an

evolutionary context, however, it is important to recognise that these morphological differences do not indicate how reproductive biology has evolved (Felsenstein 1985b). In the Lamiaceae, the ornithophilous condition has been derived from entomophily in several unrelated genera, corresponding with the same floral characteristics (Huck 1992b). As previously shown for similarly constructed flowers of *Penstemon* and *Keckiella*, the evolution of ornithophily may be a process of constrained evolution, where no evolutionary shifts have gone backwards. Since the phylogeny has recently become available, I would like to investigate how bird pollination has evolved in *Prostanthera*.

The evolution of pollination has been studied using a phylogeny mapped with pollination syndrome states (Baum *et al.* 1998; Weller *et al.* 1998; Givnish *et al.* 2000; Hartmann *et al.* 2002; Perret *et al.* 2003; Ollerton *et al.* 2009b; Alcantara and Lohmann 2010), or with quantitative floral characteristics that define a pollination syndrome (Goldblatt and Manning 1996; Bruneau 1997; Weller *et al.* 1998; Beardsley *et al.* 2003; Dupont *et al.* 2004; Ree 2005; Friedman and Barrett 2008). Flower shape is an important characteristic of a pollination syndrome, but until recently, its objective quantification has been limited. One investigation into the floral evolution of *Saintpaulia* and *Streptocarpus* measured 44 floral characteristics in order to quantify floral shape and then reconstruct the ancestral states (Harrison *et al.* 1999). In Chapter 3 I demonstrated that relative warps analysis is another method that can quantify differences in floral shape and then also permit the visualisation of these changes. Recent advances now permit landmark data to be mapped onto phylogenies so that ancestral shapes may be reconstructed and visualised (Klingenberg 2008). The application of this technology has been limited primarily to studies of skeletal shape

change in humans and other vertebrates (Slice 2007). However, relative warps analysis has been successfully used for a variety of plant specimens (Jensen *et al.* 2002; Gómez *et al.* 2006; Jacques and Zhou 2010) and so the reconstruction of ancestral shapes provides a new opportunity to investigate morphological evolution in plants.

The evolution of pollination in *Prostanthera* is investigated by interpreting pollination syndromes from ancestral shapes inferred using morphological and phylogenetic data. The landmark data for 17 species representing the phylogenetic diversity of *Prostanthera* was acquired from the morphological study conducted in Chapter 3. Chloroplast and nuclear sequence data were acquired for the very same subset from the larger phylogenetic study of *Prostanthera* (Chapter 2). The aim of this chapter is to understand the ancestral pollination syndrome of *Prostanthera* and the subsequent morphological changes that have taken place to produce a new pollination syndrome.

Materials and methods

Specimens

Seventeen species of *Prostanthera* used for this study are listed in (Table 5.1).

Morphological data were acquired from the corresponding samples used in Chapter 3.

The chloroplast (*trnT-F* and *ndhF-rpl32*) and nuclear (ETS) sequence data were acquired from the corresponding samples used in Chapter 2. The outgroup *Westringia* used the morphological data of *W. senifolia* and the sequence data of *W. longifolia*.

Phylogenetic analysis

The phylogenetic analysis followed the same procedures listed in Chapter 2. This study analysed nuclear (ETS) and plastid (*trnT-F* and *ndhF-rpl32*) nucleotide sequence as a concatenated matrix (Appendix 5.1). Maximum parsimony (MP), and bootstrap analysis were conducted using PAUP*4.0b10 (Swofford 2002). A bootstrap (BS) value of 95% - 100% is considered strong support, 75-95% is considered moderate support, and 50-74% is considered weak support. Congruence amongst data sets (*trnT-F*, *ndhF-rpl32*, ETS) was examined using the incongruence length difference test (ILD). A reverse constraint analysis was used to test the results of the heuristic search. Bayesian inference (BI) analysis was conducted with Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001). Strong support is considered as a prior probability (PP) of 95% and above. The search was conducted with the transversional model “TVM+G” as indicated by Modeltest (Posada and Crandall 1998). The number of substitution types was set to 6 and the rate variation across sites was set as gamma-distributed.

Morphometric analysis

Using the same procedures as in Chapter 3, TPSdig (Rohlf 2006) was used to select ten landmarks on three replicate flowers of each species. A consensus of the three replicates was used as the representative shape file for each species (Appendix 5.2). Relative warps analysis was performed on this data using MorphoJ version 1.01c (Klingenberg 2008). This program allowed relative warps analysis to be conducted on separate components of shape change using the methods described by Klingenberg *et al.* (2002). Shape change consists of asymmetric and symmetric components for shapes with object symmetry (Klingenberg *et al.* 2002). This includes the bilaterally symmetric flowers of

Table 5.1. Species of *Prostanthera* and *Westringia* used for phylogenetic and morphological analyses. The data provided from each voucher are listed as: D= nucleotide sequence; M= morphological. Abbreviations: B.G.= botanic garden; N.P.= national park; N.R.= nature reserve; S.F.= state forest.

Study Species	Locality	State	Voucher #	Data
<i>Prostanthera aspalathoides</i> Benth.	Gubbatta N.R.	NSW	NSW803944	M/D
	Taleeban N.R.	NSW	NSW803918	M
	Hiawatha S.F.	NSW	NSW803950	M
<i>P. chlorantha</i> (F.Muell.) Benth.	Currency Creek	SA	NSW803952	M
	Kangaroo Island	SA	NSW743683	D
<i>P. cruciflora</i> J.H.Willis	Mount Kaputar N.P.	NSW	NSW803951	M
	Mount Kaputar N.P.	NSW	AE922944	D
<i>P. cuneata</i> Benth.	Mt. Buffalo N.P.	VIC	NSW844231	M/D
<i>P. lasianthos</i> Labill.	Blue Mountains N.P.	NSW	NSW799707	M/D
<i>P. linearis</i> R.Br.	Brisbane Waters N.P.	NSW	NSW784600	M/D
<i>P. monticola</i> B.J.Conn	Mount Buffalo N.P.	VIC	NSW803943	M/D
<i>P. nivea</i> Benth. var. <i>induta</i>	Warrumbungles N.P.	NSW	NSW803949	M/D
<i>P. nivea</i> Benth. var. <i>nivea</i>	Mt. Kaputar N.P.	NSW	NSW803947	M
	Central Tilba	NSW	NSW844232	D
<i>P. ovalifolia</i> R.Br.	Mendooran	NSW	NSW803953	M
	Whian Whian S.F.	NSW	NSW4200941	D
<i>P. porcata</i> B.J.Conn	Buddawang N.P.	NSW	NSW779742	M
	Deua N.P.	NSW	Eurobodella B.G.#35	D
<i>P. ringens</i> Benth.	Pilliga S.F.	NSW	NSW803945	M
	Goonoo S.F.	NSW	NSW844195	D
<i>P. rotundifolia</i> R.Br.	Mt. Buffalo	VIC	NSW779804	M
	East Gippsland	VIC	NSW494368	D
<i>P. saxicola</i> R.Br. var. <i>montana</i>	Blue Mountains	NSW	NSW803916	M/D
<i>P. serpyllifolia</i> (R.Br.)Briq. subsp. <i>serpyllifolia</i>	Pulletop N.R.	NSW	NSW813913	M/D
<i>P. sieberi</i> Benth.	Royal N.P.	NSW	NSW844227	M/D
	Illawarra N.R.	NSW	NSW803920	M
<i>P. staurophylla</i> F.Muell.	Mt. Mackenzie N.R.	NSW	NSW785873	M/D
<i>P. striatiflora</i> F.Muell.	Mt. Yardi	SA	NSW449546	M/D
<i>Westringia longifolia</i> R.Br.	Tahmoor	NSW	NSW4166185	D
<i>Westringia senifolia</i> F.Muell.	Mt. Buffalo N.P.	VIC	NSW799706	M

Prostanthera. Changes that are replicated across a shapes' plane of symmetry are represented by the symmetric shape component. Changes that are not replicated across this plane are represented by the asymmetric shape component. It is recommended that separate analyses are conducted on bilaterally symmetric shapes because asymmetries can distort the important changes in symmetric shape and they can play an important role in the evolution of a bilaterally symmetric shape (Slice 2007; Mitteroecker and Gunz 2009).

Ancestral state reconstruction

MorphoJ version 1.01c (Klingenberg 2008) was used to conduct the following ancestral state reconstruction and evolutionary principal components analysis (EPCA). Ancestral flower shape at internal nodes was reconstructed by mapping the morphometric data onto the MP and BI trees. Morpho J uses squared-change parsimony to reconstruct ancestral shapes (Maddison 1991). This algorithm minimizes the sum of the squared changes on branches of a phylogeny. It emulates a Brownian motion model to overcome the problem of non-independence due to phylogenetic similarities (Felsenstein 1985b; Rohlf 2001). The analysis treats polytomies as multiple descents from the same ancestor (i.e. 'hard') rather than a result of poor resolution. Evolutionary principal components analysis (EPCA) was used to investigate patterns in shape change within the MP and BI trees. This is a principal components analysis of shape change from one node to the next on each branch of the phylogeny.

Results

Phylogenetic analysis

The incongruence length difference (ILD) test does not reject the data as significantly homogenous (p-value = >0.05) for any combination of the chloroplast and nuclear markers in the dataset (Table 5.2).

The results of a chi-square test on the base frequencies for each marker indicate that the frequencies do not significantly stray from compositional homogeneity (Table 5.3). This indicates that compositional bias does not play a significant role in these data. Tree information and statistics are listed for the maximum parsimony (MP) and Bayesian inference (BI) analyses (Table 5.4). The reverse constraint analysis found no trees with the same or shorter number of steps than the most parsimonious tree.

All trees recovered from BI and MP analyses have a similar topology to the results of the combined data set (Fig. 5.1). The MP tree resolves *Prostanthera* as monophyletic with strong support (BS= 100%) but the BI tree does not. Both trees recover all species of section *Klanderia* at a poorly resolved internal node; in the BI tree they are placed in a derived clade with *P. cuneata*. Three areas of strong support are consistent between both trees. *Prostanthera lasianthos* and *P. linearis* form a clade sister to most of *Prostanthera* (BS= 97%, PP= 1.0). *Prostanthera aspalathoides*, *P. chlorantha*, and *P. serpyllifolia* form a derived clade amongst section *Klanderia* (BS= 100%, PP= 1.0). *Prostanthera cruciflora*, *P. ovalifolia*, *P. rotundifolia*, and *P. sieberi* belong to section *Prostanthera* and form another derived clade (BS= 100%, PP= 1.0).

Table 5.2. P-values for the incongruence length difference test for all combinations of the chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) markers of *Prostanthera*.

Marker	<i>trnT-F</i> + <i>ndhF-rpl32</i>	<i>trnT-F</i> + ETS	<i>ndhF-rpl32</i> +ETS	All
p-value	0.474	0.301	0.099	0.089

Table 5.3. Base frequencies of the individual data sets and concatenated data set of chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) markers.

<i>Base frequencies and X² test</i>	Plastid		Nuclear	All Markers
	<i>trnT-F</i>	<i>ndhF-rpl32</i>	ETS	
A	0.31814	0.33335	0.23748	0.30822
C	0.16727	0.15785	0.27376	0.18269
G	0.16179	0.12990	0.29535	0.17677
T	0.35279	0.37890	0.19342	0.33232
X ²	1.597066	8.631344	6.050376	3.151423
degrees freedom	51	51	51	51
p-value	1.00	1.00	1.00	1.00

Table 5.4. Statistics for maximum parsimony analysis and Bayesian inference analysis of the concatenated data set of chloroplast (*trnT-F*, *ndhF-rpl32*) and nuclear (ETS) markers.

Total number of taxa	18	Tree length	291
Number of ingroup taxa	17	Reverse constraint analysis score	292
Aligned length	2695	Consistency index	0.577
Total indels	134	Retention index	0.626
Parsimony informative indels	40	Rescaled consistency index	0.361
Parsimony informative characters	95	nodes resolved from strict consensus	12
Total parsimony informative characters	135	nodes resolved with Bootstrap values $\geq 75\%$	6
		nodes resolved with prior probability ≥ 0.95	4

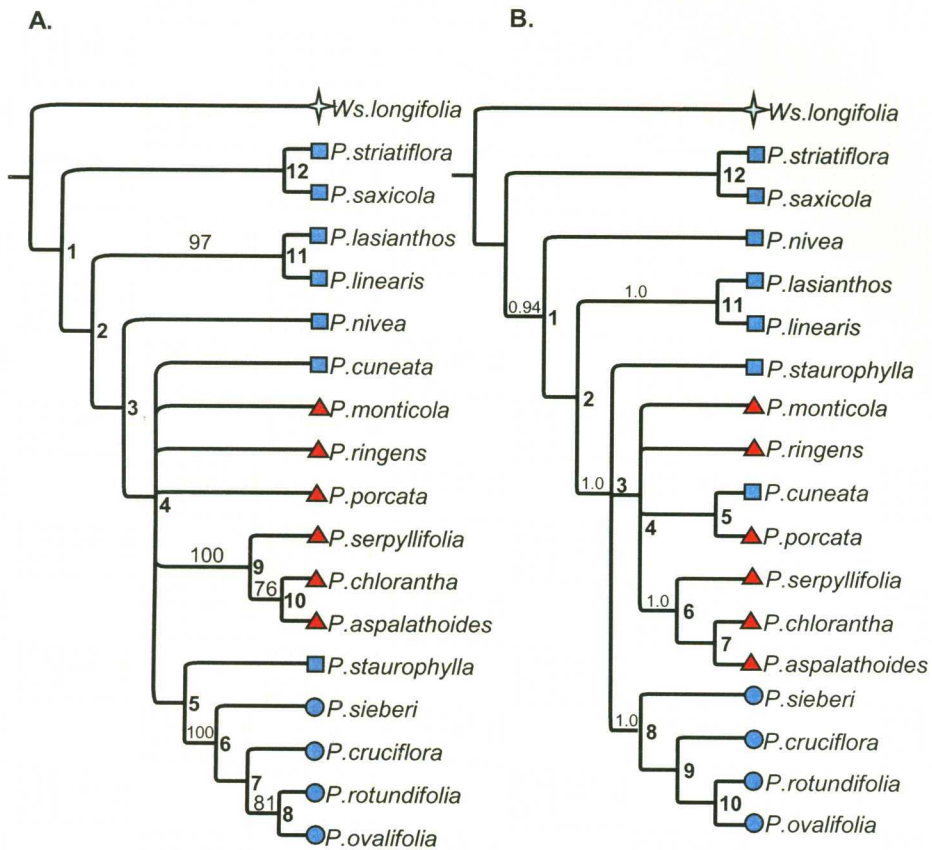


Figure 5.1. Maximum parsimony and Bayesian inference trees of the combined chloroplast (*trnT-F* and *ndhF-rpl32*) and nuclear (ETS) regions. **A:** strict consensus of 12 most parsimonious trees with bootstrap values ($\geq 75\%$) **B:** 50% majority rule from Bayesian analysis showing posterior probabilities (≥ 0.95). Node numbers are given to the right of their respective node. Symbols represent groups identified from Chapter 3 and colours represent sectional classification. triangle= group 1; circle= group 2a; squares= group 2b; red = *Prostanthera* section *Klanderia*; blue = *Prostanthera* section *Prostanthera*.

