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A phylogenetic study of the tribe Podalyrieae (Fabaceae)

by

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I declare that this dissertation has been composed by myself and the work contained within, unless otherwise stated, is my own.

Boatwright

J.S. Boatwright (February 2006)

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Abstract

The tribe Podalyrieae is a group of Papilionoid legumes that are largely endemic to the Cape Floristic Region of southern Africa. A phylogenetic study of the tribe was undertaken using gene sequences obtained from the internal transcribed spacer (ITS) as well as the plastid gene *rbcL*. Although the resolution was poor in the resulting trees, several groupings were noted within the tribe. The subtribe Xiphothecinae remains relatively unchanged and consists of the genera *Amphithalea* and *Xiphotheca*. The subtribe Podalyriinae was found to be paraphyletic. A close relationship was observed between the genera *Liparia* and *Podalyria* with *Stirtonanthus* as sister. Additional chloroplast genes (*trnL-F* and *trnS-trnG*) were sequenced to obtain better resolution within this group. While *Podalyria* and *Stirtonanthus* are monophyletic, the monophyly of *Liparia* is still uncertain. *Virgilia* and *Calpurnia* are closely related and *Cyclopia* retains its isolated, monophyletic position sister to the tribe. The species of *Cadia* included in the phylogenetic analysis formed a sister grouping to the tribe Podalyrieae and the inclusion of this genus in Podalyrieae is discussed. A date for the root node of the tribe was estimated at 28.55 MYA, using non-parametric rate smoothing (NPRS), indicating a major radiation to have taken place during the Pliocene. By means of independent contrasts it was determined that the rate of molecular evolution is higher in reseeders than resprouters, perhaps due to more reproductive cycles in these individuals, that would in turn affect the rate of DNA substitution.

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List of abbreviations

ACCTRAN	accelerated transformation
AIC	Akaike Information Criterion
bp	base pair
BP	bootstrap percentage
BSA	bovine serum albumin
CAIC	Comparative Analysis by Independent Contrasts
CFR	Cape Floristic Region
CI	consistency index
cp	chloroplast
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DELTRAN	delayed transformation
dGTP	2'-deoxyguanosine 5'triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
eg	for example
ETS	external transcribed spacer
g	gram(s)
ie	in explanation
IGS	intergenic spacer
ILD	incongruence length difference test
IR	inverted repeat
ITS	internal transcribed spacer
LR	likelihood ratio
LSC	large single-copy region
m	meter(s)
$MgCl_2$	Magnesium chloride
min	minute(s)
ML	maximum likelihood
ml	milliliter(s)
mM	millimolar(s)

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MP	maximum parsimony
MYA	million years ago
nc	nuclear
ng	nanogram(s)
No	number
NPRS	non-parametric rate smoothing
nst	number of substitutions
°C	degrees Celsius
PAUP	phylogenetic analysis using parsimony
PCR	polymerase chain reaction
pers comm.	personal communication
PP	posterior probability
rDNA	ribosomal deoxyribonucleic acid
RI	retention index
sec	second(s)
SSC	small single-copy region
SW	successive weighting
TBR	tree bisection and reconnection
TL	tree length
ts	transition(s)
tv	transversion(s)
v	version
µl	microliter(s)
µM	micromolar(s)

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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

1.1 General introduction

The Cape Floristic Region (CFR) is among the most botanically diverse regions on earth and is regarded as one of the six floral kingdoms of the world (Good, 1964; Takhtajan, 1969). It comprises less than 5% of the total area of South Africa, but contains an estimated 9030 species of vascular plants (8920 of which are flowering plants) with an endemism of 69% (Goldblatt and Manning, 2002). Two of the most notable features of this flora, is the great species richness and high levels of endemism (Good, 1953; Goldblatt, 1978; Linder, 2003). Reasons for this diversity have been associated with numerous factors that create a variety of habitats and niches to be filled e.g. environmental and climatic conditions (including climatic history); diversity of soils; and fire (Goldblatt and Manning 2002; Linder 2003). Fynbos is one of the vegetation types in the CFR and is defined by structural, floristic and phytogeographical criteria. Many definitions exist to describe fynbos. Campbell (1985) summarises the essential features of this vegetation as the presence of restioids, ericoids and proteoids (Figures 1.1 and 1.2).