Morphometric analysis

In relative warps analysis of the symmetric component, two groups corresponding to section *Prostanthera* and section *Klanderia* are recovered in the ordination along relative warp one (RW1) (Fig. 5.2A). Two subgroups of section *Prostanthera* are divided along RW2. Together, RW1 and RW2 account for 79.1% (65.2% and 13.9%, respectively) of the symmetric component of shape.

The deformation of a thin-plate spline by each relative warp is illustrated in figure 5.3A. The deformations of RW1 are a shift in size proportion between the adaxial median lobe-pair (refer to Chapter 1 for definition) and the abaxial lobes (abaxial lobe and lateral lobes) of the flower (Fig. 5.3A, RW1). A positive amplitude (indicated by arrows) displaces the landmarks of the adaxial median lobe-pair upwards, increasing the size of the adaxial lobes. Arrows also indicate that the landmarks representing the lateral lobes are displaced downwards as well as inwards, decreasing the size of the abaxial lobes.

The deformations of RW2 are a shift between a bilaterally symmetric shape and a more radially symmetric shape based on changes in orientation of the lateral lobes and the adaxial median lobe-pair (Fig. 5.2A, RW2). A positive amplitude (indicated by arrows) displaces the landmarks representing the lobe tips of the adaxial median lobe-pair upwards, increasing the size of each lobe. Arrows also indicate that the landmarks representing the tips of each lateral lobe are displaced upwards.

No pattern corresponding to sectional classification is observed in the ordination of the asymmetric shape component (Fig. 5.2B). Relative warp 1 and RW2 account for 57.5%

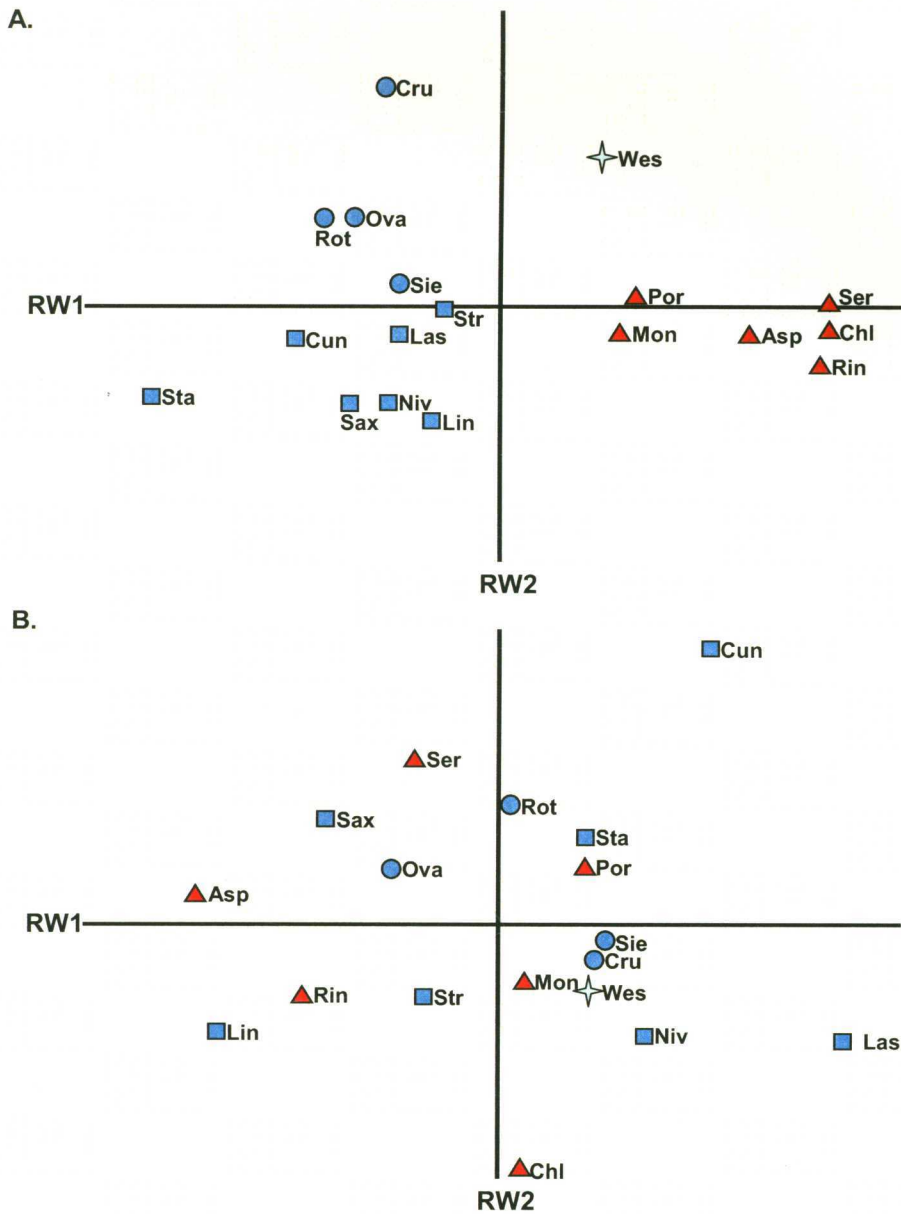


Figure 5.2. Ordination from relative warps analysis of flower shape for (A) the symmetric component and (B) the asymmetric component. Symbols represent groups identified from Chapter 3 and colours represent sectional classification. Triangle= group 1; circle= group 2a; squares= group 2b; star= *Westringia*; red = section *Klanderia*; blue = section *Prostanthera*. Species abbreviations are as follow: Asp=*P. aspalathoides*; Chl=*P. chlorantha*; Cru=*P. cruciflora*; Cun=*P. cuneata*; Las=*P. lasianthos*; Lin=*P. linearis*; Mon=*P. monticola*; Niv=*P. nivea*; Ova=*P. ovalifolia*; Por=*P. porcata*; Rin=*P. ringens*; Rot=*P. rotundifolia*; Sax=*P. saxicola*; Ser=*P. serpyllifolia*; Sie=*P. sieberi*; Sta=*P. staurophylla*; Str=*P. striatiflora*; Wes=*Westringia*.

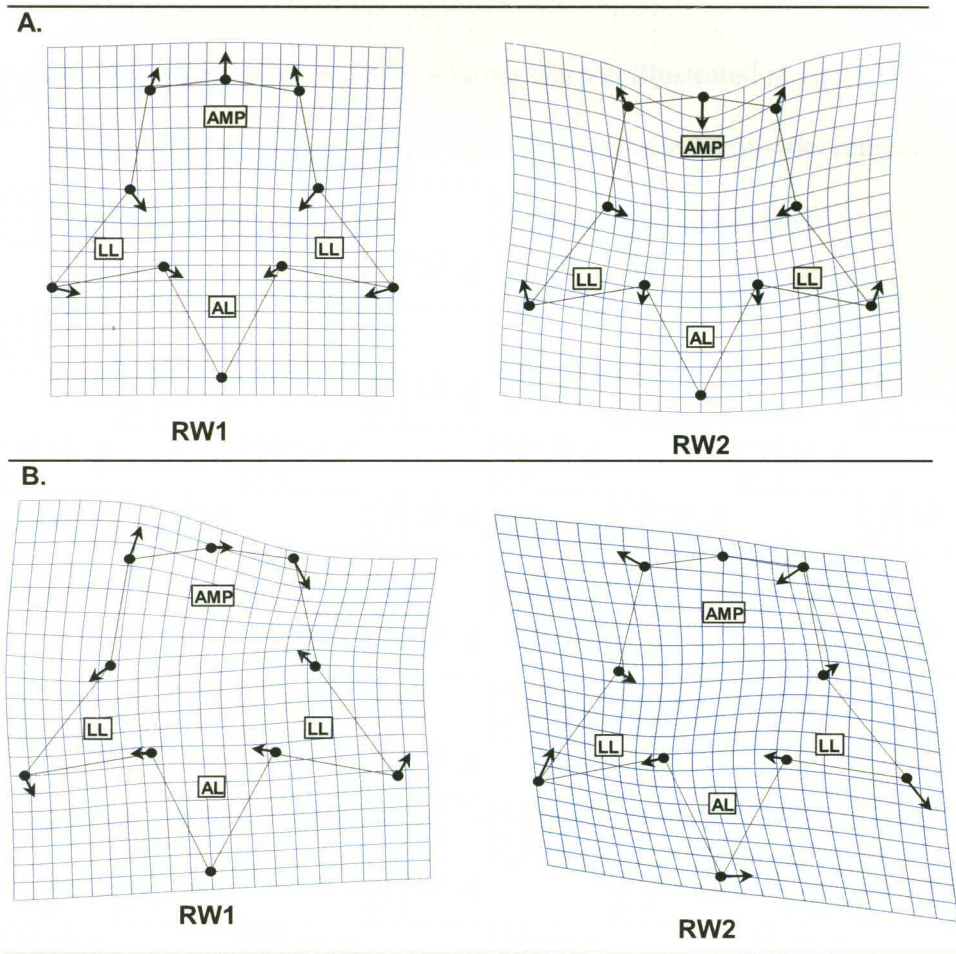


Figure 5.3. Thin-plate splines demonstrating the deformations of relative warp 1 (RW1) and relative warp 2 (RW2) from relative warps analysis of the (A) symmetric data set and the (B) asymmetric data set. Direction and length of arrows indicate the magnitude of positive amplitude deformation on each landmark. Abbreviations address the components of the flower. AL= abaxial lobe; AMP= adaxial median lobe-pair; LL= lateral lobe.

(34.9% and 22.6%, respectively) of the asymmetric component of shape. The deformation of a thin-plate spline by each relative warp is illustrated in figure 5.3B. A positive amplitude (indicated by arrows) of RW1 displaces the landmarks representing one lateral lobe upward while those of the other are displaced downwards.

Simultaneously, the landmarks of the two adaxial lobes are displaced in an opposite direction from each other. A positive amplitude (indicated by arrows) of RW2 displaces the landmarks representing one lateral lobe downward while those of the other are displaced upwards. Simultaneously, the landmarks representing the adaxial lobes are displaced in a similar direction to one side of the shape.

Evolutionary analysis

The MP strict consensus tree and BI tree were separately mapped onto the ordination of relative warps analysis using unweighted squared-change parsimony (Fig. 5.4A and Fig. 5.4B, respectively). For each ordination, an evolutionary principal components analysis (EPCA) was conducted for the changes along each of the branches (Fig. 5.5).

The reconstructed ancestral state of *Prostanthera* in BI and MP trees is a narrow, bilaterally symmetric flower with large distinctive adaxial lobes (Fig. 5.4). From the tree root to node 3, floral shape widens and the adaxial lobes become indistinguishable. These changes continue towards members of group 2b. From node 3 to node 4 the shape of the flower narrows and the adaxial lobes become larger than the abaxial lobes; this change continues from node 4 to all taxa of section *Klanderia*. An exception to this is in the BI tree where there is a fluctuation in floral width from node 4 through to *P. porcata*. From node 4 to node 5 in the BI and MP trees the adaxial median lobe-pair

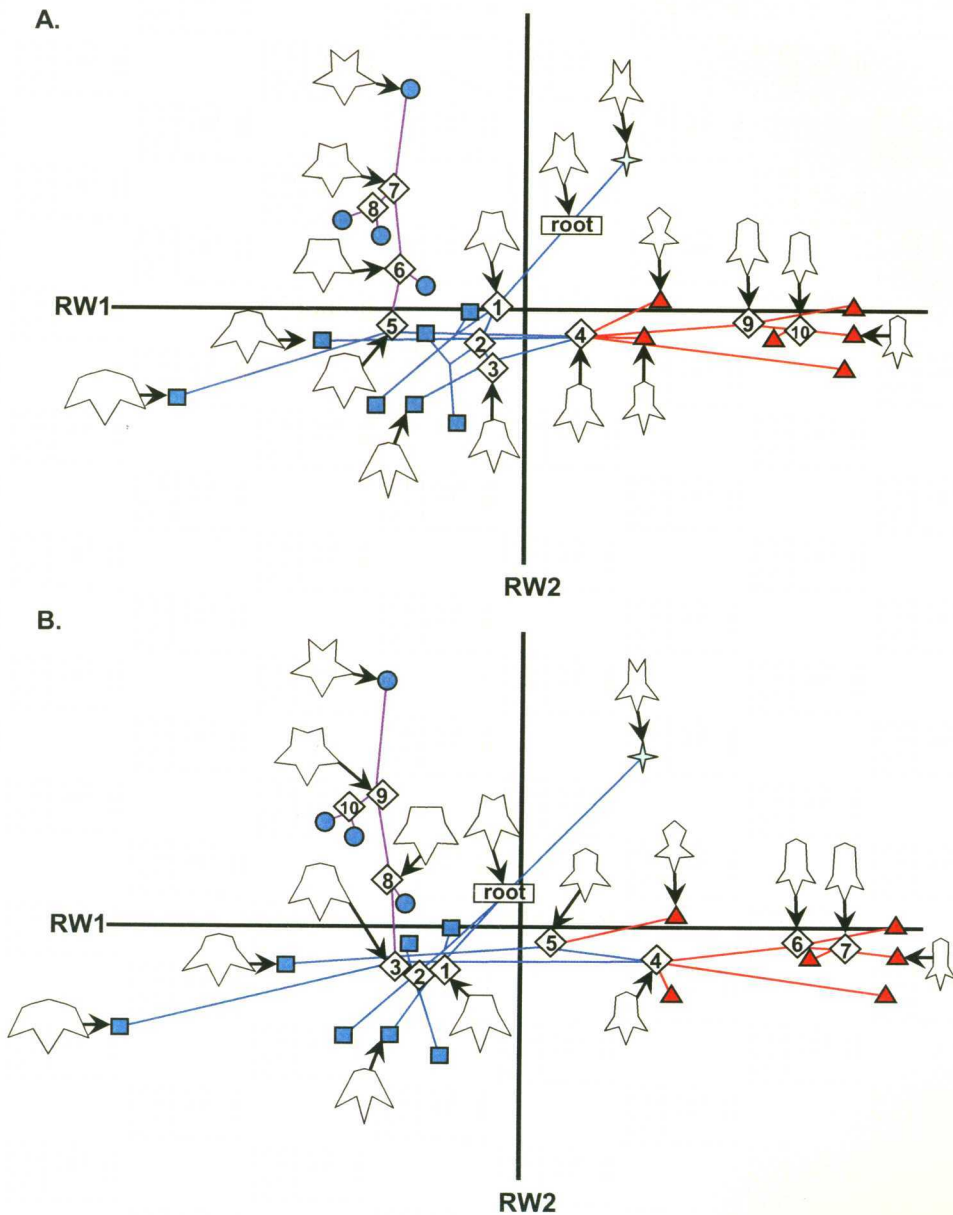


Figure 5.4. The phylogeny of *Prostanthera* constructed from maximum parsimony (A) or Bayesian (B) methods is mapped on the relative warps ordination of the symmetric component. OTU's of the ordination form the terminal nodes and are coded with the symbols that distinguish groups described in Chapter 3 and are coloured corresponding to sectional classification. Internal branches are also coloured in this fashion. Internal node numbers correspond with numbering in Figure 5.1. Reconstructed shape outlines are drawn for most internal nodes. Triangle= group 1; circle= group 2a; square= group 2b; Star= *Westringia*; red= section *Klanderia*; blue= section *Prostanthera* (group 2b); purple= section *Prostanthera* (group 2a).

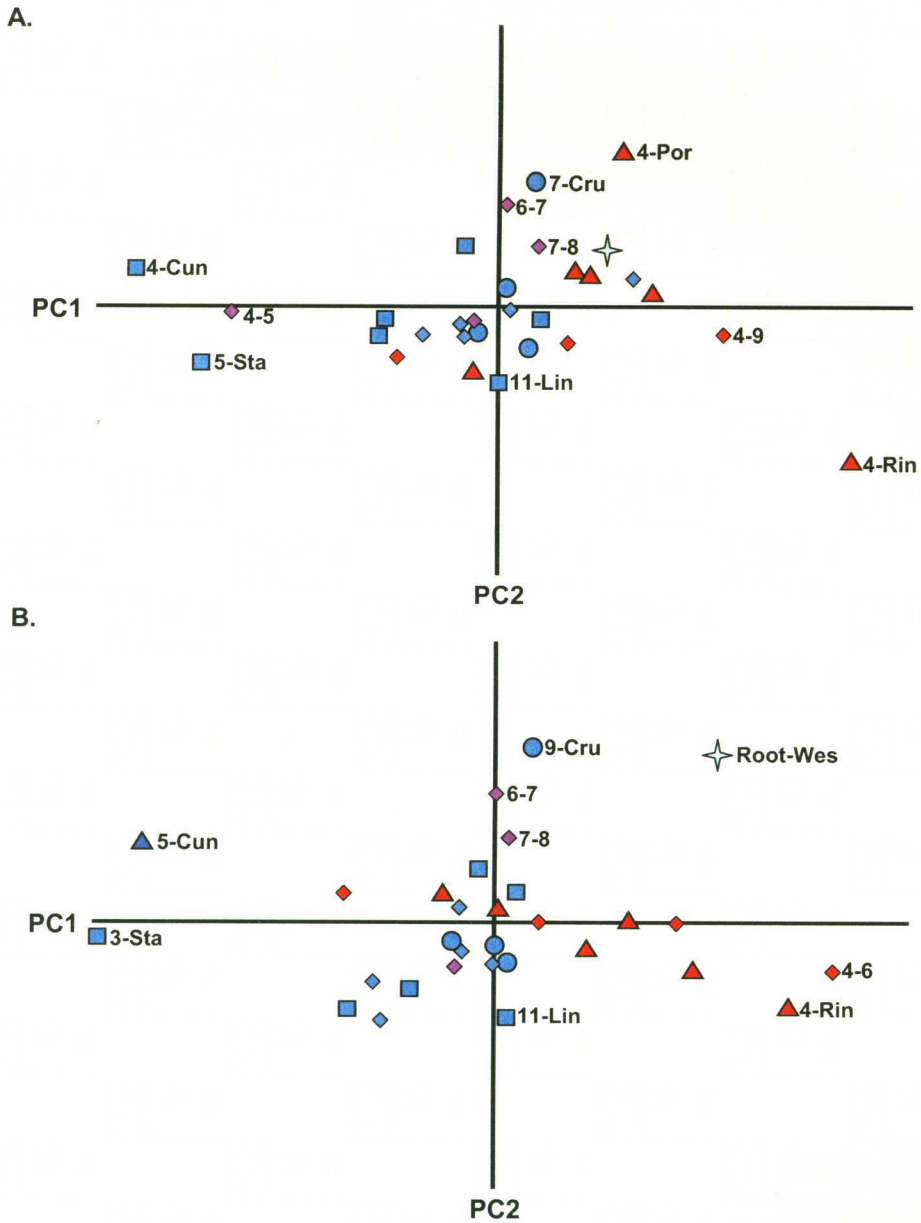


Figure 5.5. Evolutionary principal components analysis of the phylogeny of Figure 5.4. (A) uses the strict consensus tree from maximum parsimony analysis and (B) uses the 50% majority rule tree from Bayesian analysis. Branches are labeled using node labels and taxon abbreviations described from figure 5.2. Symbols represent groups identified from Chapter 3 and colours represent sectional classification. Triangle= group 1; circle= group 2a; square= group 2b; star= *Westringia*; red= section *Klanderia*; blue= section *Prostanthera* (group 2b); purple= section *Prostanthera* (group 2a).

becomes smaller than the abaxial lobes as the flower shape broadens. This change continues to *P. cuneata* and *P. staurophylla*. Both trees demonstrate similar changes from group 2b towards the taxa of Group 2a: the lobes of the adaxial median lobe-pair become larger and the orientation of the lateral lobes changes from pointing downward to horizontal. This occurs at node 5 in the MP tree whereas it occurs at node 3 in the BI tree.

Evolutionary principal components analysis (EPCA) of branch changes for the MP tree has a principal component one (PC1) and principal component two (PC2) that represent 76.0% (63.1% and 12.8%, respectively) of the variation (Fig. 5.5A). The positive direction of PC1 corresponds to negative change in RW1. Most branches supporting section *Klanderia* species have positive PC1 values and most branches supporting section *Prostanthera* have negative PC1 values. Two branches (branches 4-Rin and 4-9) have distinctively large positive PC1 values and three branches (4-Cun, 5-Sta, and 4-5) have distinctively large negative values of PC1. The positive direction of PC2 corresponds to a positive change in RW2. Most branches supporting species from Group 2a (as defined from Chapter 3) have positive PC2 values.

The EPCA using the BI tree has a PC1 and PC2 that represent 76.7% (62.0% and 14.6%, respectively) (Fig. 5.5B). The change along PC1 and PC2 is similar to the change described in the ordination using the MP tree. Most branches supporting section *Klanderia* species have positive PC1 values and most branches supporting section *Prostanthera* have negative PC1 values. The two branches 4-Rin and 4-6 have the largest positive PC1 values and the two branches 5-Cun and 3-Sta have the largest negative values of PC1. The positive direction of PC2 corresponds to a positive change

in RW2. Branches that support species from Group 2a and *Westringia* have positive PC2 values.

Discussion

The acquisition of morphological and molecular data in previous analyses provides the opportunity to investigate how pollination has evolved in *Prostanthera*. The reconstruction of ancestral shapes suggests that ancestral *Prostanthera* was entomophilous and has recently evolved ornithophilous species. An entomophilous flower shape more radially symmetric than the ancestral flower shape evolved through a change in orientation and reflexion of the adaxial and lateral lobes.

Phylogenetic analysis

Phylogenetic analyses produced a topology consistent with the relationships inferred by MP and BI phylogenies in Chapter 2. The consistencies include that *P. lasianthos*, *P. linearis*, *P. nivea*, *P. saxicola*, and *P. striatiflora* all emerge from the base of the phylogeny, most section *Klanderia* species are derived from a similar node nested within the phylogeny, and *P. cruciflora*, *P. ovalifolia*, *P. rotundifolia*, and *P. sieberi*, form a group resembling clade F from the results of Chapter 2.

Morphological analysis and interpretation of pollination syndromes

Relative warps ordination of the asymmetric shape component demonstrates no pattern corresponding to sectional classification or relationships recovered from phylogenetic analysis. The relative warps in this analysis also only account for an approximate half

of the shape change. These results support that floral asymmetry does not describe relationships within *Prostanthera*.

Relative warps ordination of the symmetric shape component distinguishes section *Klanderia* from section *Prostanthera* as a narrower flower shape with smaller abaxial lobes and a larger adaxial median lobe-pair. These characteristics are representative of many ornithophilous characters that exclude insect visitation (Faegri and Van der Pijl 1979; Cronk and Ojeda 2008). A narrow width of the flower corresponds to a narrow tube width that prevents insects from crawling inside the flower. The reduction of abaxial lobes denies an insect a suitable landing pad to gain entry to the flower. A large adaxial median lobe-pair increases the distance from corolla mouth to nectary and makes it difficult for insects without long mouthparts to reach a nectary. Section *Prostanthera* has flowers with a shorter, wider floral tube and large landing pads so that insects can land on and enter the corolla. Based on the quantified floral visitation by pollinators in Chapter 4, these distinct morphologies are supported as predominantly ornithophilous and entomophilous syndromes, respectively.

Relative warps analysis of the symmetric shape component also demonstrates the reorientation of lateral lobes and adaxial median lobe-pair between two clades within section *Prostanthera*. The flower shape of group 2a is more radially symmetric and the flower shape of group 2b is more bilaterally symmetric (see Fig. 3.1a and Fig. 3.1b for a comparison with flower images). The change in the shape of the adaxial median lobe-pair has previously been linked to the reflexion of each lobe (Chapter 3), which creates a more open and accessible corolla tube. It appears that these differences indicate two different pollination syndromes. A bilaterally symmetric flower corresponds with

melittophily based on its powerful attracting cue to bees (Westerkamp and Claßen-Bockhoff 2007). A radially symmetric flower with an open corolla tube corresponds with general entomophily since it provides few restrictions to visitors. However, based on the quantification of pollinators (Chapter 4), these morphologies both receive a wide diversity of floral visitors.

Evolution of pollination

The inferred ancestral shape of *Prostanthera* is a bilaterally symmetric flower with a wide floral tube and a large abaxial lobe. This suggests that ancestral *Prostanthera* was likely generalist entomophilous. An ornithophilous syndrome subsequently evolved as the width of the floral tube decreased and the abaxial lobes reduced and/or became reflexed. Similar floral changes from an entomophilous pollination syndrome are inferred for the evolution of ornithophilous taxa within the Antirrhinae, *Mimulus*, *Penstemon*, and *Salvia* (Ghebrehiwet *et al.* 2000; Beardsley *et al.* 2003; Wester and Claßen-Bockhoff 2007; Wilson *et al.* 2007). Change to ornithophily is likely to have occurred at an accelerated rate since the EPCA demonstrates the largest amount of change from the ancestor of section *Klanderia* (Fig. 5.4, node 4) to most nodes in section *Klanderia*. This corresponds to the rapid radiation of ornithophilous species inferred from the short branch lengths by BI trees in Chapter 2. The results also infer that a more radially symmetric floral morphology has recently evolved. The BI tree infers that this morphology evolved through the reflexion of the adaxial median lobe-pair and a change in orientation in lateral lobes of the ancestral flower. Since the MP tree is poorly resolved it does not reject that this inferred entomophily evolved from ornithophily. However, a reverse change from ornithophily to this entomophilous syndrome seems unlikely since this process is rarely observed and is regarded as

constrained evolution (Bruneau 1997; Wilson *et al.* 2007). It is then likely that the recently derived flower type evolved from the bilaterally symmetric flower morphology. The visitation data of Chapter 4 reject that the bilaterally symmetric flower morphology is melittophilous. However, as was discussed in Chapter 4, present pollinator guilds may not necessarily be representative of those in the past. Therefore, the current radially symmetric flower morphology could have evolved from a melittophilous syndrome under the pollinator selection pressure of a general suite of insects that are now observed visiting species of *Prostanthera*. The conflict with this speculation, however, is that the bilaterally symmetric flowers are still observed. This could be due to a change in the “most effective” pollinator rather than the diversity of pollinators (Stebbins 1970).

It is difficult to infer the timing of floral evolution for *Prostanthera* since the fossil record is poor in the Lamiaceae and non-existent in the Prostantheroideae (Crane *et al.* 2004; Harley *et al.* 2004; Crepet 2008). The correlation between pollinators and floral type suggests, however, that ancestral *Prostanthera* did not evolve in Australia if it had a putative melittophilous syndrome. The arrival of bees to Australia appears to have occurred only 35 million years ago (mya) (Leys *et al.* 2002) whereas ancestral Lamiaceae is suggested to have evolved in the early Paleocene (65 mya) (Friis and Crepet 1994). It is possible that when an ancestral lineage of Lamiaceae entered Australia it encountered a pollinator selection pressure not so heavily influenced by bees. Even if bees had arrived beforehand their efficiency as pollinators was likely compromised by the cooling trend in Australia that had already started around the beginning of the Oligocene (35mya) (Martin 2006). Solitary bees in Australia function mainly at the highest ambient temperatures concentrated in the middle of the day (Paton

1993). The social bees (Apidae) that have the behavioural mechanisms to function for longer are poorly represented in Australia (Ford 1985b). Pollinators that were more reliable at cooler temperatures were better pollinators and influenced floral change. Flies are an important pollinator for many plants in colder extremes such as tundra or Alpine areas where bees may even be absent (Kevan and Baker 1983; Inouye and Pyke 1988; McCall and Primack 1992; Hingston and McQuillan 2000). Birds are another ideal pollinator in cooler conditions or inclement weather because they are homeothermic (Stiles 1975; Ford *et al.* 1979; Ford 1985b). Based on the superficial floral shapes across the Westringieae it appears that similar selection pressures have influenced similar evolutionary trends in other genera as well as *Prostanthera*. The ornithophilous flower shape appears to have independently evolved in *Hemigenia*, *Hemiandra*, and *Microcorys* (Guerin 2005). *Westringia* is also very similar in appearance to the generalist entomophilous flower of *P. cruciflora* (Chapter 4) and relative warps analysis show that it has similar deformations of shape. An extension of this phylogenetic and morphological investigation to the entire tribe would provide a better understanding of the processes influencing what appears to be multiple convergences on these two pollination syndromes.

A limitation to this study is that the inference of the ancestral floral shape of *Prostanthera* is based on the analysis of *Prostanthera* and *Westringia*. Only a single species of *Westringia* represents an entire clade comprising *Westringia*, *Hemigenia*, *Hemiandra*, and *Microcorys*. It seems possible that the ancestral shape should be represented by these results since *Hemigenia* and *Microcorys* flowers appear similar to *Westringia* (Guerin 2005). However, a more accurate reconstruction of the ancestral *Prostanthera* shape will require data from all genera in the Westringieae. Similarly, a

reconstruction of the ancestral shape for the Westringieae will require good sampling from the Chloantheae.