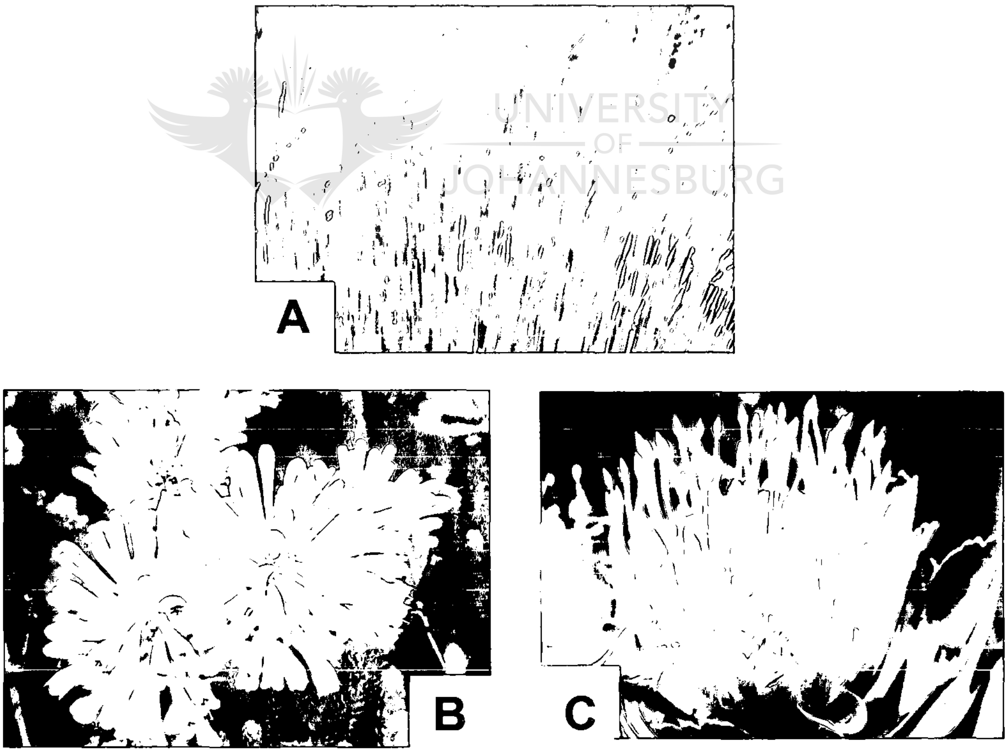

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Figure 1.1 Fynbos vegetation is characterised by the presence of restiods, ericoids and proteoids. A: *Chondropetalum tectorum* (L.f.) Raf. (Restionaceae); B: *Erica sessiliflora* L.f. (Ericaceae); C: *Leucospermum conocarpodendron* (L.) Beuk. (Proteaceae).