Conclusions

This study is the first to use ancestral shape reconstruction with landmark data in order to infer the evolution of pollination syndromes. The inferred floral shape of ancestral *Prostanthera* was entomophilous and through a rapid change in morphology an ornithophilous clade has evolved with another entomophilous clade characterised by a more radially symmetric flowers. Poor resolution in the phylogeny prevents a clearer understanding about how the clades with new pollination syndromes evolved in relation to each other.

Chapter 6

Discussion and conclusions



Flowers of *Prostanthera ovalifolia*.

The combined advances in morphological and molecular analyses provide a phylogenetic framework in which the evolution of pollination can be investigated. Although, the monophyly of *Prostanthera* (including *Wrixonia*) has been confirmed by my studies, the traditional infra-generic classification of the genus is not supported. The detailed observations of pollination biology have demonstrated new floral types that provide support for various pollination syndromes. Ornithophily has evolved twice within *Prostanthera* and a new entomophilous pollination mechanism is recognised, distinct from that observed in *Prostanthera sensu stricto*. This chapter discusses these results and their importance to a contemporary understanding of systematics and pollination.

Phylogenetic considerations for *Prostanthera*

The phylogeny of genera within the Westringieae, subfamily Prostantheroideae, is relatively unknown (Olmstead *et al.* 1998; Conn *et al.* 2009). A phylogeny of *Hemigenia* and *Microcorys* (both Westringieae), using *trnT-F* marker and morphological data, concludes that although the tribe is monophyletic, both genera are polyphyletic (Guerin 2008). The phylogeny of *Prostanthera* based on chloroplast (*trnT-F* and *ndhF-rpl32*) and nuclear ETS markers provide additional support for the monophyly of the tribe. Furthermore, it demonstrates that *Prostanthera* is a monophyletic clade sister to *Westringia*, *Hemiandra*, *Hemigenia sensu lato*, and *Microcorys sensu lato*. The diatypic genus *Wrixonia* F.Muell., however, nests within *Prostanthera*, in an unresolved clade with section *Klanderia* (F.Muell.) Benth. Mueller (1876) erected the genus *Wrixonia* to accommodate *W. prostantheroides* F.Muell. However, in doing so, he expressed doubt about the distinctiveness of his new genus, '*Planta, si intra Prostantheram inclusa, P. Wrixoni dicenda*' (Mueller 1876). Since then

the genus has been maintained because *W. prostantheroides* and *W. schultzii* are easily distinguished from the rest of Prostantheroideae by their adaxial staminodes. Although staminodes are present in other genera of the Prostantheroideae (*Hemigenia* and *Microcorys*), they are clearly not homologous since they are formed from the reduction of a locule rather than the reduction of an entire stamen, as is the case in *Wrixonia*. The analysis of *Wrixonia* and *Prostanthera* using molecular data demonstrates that staminal traits do not support two monophyletic groups. Morphological variation of anthers within the Labiate taxon *Salvia* have led to the conclusion it is polyphyletic, consisting of three clades that have independently evolved similar anther morphology (Walker and Sytsma 2007). However, it is difficult to divide *Prostanthera* under a rank-based phylogenetic nomenclature since the phylogeny is a grade. Therefore, the genus *Wrixonia* is reduced to the synonymy of *Prostanthera* and its species are transferred.

Prostanthera prostantheroides (F.Muell.) T.C.Wilson, *comb. nov.*

Basionym: *Wrixonia prostantheroides* F.Muell. *Fragmenta Phytographiae Australiae* 10: 18 (1876)

Type: *J. Young s.n.*, 27 Oct 1875, 'in vicinia montis Churchman,' Western Australia (MEL502314).

Prostanthera schultzii (F.Muell. ex Tate) T.C.Wilson, *comb. nov.*

Basionym: *Prostanthera schultzii* F.Muell. ex Tate, *Botany of the Horn Expedition* 173 (1896).

Synonym: *Wrixonia schultzii* (F.Muell. ex Tate) Carrick, *Journal of the Adelaide Botanic Garden* 1: 28–30 (1976).

Type: *R. Tate s.n.*, Jun 1894, 'summit of Mount Sonder,' Western Australia
(MEL43620).

The description of *Prostanthera*, as provided by Conn (2004), needs to be modified to include the presence of staminodes as found in the above two species. The key to genera (Conn 2004, p. 207) is here modified to accommodate the reduction of *Wrixonia* to the synonymy of *Prostanthera*:

1. Calyx 2-lobed or 2-lipped; anthers 2-theous; stamens 4 or 2; leaves and branchlets frequently aromatic when crushed *Prostanthera*
- Calyx 5-lobed, sometimes almost 2-lipped; anthers 1-theous; stamens 4 or 2; leaves and branchlets non-aromatic 2
2. Stamens 4, all fertile 3
- Stamens 2; staminodes 2 4
3. Anther-connective terminating in a minute lobe (sterile theca), not readily visible *Hemiandra*
- Anther-connective elongated and distinct, abaxial staminal pair terminating in a short, dilated, crested end and adaxial staminal pair attenuated and glabrous, or with an imperfect theca at the end *Hemigenia*
4. Connective of fertile stamens elongated, extending beyond the insertion of the filalament into a short, dilated and bearded end *Microcorys*
- Connective of fertile stamens not or only minutely extended beyond the insertion of the filament *Westringia*

Although *Hemigenia* and *Microcorys* are both polyphyletic (Guerin 2008), insufficient detail is provided to evaluate the full implications on the taxonomy of these genera. Therefore, the above key to genera continues to reflect the broad generic concepts assumed by Conn (2004) .

The infrageneric relationships of *Prostanthera* are poorly resolved in many areas of the phylogeny from each of the three molecular markers (*trnT-F*, *ndhF-rpl32*, and ETS). However, *trnT-F* and ETS markers provide sufficient resolution to make several conclusions about the infra-generic structure of *Prostanthera*. Until now, Bentham's (1870) infrageneric classification of *Prostanthera* has formed the framework for newly described species. The phylogeny supports Bentham's (1870) decision to reduce *Cryphia* R.Br. and *Klanderia* F.Muell. to the synonymy of *Prostanthera* as section *Klanderia* (F.Muell.) Benth. The monophyly of this section cannot be rejected, however, since most of its members form an unresolved clade nested within section *Prostanthera*, the latter section is paraphyletic. Therefore Bentham's (1870) sectional classification is not descriptive of the evolutionary history within *Prostanthera*.

Bentham (1870) recognised three series within section *Prostanthera*, namely series *Racemosae*, series *Convexae*, and series *Subconcavae*. However, these morphologically defined series are not supported by the molecular data used here. From a nomenclatural perspective, the name of series *Racemosae* Benth. must be re-named as series *Prostanthera* because it includes *P. lasianthos* Labill. (type specimen of the genus) (refer Article 22.1, (McNeill *et al.* 2006). This series is here formally reduced to the synonymy of series *Prostanthera*:

Prostanthera Labill. section *Prostanthera* series *Prostanthera*

Synonym: *Prostanthera* Labill. section *Prostanthera* series *Racemosae* Benth., *Flora Australiensis* 5, 91, 93 (1870)

Type species: *Prostanthera lasianthos* Labill.

The phylogeny conflicts with Bentham's series classification, with no well-supported monophyletic clades corresponding to these series being consistently recovered.

However, when these series are mapped onto the molecular phylogeny, many species of series *Subconcaevae* and series *Convexae* form early divergent clades and most species of series *Prostanthera* form a recently derived clade sister to section *Klanderia*.

Therefore, the internal resolution of *Prostanthera* appears to consist of three general groupings as shown in the results of Chapter 2:

1) section *Prostanthera sensu stricto*, which consists mostly of series

Prostanthera

2) section *Prostanthera pro parte*, which consists mostly of series *Subconcaevae* and series *Convexae*

3) section *Klanderia*

However, the molecular phylogeny is poorly resolved and the structure forms a grade, and hence does not support the monophyly of these series as defined by Bentham (1870). An alternative proposal for a sectional classification might be to use the well-supported Clade E and Clade F (Chapter 2) to form two large sections, but then the remainder of the genus would be fractured into numerous small clades that are not supported by any known morphological synapomorphies. Therefore, Bentham's (1870) infrageneric classification is not well supported and should be abandoned. Since the

phylogeny is a grade with many poorly resolved regions, a new sectional classification cannot be established until better resolution is acquired.

Despite its structure as a grade, the phylogeny resolves several groups that appear to be defined by morphological characteristics. It is useful for these groups to be acknowledged should phylogenetic nomenclature be applied to *Prostanthera*. Analyses of morphological data (Chapter 2) demonstrate that representatives of Clade F have shorter anther appendages, more radially symmetric flowers, exserted stigmas, and corolla with reflexed adaxial median lobe-pair in comparison to other species of *Prostanthera*. Members of this clade are also confined to eastern Australia (Althofer 1978; Conn 1992a). Clade E, and the early divergent species of *Prostanthera*, are distinguished from other *Prostanthera* by flowers with a corolla that has a non-reflexed adaxial median lobe-pair, long anther appendages, and stigmas that are not exserted. All results demonstrate that *P. ferricola*, *P. magnifica*, and *P. striatiflora* form a well supported clade. ETS data also infers that they are closely related to *P. althoferi*, *P. behriana*, and *P. wilkeana*. An enlarged adaxial calyx lobe appears to be a distinctive unifying feature among these species (Conn 1988; Conn and Shephard 2007). Clade H contains most species recognised in the *P. calycina*-*P. microphylla*-*P. serpyllifolia* complex, section *Klanderia* (Conn 1984), but no morphological synapomorphies are known.

The phylogeny also indicates the need for taxonomic changes or clarifications of some species. *Prostanthera spinosa* F.Muell and *P. sejuncta* M.L.Will. have a very similar floral morphology and similar habit of lateral shoots terminating in spines. Recently, *P. spinosa sensu lato* was divided into three distinct species: *P. spinosa*, *P. sejuncta*, and

P. arapilensis M.L.Will. using AFLP markers. The current molecular phylogeny supports the taxonomic distinctness of *P. spinosa* and *P. sejuncta*. However, surprisingly, they are not sister taxa and so the spinose habit is homoplastic. To understand the evolutionary history of the spinose habit, *P. nudula* J.M. Black ex E.L. Robertson, another semi-spinose plant (Conn 1988), and *P. arapilensis* should also be included in future phylogenetic analyses.

Prostanthera junonis B.J.Conn is a recently recognised and endangered endemic plant of New South Wales and its affinities were unknown (Conn 1997). The molecular phylogeny suggests that *P. junonis* is closely related to *P. saxicola* R.Br. The ETS data even suggests that it is more closely related to *P. saxicola* var. *major* than *P. saxicola* var. *montana*. This suggests that either the varieties of *P. saxicola* merit the taxonomic rank of species or that *P. junonis* should be reduced to a variety of *P. saxicola*. Since data from other varieties of *P. saxicola* (*P. saxicola* var. *saxicola* and *P. saxicola* var. *bracteolata*) have yet to be included in the analysis, a revision of *P. saxicola* is not yet possible.

Prostanthera sp 'E. sensu B.J.Conn (1992)' was believed to be closely related to *P. linearis* R.Br. (Conn 1992b). However, the phylogeny infers that it is closely related to *P. lasianthos*, since they are consistently placed in a well-supported clade.

Assessment of relative warps analysis

Investigations of pollination syndromes have been hampered by the lack of a method for objectively defining floral shape, which is an important component of a pollination syndrome. Studies that have tested pollination syndromes have resorted to coding

flower shape subjectively, for example, as vestibular or tubular (Wilson *et al.* 2004), or campanulate, subcampanulate, or tubular (Martén-Rodríguez *et al.* 2009). The difficulty with implementing these categories is that apparently subtle differences in shape cannot be reliably assessed. The study of pollination in an evolutionary context has also depended on similar descriptions of shape (Goldblatt and Manning 1996; Bruneau 1997; Baum *et al.* 1998; Johnson *et al.* 1998; Weller *et al.* 1998; Ghebrehiwet *et al.* 2000; Givnish *et al.* 2000; Perret *et al.* 2003; Ree 2005). A recent study by Gómez *et al.* (2006) has employed a more contemporary analysis of shape, namely, relative warps analysis, to successfully quantify differences in floral symmetry of *Erysimum mediohispanicum* (Brassicaceae). I have found that this analytical tool is useful for identifying other characteristics of pollination syndromes in addition to floral symmetry. Furthermore, its sensitivity is similar to a multivariate analysis of flowers, with both analyses showed similar changes amongst features such as lobe reflexion and tube diameter. Multivariate analysis was advantageous since it could also employ measurements not associated with floral shape, such as colour, or anther appendage length. Although relative warps analysis could not measure these additional features, it was unique in that it was able to categorise the specific changes between radial and bilateral symmetry that were associated with changes between the two floral types (floral types 2a & 2b) of section *Prostanthera*. Further studies of pollination syndromes will profit best from the use of both techniques.

Floral morphology and pollination of *Prostanthera*

Floral characteristics have been extensively used to circumscribe flowering plants. For example, many of the genera of the Lamiaceae and, in particular, several of the genera within the subfamily Prostantheroideae are differentiated in part by androecial features

(Harley *et al.* 2004). Since differences in androecial morphology do not reflect the synonymous relationship between *Wrixonia* and *Prostanthera*, the evolution of the androecium should be evaluated for all genera in the Westringeeae. Androecial features have traditionally been used to distinguish *Hemigenia* and *Microcorys* (Carrick 1976). According to the phylogenetic relationships of these latter two genera (Guerin 2008), androecial features appear to be homoplastic.

Floral characteristics have also been used to describe the pollination for several species of *Prostanthera* (Keighery 1980; Conn 1984), yet specific characteristics that support ornithophilous or entomophilous syndromes have not been identified or quantified. Previously, two floral types were recognised, one type represented by section *Klanderia* (Conn 1984) and another type in section *Prostanthera* (Conn 1988). However, the pollination mechanism was believed to be relatively similar throughout the genus (Conn 1984). Morphometric analyses and detailed observation of phenology show that there are three distinct floral types, each with a relatively distinct pollination mechanism. Morphometric analysis and observations confirm that protandrous flowers in the section *Klanderia* have ornithophilous features (Conn 1984) and are here described as floral type 1. The corollas of floral type 1 lack insect landing ‘pads,’ have longer and narrower floral tubes than floral types 2a and 2b (see below), and are red or green. The remaining two floral types (2a & 2b) are present in section *Prostanthera*, both having corollas with entomophilous features, such as insect landing ‘pads,’ short and broad corolla tubes, and purple or white in colour. However, floral type 2a also shares many features with floral type 1: it has a pollen release mechanism that depends on large anther appendages to be dislodged. This requires a more ‘closed’ corolla tube which is necessary to hide the anthers. As the stigma becomes receptive, anthers are relocated

laterally towards the inner side of the corolla. Floral type 2b differs from floral type 2a because it has a more radially symmetric flower, a more open corolla (due to a reflexed adaxial median lobe-pair), and has reduced anther appendages. Unlike the other two floral types, the stigma of floral type 2b is exerted from the corolla and the anthers are never relocated throughout anthesis.