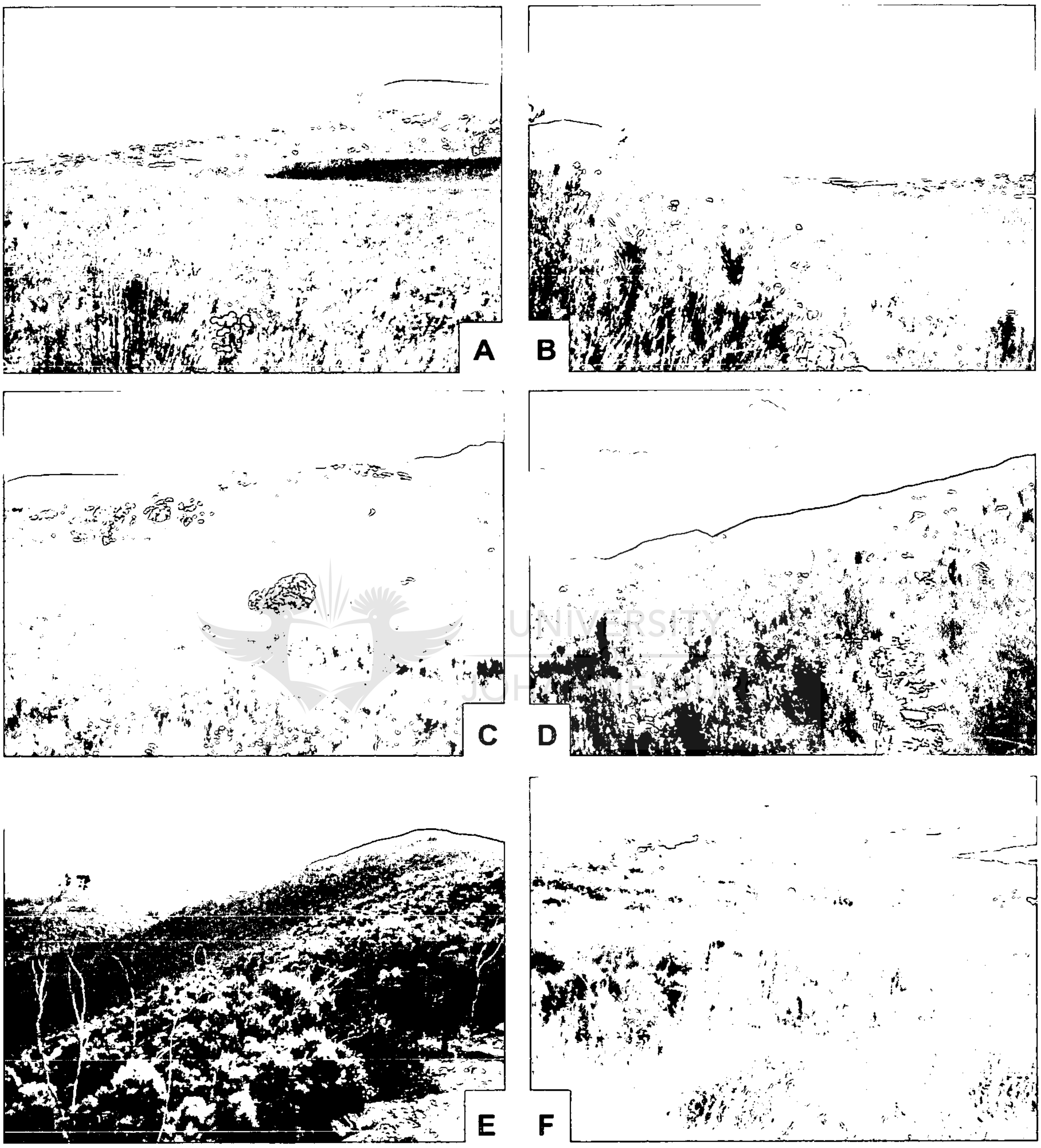

Figure 1.2 Fynbos is a sclerophyllous vegetation type occurring on sandy soils in the Cape. A-D: Fernkloof Nature Reserve; E: Potberg, De Hoop Nature Reserve; F: Swartberg Pass.

The region is primarily dominated by winter rainfall with occasional snow, restricted to the mountains, followed by hot dry summers. The winter rainfall persists in the western Cape, while rain can be expected throughout the year along the south coast, and in the east mainly in summer (Linder, 2003). This Mediterranean climate has remained dominant after its establishment in the Miocene to late Pliocene, about 3-4 million years ago (MYA). It is commonly suggested that the explosive speciation of plant taxa characteristic of fynbos was triggered by this change in climate near the Miocene-Pliocene boundary (Deacon *et al.*, 1992; Linder *et al.*, 1992).

Local fluctuations in rainfall are visible, particularly in mountainous areas, with those slopes facing the prevailing winds receiving more precipitation. The effect of limited rainfall on the vegetation is amplified by differences in soil, becoming more pronounced as rainfall decreases. Different soil types support characteristic vegetation, depending on the levels of precipitation. Forest vegetation normally occurs on deeper soils in places where precipitation is high. At lower rainfall and poorer soil quality, forest is replaced by shrubby or herbaceous vegetation, e.g. fynbos on sandy soils and renosterveld on clay soils (Goldblatt and Manning, 2002). The CFR is largely composed of alternating layers of erosion-resistant sandstone of the Table Mountain and Witteberg Groups, or fine-grained shales of the Bokkeveld Group (Goldblatt and Manning, 2000; 2002). Soils derived from this sandstone bedrock are typically coarse-grained and low in nutrients, but high in aluminium. Soil nutrients play an important role in determining the vegetation type that will occur in a specific area, e.g. the richer soils of the Cape mountains in the east leads to an intermingling of the Cape flora and tropical floristic elements (Campbell, 1983; Cowling, 1983; Linder, 2003).

Frequent fires are an important selective force in plant reproductive ecology and are generally involved in biological processes such as stimulation of flowering and germination (Le Maitre and Midgley, 1992). Fire is an important factor in the Cape floral composition, occurring frequently in cycles of between 5 and 50 years (Linder, 2003). Deacon *et al.* (1992) describe fynbos as pyrophylic or 'fire-loving' vegetation that is dominated by plants with life strategies tuned to the fire regime. Two main fire survival strategies are found. Resprouters which resprout from a woody rootstock after fire (usually with a multi-stemmed appearance) and reseeders that regenerate from seed and are typically single stemmed (Schutte *et al.*, 1995).