Pollination in *Prostanthera*

Little has been done to test Vogel's (1954) pollination syndromes, and so far, results are equivocal about their existence. Pollination syndromes are shown to exist in some plant groups, but in a limited way (Kay and Schemske 2003; Hargreaves *et al.* 2004; Wilson *et al.* 2004; Wolfe and Sowell 2006; Martén-Rodríguez *et al.* 2009). For example, observations of several species of *Penstemon* (Scrophulariaceae) show they all receive visits by hymenopterans and birds. In this genus, the difference between ornithophilous and entomophilous characteristics significantly corresponds to the difference between bird and bee visitation (Wilson *et al.* 2004). Flower characteristics such as long and narrow floral tubes, large volumes of nectar, reflexed abaxial lobes, and red colour are correlated with higher bird visitation in other plants (Schemske and Bradshaw 1999; Thomson *et al.* 2000; Kay and Schemske 2003; Castellanos *et al.* 2004; Wilson *et al.* 2004; Wolfe and Sowell 2006; Martén-Rodríguez *et al.* 2009). I show that pollination syndromes based on this trend also exist in a limited way for *Prostanthera*. The species in section *Klanderia* have most of these floral features and are correlated with larger numbers of bird visits when compared to species of section *Prostanthera*. The species of section *Prostanthera* have flowers with wide corolla tubes, smaller volumes of nectar, non-reflexed abaxial lobes, and purple or white colours. They have higher insect visitation than flowers of section *Klanderia*. Many species of *Prostanthera* match

ornithophilous or entomophilous pollination syndromes better than *Penstemon* because many species were found to be strictly visited by either birds or insects.

The pollination of a large number of Australian flora is inferred from characteristics associated with pollination syndromes (Keighery 1980); the specific characteristics themselves have never been identified or quantified. My detailed observations of pollination syndromes in *Prostanthera* verify that the ornithophilous versus entomophilous syndromes can be accurately predicted by assessments of floral structures. However, there are occasional exceptions, for example, *P. monticola*, a typical ornithophilous species, is occasionally visited by bees, and the putatively entomophilous species *P. lasianthos* is visited by insects and birds.

The most significant departure from morphologically defined pollination syndromes defined by morphology is the bird visitation observed on *P. lasianthos*, even though the floral characteristics closely resemble those of many entomophilous flowers. This species has large inflorescences that ensure the total available nectar per plant provides energy levels that are comparable to those available in ornithophilous, less floriferous species of section *Klanderia*. Brown (1995) observed this phenomenon occurring between small honeyeaters and entomophilous plants of Papua New Guinea. He suggested that the small mass and foraging efficiency of some small honeyeaters might allow them to exploit the resources from large inflorescences of entomophilous flowers found on weak branches that larger, more aggressive honeyeaters might not defend. Small honeyeaters of New Zealand are also pollinators of putatively entomophilous plants, such as *Dysoxylum spectabile* Hook.f. (Meliaceae) and *Pseudopanax arborescens* K.Koch (Araliaceae) (Anderson 2003). I demonstrate that this case exists

in Australia, between birds such as the eastern spinebill (*Acanthorhynchus tenuirostris*) and the silvereye (*Zosterops lateralis*), and *P. lasianthos*. Ornithophilous plants are normally characterised by having large quantities of nectar (Faegri and Van der Pijl 1979), which is dilute (Petanidou 2005), and contains specific types of sugars (Dupont *et al.* 2004) and amino acids (Baker and Baker 1986; Petanidou *et al.* 2006). These characteristics also need to be quantified in *P. lasianthos* to better understand its pollination syndrome. The results so far, however, suggest that flower number and foraging dynamics of pollinators may need consideration when evaluating pollination syndromes.

In section *Prostanthera*, floral type 2b appears to be a specialist of bees (melittophilous) since it has a complex pollen dispensing device and anthers are hidden within the corolla. Floral type 2a appears to be an entomophilous generalist because it has a more open, cup-like flower that might be accessible to a range of insects. However, a similar, highly diverse suite of insects visit both flower types. These observations do not support the prediction that floral type 2b represents a specialist pollination syndrome. Whilst comparing several genera in the Tasmanian flora and their visitor profiles, Hingston and McQuillan (2000) demonstrated only a few cases where putatively melittophilous flora actually receive different visitors than generalised entomophilous syndromes. Using quantitative analysis of pollinator visits, I support their conclusions. However, the traditionally defined melittophilous syndrome may correspond better with more social bees, such as *Apis* and *Bombus* (Faegri and Van der Pijl 1979), and not to more solitary bees, such as *Leioproctus* or *Lasioglossum*. Both floral types 2a and 2b of *Prostanthera* receive visitors that correspond better with the ‘small bee syndrome’ (Newstrom and

Robertson 2005), which is a generalist pollination syndrome, attracting several groups of non-social bees, flies, and other insects.

Evolution of pollination in *Prostanthera*

The floral morphology of type 2b from section *Prostanthera* is inferred as the ancestral flower morphology of *Prostanthera*. If floral morphology is interpreted as pollination syndrome then ancestral *Prostanthera* is entomophilous. Subsequently, an ornithophilous morphology evolved once to many times from the ancestral entomophilous lineage, or it evolved once and subsequently there was at least one reversion to an entomophilous morphology. It is more likely that the first situation has occurred since transitions to ornithophily appear to be very common, especially in Australia (Keighery 1980; Ford 1985b; a; Paton 1986; Cronk and Ojeda 2008). The short branch lengths in the phylogeny suggest that evolution to ornithophily occurred rapidly. This is plausible since entomophilous flowers with a floral tube, such as those of *Prostanthera*, are predisposed to becoming ornithophilous and it is shown that a switch in alleles can drastically change visitation rates between birds and insects (Schemske and Bradshaw 1999; Wilson *et al.* 2007; Cronk and Ojeda 2008).

An investigation into several genera in the Mirbelieae (Fabaceae) remains the only detailed assessment of the evolution of ornithophily (or pollination syndrome for that matter) in Australia (Crisp 1994; Crisp and Cook 2003). Several genera in the Mirbelieae have apparently recently converged by rapidly evolving long, red, tubular flowers. The evolution of ornithophily in *Prostanthera* is also inferred to have evolved recently since taxa of section *Klanderia* are nested in a clade within the genus. However, the clade representative of flower type 2a appears to have evolved more

recently than ornithophilous species. If the floral morphology of this clade is suited better to a more generalist pollination syndrome (as discussed above), this indicates an evolutionary trend less specialised on bees.

Suggestions for future research

Improving the resolution of phylogenies

The combination of three nucleotide sequence datasets does not provide enough phylogenetic resolution to infer the evolutionary history of some groups within *Prostanthera*. There are three regions in the phylogeny of *Prostanthera* that are poorly resolved: the polytomy at the 'base' of the *Prostanthera* clade, the polytomy containing most of section *Klanderia*, and the polytomy found within clade F. With regards to section *Klanderia*, it remains unresolved if it is a monophyletic group (representing a single origin of ornithophily), a polyphyletic group (representing multiple origins of ornithophily), or a paraphyletic group (representing a single origin of ornithophily with reversals to entomophily). Additional work is required to resolve the evolutionary history of this clade.

The rate at which DNA can be sequenced is increasing and techniques are currently at a state where full chloroplast genomes are being analysed (Timme *et al.* 2007). This has increased a selection of new informative markers available for phylogenetic analyses (Shaw *et al.* 2005). The new cpDNA *ndhF-rpl32* marker is not even variable enough to provide resolution within *Prostanthera*; however, its phylogeny provides resolution at a higher taxonomic level and should help to resolve the generic limits between the polyphyletic *Hemigenia* and *Microcorys* (Guerin 2008).

Different markers are likely needed to resolve each of the three aforementioned regions of the phylogeny. The cpDNA *trnS-G* spacer (Simões *et al.* 2006), *matK* gene, and *ndhF* gene (Conn *et al.* 2009) sufficiently resolve phylogenies at the generic level and might improve the resolution at the 'base' of *Prostanthera*. Faster evolving markers are needed to improve the resolution of the polytomies of Clade D or within Clade F, which contain morphologically similar species. Low copy nuclear COS genes may be one solution since they work well for the sister family Scrophulariaceae, and are shown to be faster evolving than markers such as the *matK* (Li *et al.* 2008). Polymorphic DNA-based markers, such as Amplified Fragment Length Polymorphisms (AFLPs), might be an alternative to improve resolution of relationships amongst specific closely related species, such as the ornithophilous species of clade H (Zhang *et al.* 2001; Beardsley *et al.* 2003). Recently, AFLPs have been successfully used to distinguish between *P. arapilensis*, *P. sejuncta*, and *P. spinosa* (Williams *et al.* 2006). Although AFLPs may provide a potential source of phylogenetic information, there are properties of restriction fragment data that limit phylogenetic interpretation (Koopman 2005). Several problems that may arise include non-independence of fragments, difficulty of homology assignment of fragments, or asymmetry in the probability of losing and gaining fragments.

Unfortunately, there are no fossil records for the Westringieae and few records for the Lamiaceae (Harley *et al.* 2004; Crepet 2008). This has hampered attempts to estimate dates for divergence in the Lamiaceae. A molecular clock might be calibrated, however, using the phylogeny of the Lamiales (Wagstaff *et al.* 1998) and the mid-Eocene fossils found for *Clerodendrum* (Harley *et al.* 2004). Subsequently, a date for the divergence of *Prostanthera* might be extrapolated from the phylogeny of the Lamiales (Wagstaff *et*

al. 1998), being aware that interpretation of dates based on fossils imply minimum rather than absolute dates (Heads 2005).

A new dimension for geometric morphometrics

Landmark analysis of the corolla encountered the limitations of describing three-dimensional (3D) changes in a two-dimensional (2D) paradigm. For example, careful study was required to differentiate between changes of reflexion and size of petal lobes. A 3D analysis of floral shape will permit a better understanding of these changes. It will also reduce the need for standardising an angle at which specimens are photographed. Recent developments in geometric morphometrics include software programs that permit relative warps analyses using 3D shape (Mitteroecker and Gunz 2009). 3D shape can be acquired using laser reconstruction of an objects surface, and has been popular for use in analysing skeletal structure (Marcus *et al.* 2000; Lockwood *et al.* 2004; Drake and Klingenberg 2008; Sztencel-Jablonka *et al.* 2009). Micro-computed tomography (Micro-CT) is another tool that acquires 3D shape, using x-ray beams to create digital slices that are then stacked together to recreate a 3D image. It has the advantage of being able to digitally capture internal anatomical features in addition to surface detail, which may be of aid when searching for homologous landmark points. Until recently, Micro-CT technology has been limited to dense material such as small skulls (Willmore *et al.* 2006) or sclerophyllous plant tissue (Steppe *et al.* 2004; Tarver *et al.* 2006; Jansen *et al.* 2007). Recent application to soft tissue, such as mouse embryos (Johnson *et al.* 2006) and snails (Golding and Jones 2007) demonstrates a promising opportunity to advance the study of plant morphology.

Quantifying pollinator effectiveness

The study of pollination is limited by the time consuming processes necessary for understanding the effectiveness of a pollinator. Pollinators in this study were distinguished from visitors by careful examination of their interaction with the flower and a better understanding of the pollination mechanism. However, this completes only part of an assessment for a *bona fide* pollinator because it assumes that a visitor will actually transport adequate amounts of pollen from flower to flower, and that the result of its visitation is ultimately the fertilisation of an ovary. Furthermore, these observations cannot determine the effectiveness between pollinators. Therefore, in the case where different floral types attract similar putative pollinators, for example floral types 2a and 2b of *Prostanthera*, there is no means of distinguishing whether both types are equally effective at using the same pollinators, or whether one type is different to the other because a particular pollinator is more effective.

Pollen transfer quantification, visitation duration, and fruit set are considered to be more robust assessments of pollinator effectiveness in comparison to observations of visitors (Schemske and Horvitz 1984; Ivey *et al.* 2003). The hypothesized pollinators in this study provide the basis upon which future studies could measure their effectiveness.

Prostanthera lasianthos, for example, will provide an ideal candidate for an investigation of pollinator effectiveness since it is widely distributed in eastern Australia and is visited by birds and insects. In order to investigate pollen transfer, it will be necessary to examine pollen loads of visitors after visiting flowers (Paton and Ford 1977; Hargreaves *et al.* 2004). Pollen on stigmas of virgin flowers can also be quantified after visitations (Robertson *et al.* 2005; Larson *et al.* 2006). Fruit set is also relatively easy to measure in *Prostanthera* since fruits mature within a year (Tierney

and Gross 2001). Therefore, fruit set may also be compared between pollinated and non-pollinated flowers to measure pollination success (Gross and Mackay 1998; Ortega-Olivencia *et al.* 2005).

Conclusions

The genus *Prostanthera* provides an excellent model for investigating pollination because of its morphological diversity and pollination biology, having features characteristic of many angiosperms. The construction of a molecular phylogeny of *Prostanthera* has provided a relational framework in which the evolution of morphological features and pollination syndromes can be interpreted. Continued application of phylogenies and morphological analyses on other representative flora are needed to further elucidate the pollination of plants.

References

- Alcantara S, Lohmann LG (2010) Evolution of floral morphology and pollination system in Bignoniaceae (Bignoniaceae). *American Journal of Botany* 97:782-796
- Althofer GW (1978) *Cradle of incense: the story of Australian Prostanthera*. Society for Growing Australian Plants
- Amela García M, Gottsberger G (2009) Composition of the floral nectar of different subgenera of Argentinian *Passiflora* species. *Plant Systematics and Evolution* 283:133-147
- Anderson SH (2003) The relative importance of birds and insects as pollinators of the New Zealand flora. *New Zealand Journal of Ecology* 27:83-94
- Austin MP, Belbin L (1982) A new approach to the species classification problem in floristic analysis. *Austral Ecology* 7:75-89
- Baker H, Baker I (1986) The occurrence and significance of amino acids in floral nectar. *Plant Systematics and Evolution* 151:175-186
- Baldwin BG, Markos S (1998) Phylogenetic Utility of the External Transcribed Spacer (ETS) of 18S-26S rDNA: Congruence of ETS and ITS Trees of *Calycadenia* (Compositae). *Molecular Phylogenetics and Evolution* 10:449-463
- Barber JC, Finch CC, Francisco-Ortega J, Santos-Guerra A, Jansen RK (2007) Hybridization in Macaronesian *Sideritis* (Lamiaceae): evidence from incongruence of multiple independent nuclear and chloroplast sequence datasets. *Taxon* 56:1-15
- Barbour MG, Burk JH, Pitts WD (1987) *Terrestrial plant ecology*, second edn. The Benjamin/Cummings Publishing Company, Inc.
- Barker FK, Lutzoni FM (2002) The utility of the Incongruence Length Difference test. *Systematic Biology* 51:625-637
- Baum DA, Small RL, Wendel JF (1998) Biogeography and floral evolution of Baobabs (*Adansonia*, Bombacaceae) as inferred from multiple data sets. *Systematic Biology* 47:181-207
- Beals EW (1984) Bray-Curtis ordination: An effective strategy for analysis of multivariate ecological data. In: MacFadyen A, Ford ED (eds) *Advances in Ecological Research*, vol Volume 14. Academic Press, pp 1-55
- Beardsley PM, Olmstead RG (2002) Redefining Phrymaceae: the placement of *Mimulus*, tribe Mimuleae, and *Phryma*. *American Journal of Botany* 89:1093-1102
- Beardsley PM, Yen A, Olmstead RG (2003) AFLP Phylogeny of *Mimulus* Section *Erythranthe* and the Evolution of Hummingbird Pollination. *Evolution* 57:1397-1410
- Belbin L, Collins A (2004) PATN version 3.02 and 3.03. Blatant Fabrications Pty Ltd. Hobart, Tasmania.
- Bentham G (1870) Labiatae. In: *Flora Australiensis*, vol 5. Reeve, London, pp 70-137

- Bert W, Leliaert F, Vierstraete AR, Vanfleteren JR, Borgonie G (2008) Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda: Rhabditida). *Molecular Phylogenetics and Evolution* 48:728-744
- Bookstein FL (1989) Principal warps: thin-plate splines and the decomposition of deformations. *IEEE Transactions on pattern analysis and machine intelligence* 11:567-585
- Bookstein FL, Gunz P, Mitteroecker P, Prossinger H, Schaefer K, Seidler H (2003) Cranial integration in *Homo*: singular warps analysis of the midsagittal plane in ontogeny and evolution. *Journal of Human Evolution* 44:167-187
- Briggs BG, Johnson LAS (1979) Evolution in the Myrtaceae - evidence from inflorescence structure. *Proceedings of the Linnean Society of New South Wales* 102
- Brown ED, Hopkins MJG (1995) A test of pollinator specificity and morphological convergence between nectarivorous birds and rainforest tree flowers in New Guinea. *Oecologia* 103:89-100
- Brown EM, Burbidge AH, Dell J, Edinger D, Hopper SD, Willis RT (1997) Pollination in Western Australia: a database of animals visiting flowers. *Hanbook No. 15*, . WA Naturalists' Club, Perth
- Brown R (1810) *Prodromus Florae Novae Hollandiae*. J. Johnson & Co., London
- Bruneau A (1997) Evolution and homology of bird pollination syndromes in *Erythrina* (Leguminosae). *American Journal of Botany* 84:54-71
- Buchmann SL, Nabhan GP (1996) *The Forgotten Pollinators*. Island Press/Shearwater Books, Washington D.C.
- Bybee SM, Ogden TH, Branham MA, Whiting MF (2008) Molecules, morphology and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. *Cladistics* 24:477-514
- Cantino PD (1992) Evidence for a polyphyletic origin of the Labiatae. *Annals of the Missouri Botanical Garden* 79:361-379
- Carrick J (1976) Studies in Australian Lamiaceae Part 1 the Genus *Wrixonia* (Prostantheroideae). *Journal of the Adelaide Botanic Gardens* 1:27-34
- Carthew SM (1993) An assessment of pollinator visitation to *Banksia spinulosa*. *Austral Ecology* 18:257-268
- Castellanos MC, Wilson P, Thomson JD (2004) 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* 17:876-885
- Chase MW, Hills HH (1991) Silica Gel: An Ideal Material for Field Preservation of Leaf Samples for DNA Studies. *Taxon* 40:215-220
- Christidis L, Schodde R (1993) Relationships and radiations in the meliphagine honeyeaters, *Meliphaga*, *Lichenostomus* and *Xanthotis* (Aves: Meliphagidae): protein evidence and its integration with morphology and ecogeography. *Australian Journal of Zoology* 41:293-316

- Clarke KR, Green RH (1988) Statistical design and analysis for a 'biological effects' study. *Marine Ecology Progress Series* 46:213-226
- Claßen-Bockhoff R (2007) Floral construction and pollination biology in the Lamiaceae. *Annals of Botany* 100:359-360
- Claßen-Bockhoff R, Speck T, Tweraser E, Wester P, Thimm S, Reith M (2004) The staminal lever mechanism in *Salvia* L. (Lamiaceae): a key innovation for adaptive radiation? *Organisms Diversity & Evolution* 4:189-205
- Claßen-Bockhoff R, Wester P, Tweraser E (2003) The staminal lever mechanism in *Salvia* L. (Lamiaceae): A review. *Plant Biology (Stuttgart)* 5:33-41
- Combs JK, Pauw A (2009) Preliminary evidence that the long-proboscid fly, *Philoliche gulosa*, pollinates *Disa karooica* and its proposed Batesian model *Pelargonium stipulaceum*. *South African Journal of Botany* 75:757-761
- Conn BJ (1984) A taxonomic revision of *Prostanthera* Labill. section *Klanderia* (F.v. Muell.) Benth. (Labiatae). *Journal of the Adelaide Botanic Gardens* 6:207-348
- Conn BJ (1988) A Taxonomic Revision of *Prostanthera* Labill. Section *Prostanthera* Labiatae 1. the Species of the Northern Territory South Australia and Western Australia. *Nuytsia* 6:351-411
- Conn BJ (1992a) Lamiaceae. In: Harden GJ (ed) *Flora of New South Wales*, vol 3. Royal Botanic Gardens and Domain Trust, Sydney
- Conn BJ (1992b) Relationships within the tribe Prostanthereae (Labiatae). In: R.M. H, T. R (eds) *Advances in Labiate science*. Royal Botanic Gardens, Kew, pp 55-64
- Conn BJ (1994) Lamiaceae. In: Walsh NG, Entwisle TJ (eds) *Flora of Victoria*, vol 4. Inkata Press, Melbourne, pp 418-459
- Conn BJ (1997) Four rare and/or threatened new species of *Prostanthera* Section *Prostanthera* (Lamiaceae) from New South Wales. *Telopea* 7:231-244
- Conn BJ (1998) Contributions to the systematics of *Prostanthera* (Labiatae) in south-eastern Australia. *Telopea* 7:319-332
- Conn BJ (2004) IV. Subfam. Prostantheroideae. In: Kadereit JW (ed) *Flowering plants-Dicotyledons, Lamiales (except Acanthaceae including Avicenniaceae)*, vol 7 Springer-Verlag, Berlin, pp 167-275
- Conn BJ (2006) New species of *Prostanthera* section *Prostanthera* (Labiatae) from New South Wales. *Telopea* 11:252-259
- Conn BJ, Shephard KA (2007) *Prostanthera ferricola* (Lamiaceae), a new species from Western Australia. *Nuytsia* 17:147-151
- Conn BJ, Streiber N, Brown EA, Henwood MJ, Olmstead RG (2009) Infrageneric phylogeny of Chloantheae (Lamiaceae) based on chloroplast ndhF and nuclear ITS sequence data. *Australian Systematic Botany* 22:243-256

- Consiglio TK, Bourne GR (2001) Pollination and breeding system of a neotropical palm *Astrocaryum vulgare* in Guyana: a test of the predictability of syndromes. *Journal of Tropical Ecology* 17:577-592
- Crane PR, Friis EM, Pedersen KR (1995) The origin and early diversification of angiosperms. *Nature (London)* 374:27-33
- Crane PR, Herendeen PS, Friis EM (2004) Fossils and plant phylogeny. *American Journal of Botany* 91:1683-1699
- Crepet WL (2008) The fossil record of angiosperms: Requiem or renaissance? *Annals of the Missouri Botanical Garden* 95:3-33
- Crepet WL, Niklas KJ (2009) Darwin's second 'abominable mystery': Why are there so many angiosperm species? *American Journal of Botany* 96:366-381
- Crisp MD (1994) Evolution of bird-pollination in some Australian legumes (Fabaceae). In: *Linnean Society Symposium Series; Phylogenetics and ecology*. Academic Press Ltd.; Academic Press, Inc., pp 281-309
- Crisp MD (1996) Convergent evolution of bird pollination in Western Australian Fabaceae and its taxonomic implications. *Surrey Beatty & Sons, Norton, NSW*
- Crisp MD, Cook LG (2003) Phylogeny and evolution of anomalous roots in *Daviesia* (Fabaceae:Mirbelieae). *International Journal of Plant Sciences* 164:603-613
- Cronk Q, Ojeda I (2008) Bird-pollinated flowers in an evolutionary and molecular context. *Journal of Experimental Biology* 59:715-727
- Cruden RW (1972) Pollinators in high elevation ecosystems relative effectiveness of birds and bees. *Science (Washington D C)* 176:1439-1440
- CSIRO DoE (1991) *The Insects of Australia: A textbook for students and research workers*. Melbourne university press, Carlton, Victoria
- Cunningham CW (1997a) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* 14:733-740
- Cunningham CW (1997b) Is congruence between data partitions a reliable predictor of phylogenetic accuracy? *Systematic Biology* 46:464-478
- Da Silva FO, Viana BF, Jacobi CM (2005) Floral biology of *Eriope blanchetii* (Lamiaceae) in coastal sand dunes of NE Brazil. *Austral Ecology* 30:243-249
- Darwin CH (1862) On the various contrivances by which British and foreign orchids are fertilized by insects. *John Murray*
- de Merxem DG, Borremans B, de Jäger ML, Johnson T, Jooste M, Ros P, Zenni RD, Ellis AG, Anderson B (2009) The importance of flower visitors not predicted by floral syndromes. *South African Journal of Botany* 75:660-667
- Dollin A, Batley M, Robinson M, Faulkner B (2000) Native bees of the Sydney region, a field guide. *Australian Native Bee Research Centre, Richmond, NSW*

- Drake AG, Klingenberg CP (2008) The pace of morphological change: historical transformation of skull shape in St Bernard dogs. *Proceedings of the Royal Society B: Biological Sciences* 275:71-76
- Driskell AC, Christidis L (2004) Phylogeny and evolution of the Australo-Papuan honeyeaters (Passeriformes, Meliphagidae). *Molecular Phylogenetics and Evolution* 31:943-960
- Dunbar-Co S, Wieczorek AM, Morden CW (2008) Molecular phylogeny and adaptive radiation of the endemic Hawaiian *Plantago* species (Plantaginaceae). *American Journal of Botany* 95:1177-1188
- Dupont YL, Hansen DM, Rasmussen JT, Olesen JM (2004) Evolutionary changes in nectar sugar composition associated with switches between bird and insect pollination: the Canarian bird-flower element revisited. *Functional Ecology* 18:670-676
- Edwards CE, Soltis DE, Soltis PS (2006) Molecular phylogeny of *Conradina* and other scrub mints (Lamiaceae) from the southeastern USA: Evidence for hybridization in Pleistocene refugia? *Systematic Botany* 31:193-207
- Faegri K, Van der Pijl L (1979) *The principles of pollination ecology*. Pergamon Press, New York
- Felsenstein J (1985a) Confidence Limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791
- Felsenstein J (1985b) Phylogenies and the comparative method. *The American Naturalist* 125:1-15
- Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD (2004) Pollination syndromes and floral specialization. *Annual Review of Ecology Evolution and Systematics* 35:375-403
- Ferrero V, de Vega C, Stafford GI, Van Staden J, Johnson SD (2009) Heterostyly and pollinators in *Plumbago auriculata* (Plumbaginaceae). *South African Journal of Botany* 75:778-784
- Ford CM, Johnson SD (2008) Floral traits, pollinators and breeding systems in *Syncolostemon* (Lamiaceae). *Plant Systematics and Evolution* 275:257-264
- Ford HA (1985a) Nectar-feeding birds and bird pollination why are they so prevalent in Australia yet absent from Europe. *Proceedings of Ecological Society of Australia* 14:153-158
- Ford HA (1985b) Nectarivory and pollination by birds in southern Australia and Europe. *Oikos* 44:127-131
- Ford HA, Paton DC, Forde N (1979) Birds as pollinators of Australian plants. *New Zealand Journal of Botany* 17:509-519
- Friedman J, Barrett SH (2008) A phylogenetic analysis of the evolution of wind pollination in the Angiosperms. *International Journal of Plant Sciences* 169:49-58
- Friis EM, Crepet WL (1994) Time of appearance of floral features. In: Friis EM, Chaloner WG, Crane PR (eds) *Origins of Angiosperms and their Biological Consequences*. Cambridge University Press, Cambridge, pp 145-179

- Ghebrehiwet M, Bremer B, Thulin M (2000) Phylogeny of the tribe Antirrhineae (Scrophulariaceae) based on morphological and *ndhF* sequence data. *Plant Systematics and Evolution* 220:223-239
- Givnish TJ, Evans TM, Zjhra ML, Patterson TB, Berry PE, Sytsma KJ (2000) Molecular evolution, adaptive radiation, and geographic diversification in the amphiatlantic family Rapateaceae: Evidence from *ndhF* sequences and morphology. *Evolution* 54:1915-1937
- Goldblatt P, Manning JC (1996) Phylogeny and speciation in *Lapeirousia* subgenus *Lapeirousia* (Iridaceae: Ixioideae). *Annals of the Missouri Botanical Garden* 83:346-361
- Golding RE, Jones AS (2007) Micro-CT as a novel technique for 3D reconstruction of molluscan anatomy. *Molluscan Research* 27:123-128
- Gómez JM, Perfectti F, Camacho PM (2006) Natural selection on *Erysimum mediohispanicum* flower shape: Insights into the evolution of zygomorphy. *The American Naturalist* 168:531-545
- Gower JC (1971) A general coefficient of similarity and some of its properties. *Biometrics* 27:857-871
- Gross CL (2001) The effect of introduced honeybees on native bee visitation and fruit-set in *Dillwynia juniperina* (Fabaceae) in a fragmented ecosystem. *Biological Conservation* 102:89-95
- Gross CL, Mackay D (1998) Honeybees reduce fitness in the pioneer shrub *Melastoma affine* (Melastomataceae). *Biological Conservation* 86:169-178
- Guerin G (2005) Floral biology of *Hemigenia* and *Microcorys* (Lamiaceae). *Australian Journal of Botany* 53:147-162
- Guerin GR (2008) Evidence for polyphyly in *Hemigenia* and *Microcorys* (Lamiaceae: Westringieae). *Australian Systematic Botany* 21:313-325
- Hackett DJ, Goldingay RL (2001) Pollination of *Banksia* spp. by non-flying mammals in north-eastern New South Wales. *Australian Journal of Botany* 49:637-644
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41:95-98
- Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4:1-9
- Hargreaves AL, Johnson SD, Nol E (2004) Do floral syndromes predict specialization in plant pollination systems? An experimental test in an “ornithophilous” African *Protea*. *Oecologia* 140:295-301
- Harley RM, Atkins S, Budantsev AL, Cantino PD, Conn BJ, Grayer RJ, Harley MM, De Kok RPJ, Krestovskaja T, Morales R, Paton AJ, Ryding O, Upson T (2004) Flowering Plants, Dicotyledons: Lamiales (except Acanthaceae including Avicenniaceae). In: Kadereit JW (ed) *The Families and Genera of Vascular Plants*, vol 7 Springer-Verlag, Berlin, pp 167-275