As mentioned before the level of endemism is exceptionally high in South Africa, comparable to that of an oceanic island. Six families are endemic to the Cape: Bruniaceae, Geissolomataceae, Grubbiaceae, Peneaceae, Roridulaceae, and Stilbaceae *senso stricto* (including Retziaceae). They are all shrubs that occur mostly in montane habitats (Goldblatt, 1978; Goldblatt and Manning, 2002). The biodiversity in South Africa is not evenly distributed across the sub-continent, so that some areas are more species rich than others, the so-called hotspots. The CFR not surprisingly stands out as the most diverse of these (the richest of the Mediterranean-climate hotspots), with about 6000 endemic species (Myers, 1990; Cowling and Hilton-Taylor, 1994). Myers *et al.* (2000) identified 25 out of the possible 34 hotspots of the world as high priority for conservation, as these contain the sole remaining habitats of 44% of the Earth's plant species and 35% of its vertebrate species. The Succulent Karoo, Maputaland-Pondoland-Albany and CFR in South Africa form part of these very important hotspots (Figure 1.3).

Asteraceae and Fabaceae are the largest families in the CFR, together comprising about 20% of the total species (Goldblatt and Manning, 2002). Fabaceae is well represented in most parts of the world and is the third largest family of flowering plants. It comprises about 727 genera and c. 19325 species. The unifying feature of the family is that the fruit is a legume, usually consisting of a single superior carpel, one locule, two to many ovules and parietal placentation. The family is currently divided into three subfamilies, the Caesalpinioideae, Mimosoideae and Papilionoideae, and 36 tribes. The subfamily Papilionoideae, of which the tribe Podalyrieae is a member, comprises 28 tribes and c. 13800 species (Lewis *et al.*, 2005).

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1.2 Objectives of the study

This study aims to investigate phylogenetic and evolutionary aspects of the tribe Podalyrieae:

1. An almost complete species-level phylogeny for the tribe will be reconstructed using DNA sequences from the internal transcribed spacer (ITS) of nuclear ribosomal DNA and the plastid gene *rbcL*, from which the major lineages and generic relationships can be assessed.
2. The relationship between *Liparia*, *Podalyria* and *Stirtonanthus* will be investigated by sequencing further plastid genes (*trnL-F* and *trnS-trnG*) for these genera and this data can later be used in pollination studies on *Liparia*.
3. The position of *Cadia* and its possible placement within Podalyrieae will be evaluated using ITS and *rbcL* sequence data.
4. The rates of molecular evolution between reseeders and resprouters will be compared to determine whether reseeders have a higher rate of molecular evolution than resprouters.
5. A date for the root node of Podalyrieae will be produced to determine when the major radiation of the tribe took place.