- Harrison CJ, Moller M, Cronk QCB (1999) Evolution and development of floral diversity in *Streptocarpus* and *Saintpaulia*. *Annals of Botany* 84:49-60
- Hartmann S, Nason JD, Bhattacharya D (2002) Phylogenetic origins of *Lophocereus* (Cactaceae) and the senita cactus-senita moth pollination mutualism. *Am. J. Bot.* 89:1085-1092
- Heads M (2005) Dating nodes on molecular phylogenies: a critique of molecular biogeography. *Cladistics* 21:62-78
- Herrera CM (1987) Components of pollinator "quality": Comparative analysis of a diverse insect assemblage. *Oikos* 50:79-90
- Hillson CJ (1959) Comparative studies of floral morphology of the Labiatae. *American Journal of Botany* 46:451-459
- Hingston AB, McQuillan PB (2000) Are pollination syndromes useful predictors of floral visitors in Tasmania? *Austral Ecology* 25:600-609
- Horskins K, Turner VB (1999) Resource use and foraging patterns of honeybees, *Apis mellifera*, and native insects on flowers of *Eucalyptus costata*. *Austral Ecology* 24:221-227
- Huck RB (1992a) Evolutionary dicta for pollination biology studies in the Lamiaceae. *American Journal of Botany* 79:148
- Huck RB (1992b) Overview of pollination biology in the Lamiaceae. In: Harley RM, Reynolds T (eds) *Advances in Labiatae science*. Royal Botanic Gardens, Kew, pp 167-181
- Huelsensbeck P, Ronquist F (2001) MR BAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754-755
- Hunter JT, Williams JB, Conn BJ (2006) Rediscovery of *Prostanthera staurophylla* F. Muell. and reinstatement of *P. teretifolia* Maiden & Betche (Lamiaceae). *Telopea* 11:117-126
- Inouye DW, Pyke GH (1988) Pollination biology in the Snowy Mountains of Australia: Comparisons with montane Colorado, USA. *Austral Ecology* 13:191-205
- Ivey CT, Martinez P, Wyatt R (2003) Variation in pollinator effectiveness in swamp milkweed, *Asclepias incarnata* (Apocynaceae). *American Journal of Botany* 90:214-225
- Jacques FMB, Zhou Z (2010) Geometric morphometrics: A powerful tool for the study of shape evolution in Menispermaceae endocarps. *Taxon* 59:881-895
- JAMESON ML, MICÓ E, GALANTE E (2007) Evolution and phylogeny of the scarab subtribe Anisopliina (Coleoptera: Scarabaeidae: Rutelinae: Anomalini). *Systematic Entomology* 32:429-449
- Jamzad Z, Chase MW, Ingrouille M, Simmonds MSJ, Jalili A (2003) Phylogenetic relationships in *Nepeta* L. (Lamiaceae) and related genera based on ITS sequence data. *Taxon* 52:21-32

- Jansen S, Pletsers A, Steppe K, Cnudde V, Masschaele B, Choat B, Sano Y, Pesacreta T, Connell S (2007) Three-dimensional imaging of wood anatomical characters using X-ray computed microtomography (Micro-CT) and atomic force microscopy (AFM). In: Botany & Plant Biology 2007 Joint Congress, Chicago, Illinois
- Jensen RJ (1990) Detecting shape variation in oak leaf morphology: a comparison of rotational-fit methods. *American Journal of Botany* 77:1279-1293
- Jensen RJ, Ciofani KM, Miramontes LC (2002) Lines, outlines, and landmarks: Morphometric analyses of leaves of *Acer rubrum*, *Acer saccharinum* (Aceraceae) and their hybrid. *Taxon* 51:475-492
- Jiménez-Pérez NdC, Lorea-Hernández F (2009) Identity and delimitation of the American species of *Litsea* Lam. (Lauraceae): a morphological approach. *Plant Systematics and Evolution* 283:19-32
- Johnson JT, Hansen MS, Wu I, Healy LJ, Johnson CR, Jones GM, Capecchi MR, Keller C (2006) Virtual Histology of Transgenic Mouse Embryos for High-Throughput Phenotyping. In: PLOS GENETICS, vol. 2, p e61
- Johnson SD, Linder HP, Steiner KE (1998) Phylogeny and Radiation of Pollination Systems in *Disa* (Orchidaceae). *American Journal of Botany* 85:402-411
- Kay MK, Schemske DW (2003) Pollinator Assemblages and Visitation Rates for 11 Species of Neotropical *Costus* (Costaceae). *Biotropica* 35:198-207
- Kearns CA, Inouye DW (1993) Techniques for pollination biologists. University Press of Colorado, Colorado
- Keighery GJ (1980) Bird pollination in south-western Australia: a checklist. *Plant Systematics and evolution* 135:171-176
- Keighery GJ (1982) Pollination syndromes and breeding systems of Western Australian arid zone plants. Peacock Publications, Frewville, South Australia
- Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. *Annual Review of Entomology* 28:407
- Klingenberg CP (2008) MorphoJ. In. Faculty of Life Sciences, University of Manchester, Manchester, UK. http://www.flywings.org.uk/MorphoJ_page.htm
- Klingenberg CP, Barluenga M, Meyer A (2002) Shape analysis of symmetric structures: Quantifying variation among Individuals and asymmetry. *Evolution* 56:1909-1920
- Knox RB, Kenrick J, Bernhardt P, Marginson R, Beresford G, Baker I, Baker HG (1985) Extrafloral nectaries as adaptations for bird pollination in *Acacia terminalis*. *American Journal of Botany* 72:1185-1196
- Koopman WJM (2005) Phylogenetic signal in AFLP data sets. *Systematic Biology* 54:197-217
- Kooyman R, Rossetto M (2006) Factors influencing species selection for littoral rainforest restoration: Do environmental gradients matter? *Ecological Management & Restoration* 7:113-119

- Larget B, Simon DL (1999) Markov Chain Monte Carlo algorithms for the bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* 16:750-759
- Larson DL, Royer RA, Royer MR (2006) Insect visitation and pollen deposition in an invaded prairie plant community. *Biological Conservation* 130:148-159
- Legendre P, Legendre L (1998) *Numerical Ecology*. Second English edition. Elsevier, Amsterdam
- Leys R, Cooper SJB, Schwarz MP (2002) Molecular phylogeny and historical biogeography of the large carpenter bees, genus *Xylocopa* (Hymenoptera: Apidae). *Biological Journal of the Linnean Society* 77:249-266
- Li M, Wundera J, Bissolia G, Scarponia E, Gazzania S, Barbaroa E, Saedlera H, Varottoa C (2008) Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species. *Cladistics* 24:727-745
- Lindqvist C, Albert VA (2002) Origin of the Hawaiian endemic mints within North American *Stachys* (Lamiaceae). *American Journal of Botany* 89:1709-1724
- Lloyd DG (1985) Progress in understanding the natural history of New Zealand plants. *New Zealand Journal of Botany* 23:707-722
- Lockwood CA, Kimbel WH, Lynch JM, Pilbeam D (2004) Morphometrics and hominoid phylogeny: Support for a Chimpanzee-Human clade and differentiation among great ape subspecies. *Proceedings of the National Academy of Sciences of the United States of America* 101:4356-4360
- Loyn RH (1985) Bird populations in successional forests of Mountain Ash *Eucalyptus regnans* in central Victoria. *Emu* 85:213-230
- Maddison WP (1991) Squared-change parsimony reconstructions of ancestral states for continuous-valued characters on a phylogenetic tree. *Systematic Zoology* 40:304-314
- Manning JC, Goldblatt P (2005) Radiation of pollination systems in the cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. *International Journal of Plant Sciences* 166:459
- Marcus LF, Hingst-Zaher E, Zaher H (2000) Application of landmark morphometrics to skulls representing the orders of living mammals. *Hystrix* 11:27-47
- Martén-Rodríguez S, Almarales-Castro A, Fenster CB (2009) Evaluation of pollination syndromes in Antillean Gesneriaceae: evidence for bat, hummingbird and generalized flowers. *Journal of Ecology* 97:348-359
- Martin HA (2006) Cenozoic climatic change and the development of the arid vegetation in Australia. *Journal of arid Environments* 66:533-563
- McCall C, Primack RB (1992) Influence of flower characteristics, weather, time of day, and season on insect visitation rates in three plant communities. *American Journal of Botany* 79:434-442
- McFarland DC (1986) Seasonal changes in the abundance and body condition of honeyeaters (Meliphagidae) in response to inflorescence and nectar availability in the New England National Park, New South Wales. *Austral Ecology* 11:331-340

- McNeill J, Barrie FR, Burdet HM, Demoulin V, Hawksworth DL, Marhold K, Nicholason DH, Prado J, Silva PC, Skog JE, Wiersma JH, Turland JN (2006) International Code of Botanical Nomenclature (Vienna Code) adopted by the Seventeenth International Botanical Congress, Vienna, Austria, July 2005. *Regnum Veg.* 146.
- Mitteroecker P, Gunz P (2009) Advances in Geometric Morphometrics. *Evolutionary Biology* 36:235-247
- Moore WS, Weibel AC, Agius A (2006) Mitochondrial DNA phylogeny of the woodpecker genus *Veniliornis* (Picidae, Picinae) and related genera implies convergent evolution of plumage patterns. *Biological Journal of the Linnean Society* 87:611-624
- Morcombe M (2000) Field Guide to Australian Birds. Steve Parish Publishing
- Mort ME, Archibald JK, Randle CP, Levsen ND, O'Leary TR, Topalov K, Wiegand CM, Crawford DJ (2007) Inferring phylogeny at low taxonomic levels: utility of rapidly evolving cpDNA and nuclear ITS loci. *Am. J. Bot.* 94:173-183
- Mueller FJHv (1876) *Fragmenta Phytographiae Australiae* 10 (83):18
- Müller K (2005) SeqState: Primer Design and Sequence Statistics for Phylogenetic DNA Datasets. *Applied Bioinformatics* 4:65-69
- Navarro L (1997) Is the dichogamy of *Salvia verbenaca* (Lamiaceae) an effective barrier to self-fertilization? *Plant Systematics and Evolution* 207:111-117
- Neal PR, Dafni A, Giurfa M (1998) Floral symmetry and its role in plant-pollinator systems: Terminology, distribution, and hypotheses. *Annual Review of Ecology and Systematics* 29:345-373
- Neustupa J, Němcová Y (2007) A geometric morphometric study of the variation in scales of *Mallomonas striata* (Synurophyceae, Heterokontophyta). *Phycologia* 46:123-130
- Newstrom L, Robertson AW (2005) Progress in understanding pollination systems in New Zealand. *New Zealand Journal of Botany* 43:1-59
- Nicolson SW (2002) Pollination by passerine birds: why are the nectars so dilute? *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 131:645-652
- Ollerton J (1996) Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. *The Journal of Ecology* 84:767-769
- Ollerton J (1998) Sunbird surprise for syndromes. *Nature* 394:726-727
- Ollerton J, Alarcon R, Waser NM, Price MV, Watts S, Cranmer L, Hingston A, Peter CI, Rotenberry J (2009a) A global test of the pollination syndrome hypothesis. *Annals of Botany* 103:1471-1480
- Ollerton J, Killick A, Lamborn E, Watts S, Whiston M (2007) Multiple meanings and modes: on the many ways to be a generalist flower. *Taxon* 56:717-728
- Ollerton J, Masinde S, Meve U, Picker M, Whittington A (2009b) Fly pollination in *Ceropegia* (Apocynaceae: Asclepiadoideae): biogeographic and phylogenetic perspectives. *Annals of Botany* 103:1501-1514

- Ollerton J, Watts S (2000) Phenotype space and floral typology: Towards an objective assessment of pollination syndromes. Det Norske Videnskaps-Akademi. I. Matematisk-Naturvidenskapelige Klasse, Skrifter, Ny Serie 39:149-159
- Olmstead RG, Reeves PA, Lepschi J (1998) Confirmation of a monophyletic Chloanthoideae (Lamiaceae) Comprising tribes Chloanthae and Prostanthereae. Lamiales Newsletter 6:7-10
- Ortega-Olivencia A, Rodriguez-Riano T, Valtuena FJ, Lopez J, Devesa JA (2005) First confirmation of a native bird-pollinated plant in Europe. Oikos 110:578-590
- Owens SJ, Ubera-Jimenez JL (1992) Breeding systems in Labiatae. In: R.M. H, Reynolds T (eds) Advances in Labiate Science. Royal Botanic Gardens, Kew, Richmond, pp 257-280
- Paton DC (1986) Evolution of bird pollination in Australia. In: Ford HA, Paton DC (eds) The dynamic partnership: birds and plants in southern Australia. Government Printer, Adelaide, pp 32-41
- Paton DC (1993) Honeybees in the Australian environment. Bioscience 43:95-103
- Paton DC (2000) Disruption of bird-plant pollination systems in southern Australia. Conservation Biology 14:1232-1234
- Paton DC, Ford HA (1977) Pollination by birds of native plants in South Australia. Emu 77:73-85
- Paton DC, Turner V (1985) Pollination of *Banksia ericifolia* Smith: Birds, mammals and insects as pollen vectors. Australian Journal of Botany 33:271-286
- Peeters PJ (2002) Correlations between leaf constituent levels and the densities of herbivorous insect guilds in an Australian forest. Austral Ecology 27:658-671
- Perkins A (2001) The phylogenetic systematics of the genus *Calochilus* (Orchidaceae). In: School of Biological Sciences, vol. PhD. University of Sydney, Sydney
- Perret M, Chautems A, Spichiger R, Kite G, Savolainen V (2003) Systematics and evolution of tribe Sinningieae (Gesneriaceae): evidence from phylogenetic analyses of six plastid DNA regions and nuclear ncpGS. Am. J. Bot. 90:445-460
- Petanidou T (2005) Sugars in mediterranean floral nectars: An ecological and evolutionary approach. Journal of Chemical Ecology 31:1065-1088
- Petanidou T, Laere AV, Ellis WN, Smets E (2006) What shapes amino acid and sugar composition in Mediterranean floral nectars? Oikos 115:155-169
- Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817-818
- Possingham HP (1989) The distribution and abundance of resources encountered by a forager. The American Naturalist 133:42-60
- Potgieter CJ, Edwards TJ (2001) The occurrence of long, narrow corolla tubes in southern African Lamiaceae. Systematics and Geography of Plants 71:493-502