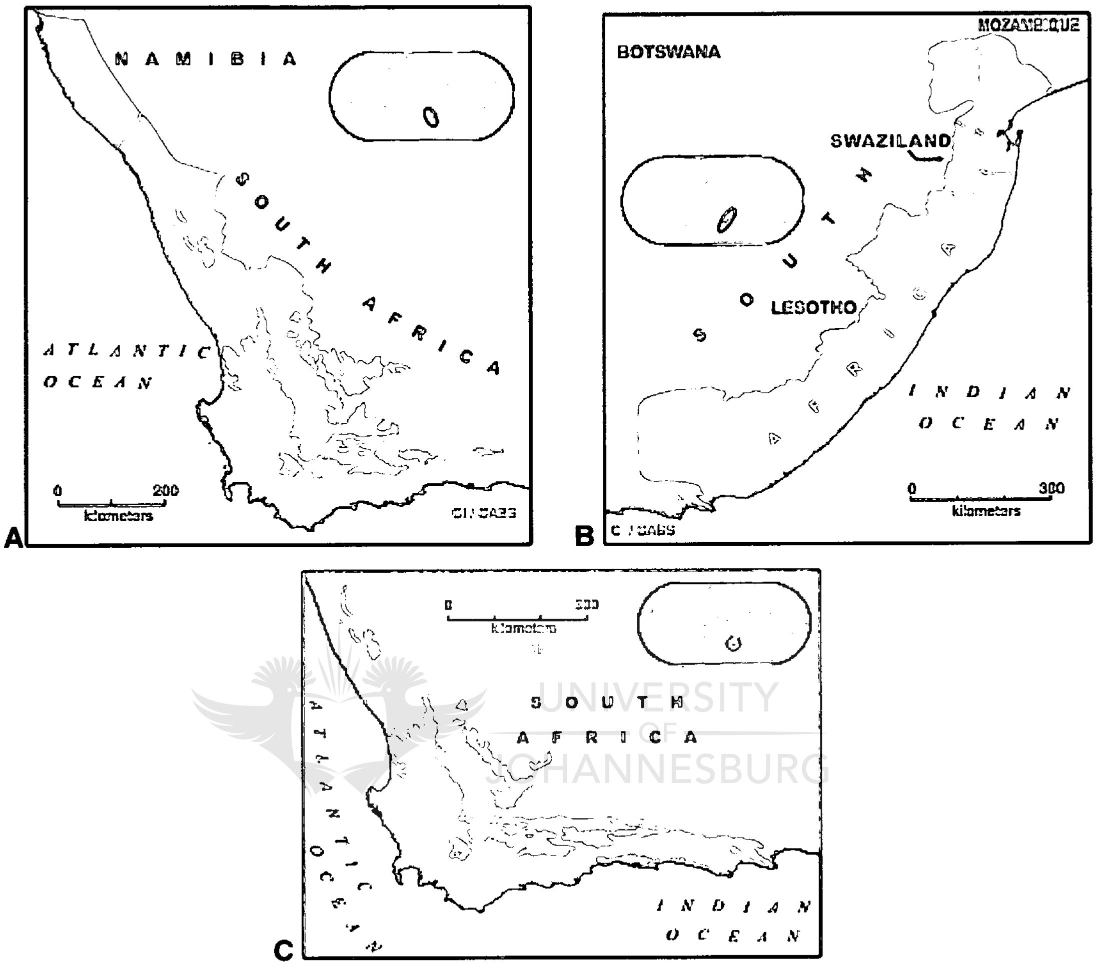

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Figure 1.3 The three hotspots that occur in South Africa. A: The Succulent Karoo (and Namibia) with the richest succulent flora on earth and a remarkably high number of endemic plant species. B: The Maputaland-Pondoland-Albany is an important center of plant endemism and stretches along the east coast of South Africa below the Great Escarpment. C: The CFR is one of the world's five Mediterranean hotspots and is home to the greatest non-tropical concentration of higher plant species in the world (from www.biodiversityhotspots.org).

CHAPTER 2

MATERIAL AND METHODS

2.1 DNA extraction and purification

Total DNA was extracted from herbarium or silica dried leaf material (0.1--0.3 g) using the 2x CTAB method of Doyle and Doyle (1987). Absolute ethanol (2.5x volume) for silica dried material and iso-propanol (2/3x volume) for herbarium material was used to precipitate DNA from the extraction products at –20°C for one and two weeks respectively. The extracted DNA was purified using QIAquick silica columns according to the manufacturer's protocol for purifying PCR products (Qiagen Inc.) Voucher information and author citations for the taxa used in the study are listed in Table 2.1.

2.2 Amplification of the gene regions

Amplification was carried out using polymerase chain reactions (PCR), in 50 μl reactions containing: 25 μl PCR Mastermix [50 units/ml *Taq* DNA Polymerase (pH 8.5), 400 μM each of dATP, dGTP, dCTP, dTTP and 3 mM $MgCl_2$ (Promega Corporation)]; 0.5 μl of both forward and reverse primers (see Table 2.2 for primer references and sequences and Figures 2.1 and 2.2 for diagrams of the gene regions); 1 μl bovine serum albumin (BSA); 1 μl dimethyl sulfoxide (DMSO) for nuclear reactions and DNA template. Sterile distilled water was added to make up a total volume of 50 μl.

Protocols listed in Table 2.3 were used in the PCR reactions. A total of 26 cycles for ITS and 28 cycles for the chloroplast genes were completed (consisting of denaturation, annealing and extension) in a GeneAmp PCR System 9700 thermal cycler. The PCR products were purified using a QIAquick PCR purification kit following manufacturer's instructions. Unamplified taxa for all the gene regions are listed in Table 2.4.