- Potgieter CJ, Edwards TJ, Van Staden J (2009) Pollination of *Plectranthus* spp. (Lamiaceae) with sigmoid flowers in southern Africa. *South African Journal of Botany* 75:646-659
- Prather LA, Monfils AK, Posto AL, Williams RA (2002) Monophyly and phylogeny of *Monarda* (Lamiaceae): Evidence from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. *Systematic Botany* 27:127-137
- Proctor M, Yeo P (1973) *The pollination of flowers*. Collins, London
- Proctor MCF, Yeo P, Lack A (1996) *The natural history of pollination*. Timber Press, Portland, Or.
- Qiagen (2001) DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit Handbook for DNA isolation from plant tissue. QIAGEN Pty Ltd, Clifton Hill, Victoria, Australia
- Raju AJS (1989) Reproductive ecology of *Ocimum americanum* L. and *O. basilicum* L. (Lamiaceae) in India. *Plant Species Biology* 4:107-116
- Raju AJS, Reddi CS (1989) Pollination biology of *Anisomeles indica* and *Anisomeles malabarica* (Lamiaceae). *Plant Species Biology* 4:157-167
- Raven PH (1972) Why Are Bird Visited Flowers Predominantly Red. *Evolution* 26:674
- Ree RH (2005) Phylogeny and the evolution of floral diversity in *Pedicularis* (Orobanchaceae). *International Journal of Plant Sciences* 166:595-613
- Reith M, Claßen-Bockhoff R, Speck T (2006) Biomechanics of *Salvia*: The role of lever and flower tube in specialization on pollinators. In: *Ecology and Biomechanics*, pp 123-145
- Renner SS, Ricklefs RE (1995) Dioecy and its correlates in the flowering plants. *American Journal of Botany* 82:596-606
- Riemann O, Ahlrichs WH (2010) The evolution of the protonephridial terminal organ across Rotifera with particular emphasis on *Dicranophorus forcipatus*, *Encentrum mucronatum* and *Erignatha clastopis* (Rotifera: Dicranophoridae). *Acta Zoologica* 91:199-211
- Robertson AW, Ladley JJ, Kelly D (2005) Effectiveness of short-tongued bees as pollinators of apparently ornithophilous New Zealand mistletoes. *Austral Ecology* 30:298-309
- Rohlf FJ (1972) An empirical comparison of three ordination techniques in numerical taxonomy. *Systematic Zoology* 21:271-280
- Rohlf FJ (1990) Morphometrics. *Annual Review of Ecology and Systematics* 21:299-316
- Rohlf FJ (1993) Relative warp analysis and an example of its application to mosquito wings. In: Marcus LF, Bello E, García-Valdecasas (eds) *Contributions to morphometrics. Monografías del museo nacional de ciencias naturales*, Madrid, pp 131-159
- Rohlf FJ (2001) Comparative methods for the analysis of continuous variables: Geometric interpretations. *Evolution* 55:2143-2160
- Rohlf FJ (2006) TPS-Dig Ver. 2.05. Department of Ecology and Evolution, State University of New York at Stony Brook

- Rohlf FJ, Marcus LF (1993) A revolution in morphometrics. *Trends in Ecology & Evolution* 8:129-132
- Rohlf FJ, Slice D (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* 39:40-59
- Rüber L, Adams DC (2001) Evolutionary convergence of body shape and trophic morphology in cichlids from Lake Tanganyika. *Journal of Evolutionary Biology* 14:325-332
- Scheen A-C, Albert VA (2009) Molecular phylogenetics of the *Leucas* group (Lamioideae; Lamiaceae). *Systematic Biology* 34:173-181
- Schemske DW, Bradshaw HD, Jr. (1999) Pollinator preference and the evolution of floral traits in Monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America* 96:11910-11915
- Schemske DW, Horvitz CC (1984) Variation among floral visitors in pollination ability: A precondition for mutualism specialization. *Science* 225:519-521
- Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, Small RL (2005) The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92:142-166
- Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* 94:275-288
- Simmons MP, Ochoterena H (2000) Gaps as Characters in Sequence-Based Phylogenetic Analyses. *Systematic Biology* 49:369-381
- Simões AO, Endress ME, Niet Tvd, Kinoshita LS, Conti E (2006) Is *Mandevilla* (Apocynaceae, Mesechiteae) Monophyletic? Evidence from Five Plastid DNA Loci and Morphology. *Annals of the Missouri Botanical Garden* 93:565-591
- Simpson K, Day N (1999) *Simpson and Day Field guide to the birds of Australia*, 6th edn. Viking Penguin Books Australia
- Slice D (2007) Geometric Morphometrics. In: *Annual review of Anthropology*, vol. 36, pp 261-281
- Slice D, Bookstein FL, Marcus LF, Rohlf FJ (1996) A glossary for geometric morphometrics. In: Marcus LF, Corti M, Loy A, Naylor GJP, Slice D (eds) *Advances in Morphometrics*. Plenum Press, New York, pp 531-551
- Steane DA, de Kok RPJ, Olmstead RG (2004) Phylogenetic relationships between *Clerodendrum* (Lamiaceae) and other Ajugoid genera inferred from nuclear and chloroplast DNA sequence data. *Molecular Phylogenetics and Evolution* 32:39-45
- Steane DA, Scotland RW, Mabberley DJ, Olmstead RG (1999) Molecular systematics of *Clerodendrum* (Lamiaceae): ITS sequences and total evidence. *American Journal of Botany* 86:98-107
- Stebbins GL (1970) Adaptive radiation of reproductive characteristics in Angiosperms, I: Pollination mechanisms. *Annual Review of Ecology and Systematics* 1:307-326

- Steppe K, Cnudde V, Girard C, Lemeur R, Cnudde J-P, Jacobs P (2004) Use of X-ray computed microtomography for non-invasive determination of wood anatomical characteristics. *Journal of Structural Biology* 148:11-21
- Stiles FG (1975) Ecology, flowering phenology, and hummingbird pollination of some Costa Rican *Heliconia* species. *Ecology* 56:285-301
- Stiles FG, Freeman CE (1993) Patterns in floral nectar characteristics of some bird-visited plant species from Costa Rica. *Biotropica* 25:191-205
- Strauss RE, Bookstein FL (1982) The Truss: Body Form Reconstructions in Morphometrics. *Systematic Zoology* 31:113-135
- Swiderski DL (1993) Morphological evolution of the scapula in tree squirrels, chipmunks, and ground Squirrels (Sciuridae): An analysis using thin-plate splines. *Evolution* 47:1854-1873
- Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (*and other methods). In, 4.0 edn. Sinauer, Sunderland, MA.
- Sztencel-Jablonka A, Jones G, Bogdanowicz W (2009) Skull Morphology of two cryptic bat species: *Pipistrellus pipistrellus* and *P. pygmaeus* — A 3D geometric morphometrics approach with landmark reconstruction. *Acta Chiropterologica* 11:113-126
- Taberlet P, Ludovic G, Guy P, Jean B (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17:1105-1109
- Tarver MR, Shade RE, Tarver RD, Liang Y, Krishnamurthi G, Pittendrigh BR, Murdock LL (2006) Use of micro-CAT scans to understand cowpea seed resistance to *Callosobruchus maculatus*. *Entomologia Experimentalis et Applicata* 118:33-39
- Tay ML, Meudt HM, Garnock-Jones PJ, Ritchie PA (2010) DNA sequences from three genomes reveal multiple long-distance dispersals and non-monophyly of sections in Australasian *Plantago* (Plantaginaceae). *Australian Systematic Botany* 23:47-68
- Temeles E, J., Rankin A, G. (2000) Effect of the lower lip of *Monarda didyma* on pollen removal by hummingbirds. *Canadian Journal of Botany* 78:1164
- Thompson DAW (1942) On growth and form. Cambridge University Press, Cambridge
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25:4876-4882
- Thomson JD, Wilson P, Valenzuela M, Malzone M (2000) Pollen presentation and pollination syndromes, with special reference to *Penstemon*. *Plant Species Biology* 15:11-29
- Tierney DA, Gross CL (2001) *Prostanthera junonis* Conn (Lamiaceae); is recovery possible? *Pacific Conservation Biology* 7:118-123
- Timme RE, Kuehl JV, Boore JL, Jansen RK (2007) A comparative analysis of the *Lactuca* and *Helianthus* (Asteraceae) Plastid genomes: identification of divergent regions and categorization of shared repeats. *American Journal of Botany* 94:302-312

- Troll W (1964) Die Infloreszenzen. Typologie und Stellung im aufbau des Vegetationskörpers, I. I Deskriptive Morphologie des Infloreszenzen; II Typologie der Infloreszenzen. Jena, Gustav Fischer
- Trusty JL, Olmstead RG, Santos-Guerra A, Sa-Fontinha S, Francisco-Ortega J (2005) Molecular phylogenetics of the Macaronesian-endemic genus *Bystropogon* (Lamiaceae): palaeo-islands, ecological shifts and interisland colonizations. *Molecular Ecology* 14:1177-1189
- Ubera-Jimenez JL, Valdés B (1983) Revisión del género *Nepeta* (Labiatae) en la Península Ibérica e Islas Baleares. *Lagascalia* 12:3-8
- Valdivia CE, Niemeyer HM (2006) Do floral syndromes predict specialisation in plant pollination systems? Assessments of diurnal and nocturnal pollination of *Escallonia myrtoidea* *New Zealand Journal of Botany* 44:135-141
- Vaughton G (1996) Pollination disruption by European honeybees in the Australian bird-pollinated shrub *Grevillea barklyana* (Proteaceae). *Plant Systematics and Evolution* 200:89-100
- Vogel S (1954) Blütenbiologische Typen als Elemente der Sipplgliederung dargestellt anhand der Flora Südafrikas. *Botanische Studien* 1:1-338
- Vogel S, Westerkamp C, Thiel B, Gessner K (1984) Ornithophily on the Canary Islands Spain. *Plant Systematics and Evolution* 146:225-248
- Vos WT, Edwards TJ, Staden J (1994) Pollination biology of annual and perennial *Leonotis* species (Lamiaceae). *Plant Systematics and Evolution* V192:1-9
- Wagstaff S, J., Hickerson L, Spangler R, Reeves PA, Olmstead RG (1998) Phylogeny in *Labiatae* s. l., inferred from cpDNA sequences. *Plant Systematics and Evolution* V209:265-274
- Walker JB, Sytsma KJ (2007) Staminal evolution in the genus *Salvia* (Lamiaceae): molecular phylogenetic evidence for multiple origins of the staminal lever. *Annals of Botany* 100:375-391
- Walker JB, Sytsma KJ, Treutlein J, Wink M (2004) *Salvia* (Lamiaceae) is not monophyletic: implications for the systematics, radiation, and ecological specializations of *Salvia* and tribe Mentheae. *American Journal of Botany* 91:1115-1125
- Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in pollination systems, and why it matters. *Ecology* 77:1043
- Weatherhead T (1986) *Boxes to Bar Hives*, 1 edn. International Colour Productions, Stanthorpe
- Weberling F (1989) *Morphology of Flowers and Inflorescences*. University Press, Cambridge
- Weller SG, Sakai A, Rankin A, Golonka A, Kutcher B, Ashby K (1998) Dioecy and the evolution of pollination systems in *Schiedea* and *Alsinidendron* (Caryophyllaceae:Alsinioideae) in the Hawaiian Islands. *Am. J. Bot.* 85:1377-1388
- Wester P, Claßen-Bockhoff R (2006a) Bird pollination in South African *Salvia* species. *Flora - Morphology, Distribution, Functional Ecology of Plants* 201:396-406

- Wester P, Claßen-Bockhoff R (2006b) Hummingbird pollination in *Salvia haenkei* (Lamiaceae) lacking the typical lever mechanism. *Plant Systematics and Evolution* 257:133-146
- Wester P, Claßen-Bockhoff R (2007) Floral diversity and pollen transfer mechanisms in bird-pollinated *Salvia* species. *Annals of Botany* 100:401-421
- Westerkamp C (1991) Honeybees are poor pollinators — why? *Plant Systematics and Evolution* 177:71-75
- Westerkamp C, Claßen-Bockhoff R (2007) Bilabiate flowers: The ultimate response to bees? *Annals of Botany* 100:361-374
- Whitten WM (1981) Pollination ecology of *Monarda didyma*, *M. clinopodia*, and hybrids (Lamiaceae) in the southern Appalachian Mountains. *American Journal of Botany* 68:435-442
- Williams ML, Drinnan AN, Walsh NG (2006) Variation within *Prostanthera spinosa* (Lamiaceae): evidence from morphological and molecular studies. *Australian Systematic Botany* 19:467-477
- Willmore KE, Zelditch ML, Young N, Ah-Seng A, Lozanoff S, Hallgrímsson B (2006) Canalization and developmental stability in the *Brachyrrhine* mouse. *Journal of Anatomy* 208:361-372
- Wilson P, Castellanos MC, Hogue JN, Thomson JD, Armbruster WS (2004) A multivariate search for pollination syndromes among penstemons. *Oikos* 104:345-361
- Wilson P, Wolfe AD, Armbruster WS, Thomson JD (2007) Constrained lability in floral evolution: counting convergent origins of hummingbird pollination in *Penstemon* and *Keckiella*. *New Phytologist* 176:883-890
- Wolfe LM, Sowell DR (2006) Do pollination syndromes partition the pollinator community? A test using four sympatric morning glory species. *International Journal of Plant Sciences* 167:1169-1175
- Yoder AD, Irwin JA, Payseur BA (2001) Failure fo the ILD to determine data combinability for Slow Loris phylogeny. *Systematic Biology* 50:408-424
- Zborowski P, Storey R (2003) A field guide to insects in Australia. New Holland Publishers, Sydney
- Zhang L-B, Comes HP, Kadereit JW (2001) Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *American Journal of Botany* 88:2331-2345
- Zhang L, Barrett SCH, Gao J-Y, Chen J, Cole WW, Liu Y, Bai Z-L, Li Q-J (2005) Predicting mating patterns from pollination syndromes: the case of "sapromyiophily" in *Tacca chantrieri* (Taccaceae). *Am. J. Bot.* 92:517-524