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2.3 Cycle sequencing

Cycle sequencing reactions were performed in 10 μl reactions consisting of: 40 ng cleaned PCR product; 0.5 μl Big Dye Terminator v. 3.1; 0.3 μl primer; 2.0 μl sequencing buffer; 0.5 μl DMSO (for nuclear reactions); and sterile distilled water to make up a final volume of 10 μl. The cycle sequencing thermal profile consists of 26 cycles of 10 sec denaturation at 96°C, 5 sec annealing at 50°C and 4 min at 60°C in a thermal cycler (GeneAmp PCR system 9700). The products were purified using ethanol precipitation to remove any excess dye terminator. Cleaned cycle sequencing products were then directly sequenced on a 3130 *xl* Genetic Analyzer (Applied Biosystems Inc.).

2.4 Phylogenetic analysis

2.4.1 Choice of outgroups

Representatives of the 'core' genistoids were chosen as outgroups in the separate and combined analyses of ITS and *rbcL*. This choice was based on the close relationship that exists between Podalyrieae and other genistoid tribes, especially Crotalarieae and Genisteae. In the analysis of *Liparia*, *Podalyria* and *Stirtonanthus*, the genera *Amphithalea* and *Xiphotheca* were chosen as outgroups due to the close relationship that was noted (Chapter 3). Voucher information, author citations and GenBank accession numbers for outgroups used in the study are listed in Table 2.4--2.6.

2.4.2 Maximum parsimony analysis (MP)

Complimentary strands of the sequenced genes were assembled and edited using Sequencher v. 3.1.2. (Gene Codes Corporation) and aligned manually. Insertions and deletions of nucleotides (indels) were scored as missing data and thus did not contribute to the analysis. Cladistic analyses for both the separate (ITS and *rbcL*) and combined (ITS and *rbcL*; for *Liparia*, *Podalyria* and *Stirtonanthus* ITS, *rbcL*, *trnL-F* and *trnS-trnG*) matrices were performed on a Macintosh G4 using the parsimony algorithm of the software package PAUP v. 4.0bl (Swofford, 1998). Tree searches were performed using a heuristic search with 1000 random sequence additions, tree bisection-reconnection (TBR) branch swapping and the MULPARS option 'on' only for ITS analysis (Chapter 3) and the combined ITS and *rbcL* analysis. All character transformations were treated as equally likely (Fitch parsimony; Fitch, 1971). To reduce the time spent on swapping, 10 trees per replicate were saved. Trees collected in the 1000 replicates were used as starting trees for another search without a tree limit. Delayed transformation character optimisation (DELTRAN) was used to illustrate branch lengths throughout [due to reported errors with accelerated transformation optimisation (ACCTRAN) in PAUP v. 4.0bl]. Internal support was estimated with 1000 bootstrap replicates using TBR and holding a total of 10 trees per replicate (Felsenstein, 1985). Only those clades of greater than 50% frequency are reported. The following scale for support percentages was used: 50--74%, low; 75--84%, moderate; 85--100%, strong.

Congruence of the separate datasets was assessed by visual inspection of the individual bootstrap consensus trees. The bootstrap trees were considered incongruent only if they displayed 'hard' (i.e. with high bootstrap support) rather than 'soft' (i.e. with low bootstrap support) incongruence (Seelanan *et al.*, 1997; Wiens, 1998). 'Conguence tests' such as ILD can be unreliable (Reeves *et al.*, 2001; Yoder *et al.*, 2001) and none of these methods were used.

2.4.3 Successive weighting (SW)

Successive approximations weighting (Farris, 1969) was used in the combined analyses to down-weight base positions that changed excessively to determine the effects of such characters on the tree topology (Chase *et al.*, 2000). For SW the 'reweight characters' command based on the RI, using the maximum value (best fit) criterion and a base weight of 1 was used. The shortest Fitch trees were used as the basis for calculating the initial weights and the search-reweighting process was repeated until the same tree length was obtained twice in succession.

2.4.4 Maximum likelihood analysis (ML)

Methods of phylogenetic inference rely on their underlying models to make assumptions about the processes of DNA substitution. It is because of this fact that all models of evolution should be explored before a choice is made as to which model to use on a specific dataset. In order to do this, a test was performed using MODELTEST v. 3.06, which uses log likelihood scores to estimate which model of DNA evolution best fits the dataset at hand (Posada and Crandall, 1998). A choice is made among 56 possible models specified in MODELTEST ('modelblock' for PAUP). A matrix containing one tree, generated from the heuristic searches, was saved in NEXUS format and read into MODELTEST. Tests were performed using the algorithms in 'modelblock'. Bayesian analysis (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003: MRBAYES) was performed, using MRBAYES v. 2.01, for the combined matrices as stated before. The TIM+I+G model of substitution, indicated by MODELTEST [Akaike Information Criterion (AIC)] as the best fitting model, was used following the procedure set out in the manual. Settings for this model in PAUP v. 4.0bl were nst=6, rates=invgamma, basefrequency=emperical, clock=unconstrained and number of generations=1000000. The resulting trees were plotted against their likelihoods in order to determine where the likelihoods converge on a maximum value. All the trees before this convergence were discarded as the 'burn-in' phase. The remaining trees were imported into PAUP v. 4.0bl and a majority rule consensus tree was produced showing the frequency (i.e. posterior probabilities or PP) of all observed bi-partitions.

2.5 Comparison between the rates of molecular evolution in reseeders and resprouters

The software package CAIC (Comparative Analysis by Independent Contrasts; Purvis and Rambaut, 1995) can be used to analyse comparative data that includes continuous variables. The program can be used among other things to compare rates of evolution among clades or characters. In this study, an analysis was performed to compare the rates of molecular evolution of reseeding versus resprouting individuals in Podalyrieae.

The resulting tree from the Bayesian analysis for the combined ITS and *rbcL* matrix was imported into TREE EDIT v. 1.0a 4.61 (Rambaut and Charleston, 2000) and exported into CAIC format, which resulted in plain text, coded phylogeny and branch length files. A table (tab delimited) was compiled containing branch lengths (continuous variable) from the Bayesian analysis and reseeding/resprouting information (categorical variable), gained from Schutte *et al.* (1995) and Schutte (pers. comm.), scored as 0 for resprouting and 1 for reseeding, for each species in the dataset. All this data was read into CAIC v. 2.6.9 following the procedure set out in the user's guide. The 'brunch' function of the CAIC program was used, as this is suitable for characters that have two states. The statistical results from the analysis done by CAIC were then used to perform a sign-test in order to compare the rates of molecular evolution between reseeders and resprouters. A similar analysis was performed on a dataset of *Protea* L., obtained from the Royal Botanic Gardens in Kew to test whether the higher diversification rates in reseeding species of *Protea*, as was mentioned by Reeves (2001), could be due to higher rates of molecular evolution in the reseeders. The results in this analysis were compared to those obtained for Podalyrieae.

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2.6 Age estimation of the root node of Podalyrieae

Additional sequences were obtained from GenBank and combined with a subset of the data for Podalyrieae to compile a genistoid ITS matrix that could be used in a high-level analysis to date the node of Podalyrieae (voucher information, literature references, author citations and GenBank accession numbers are listed in Table 2.7). Non-parametric rate smoothing (NPRS) was used, which is applied when evolutionary rates vary across lineages (Sanderson, 1997). This algorithm assumes that evolutionary rates are auto-correlated in time and limits the speed with which rates can change from an ancestral to a descendant lineage. A likelihood ratio (LR) test was used to test for rate heterogeneity among the lineages in the dataset. If significant rate heterogeneity is indicated by the test, these differences in branch lengths should be smoothed using NPRS. With this approach, an ultrametric tree was produced in TREE EDIT v. 1.0a 4.61 (Rambaut and Charleston, 2000) without assuming a molecular clock. An estimate of the local rate of molecular evolution for each branch is constructed and the difference between that local rate estimate and its descendants' local rate then minimised (Sanderson, 1997). *Diplotropis*, a Sophoroid fossil described by Herendeen and Dilcher (1990) with a known date of 56 MYA, was used to calibrate the tree (Edwards *et al.* unpublished). To compute an error estimate for the root node of Podalyrieae, the NPRS procedure was applied to 100 bootstrapped matrices.

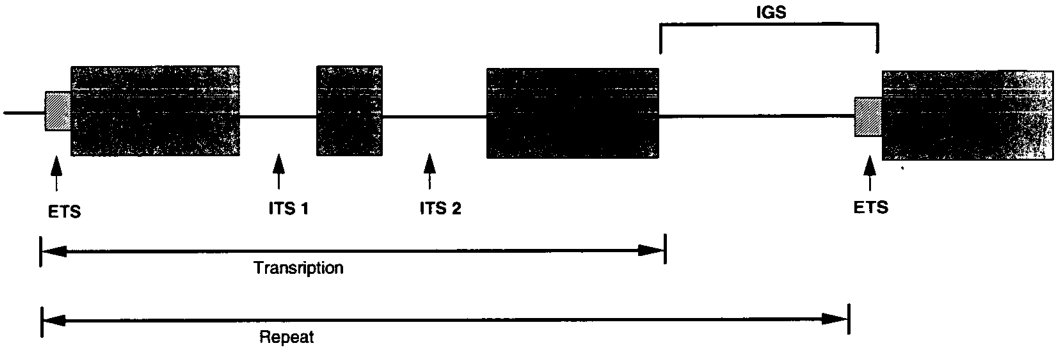

Figure 2.1 Schematic diagram of rDNA repeat in plants. The ribosomal rRNA genes are 18S, 5.8S and 26S. ITS-1 and ITS-2 are the two internal transcribed spacer regions. IGS is the intergenic spacer; ETS is the external transcribed spacer (from Soltis and Soltis, 1998).

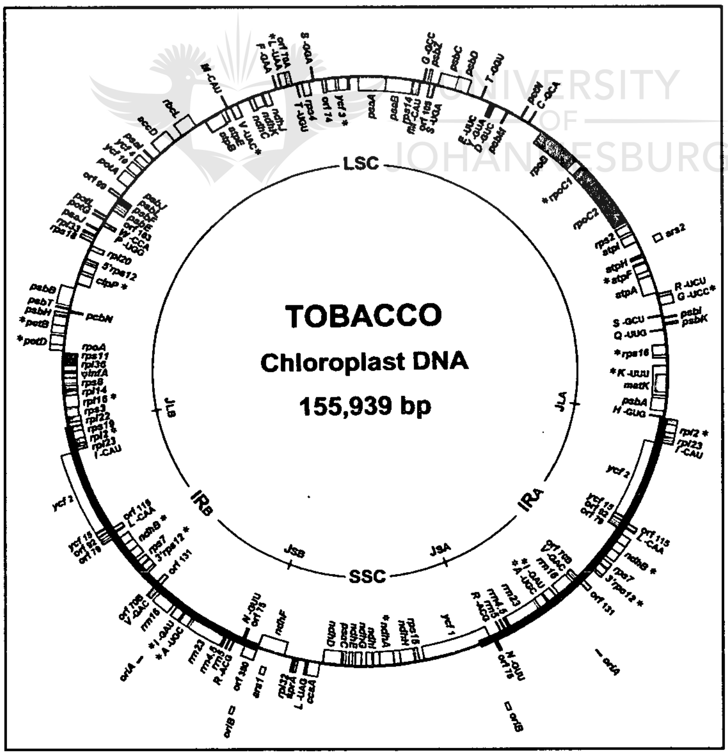

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Figure 2.2 Diagram of the chloroplast genome of tobacco showing the two inverted repeats (IR_A and IR_B), large single-copy (LSC) and the small single-copy (SSC) region (from Wakasugi *et al.*, 2001).

Table 2.1 Sources of plant material and vouchers used in this study (*Private collection of A.L. Schutte; [1]Van der Bank *et al.*, 2002; X= sequences submitted to GenBank, but accession numbers still outstanding).

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
Podalyrieae						
***Amphithalea* Eckl. & Zeyh.**						
A. alba Granby	De Hoop Nature Reserve	*Van Wyk 2125**	X	AM 180171		
A. axillaris Granby	Outeniqua Mountains, Saagtandberg	*Vlok & Schutte 17**	X	AM 180172		
A. biovulata (Bolus) Granby	Hermanus	*M. Johns s.n.*, JRAU	X	AM 180173		
A. ciliaris Eckl. & Zeyh.	Langeberg Mountains	*Vlok & Schutte 193**	X	AM 180174		X
A. cuneifolia Eckl. & Zeyh.	Jonkershoek Nature Reserve	*Vlok & Schutte 381**	X	AM 180176		
A. dahlgrenii (Granby) A.L. Schutte	Langeberg Mountains, Keeromsberg	*Vlok & Schutte 221**	X	AM 180175		
A. ericifolia L.	Bredasdorp, Heuningberg Nature Reserve	*Vlok & Schutte 368**	X	AM 180177		
A. flava (Granby)A.L. Schutte	Bonniedale	*Schutte 652**	X	AM 180178		
A. fourcadei Compton	Plettenberg Bay	*Vlok & Schutte 394**	X	AM 180179		
A. imbricata (L.) Druce	Table Mountain	*N.A. Helme 3426*, NBG	X	AM 180180		X
A. intermedia Eckl. & Zeyh.	Swartberg Mountains	*Schutte 828**	X	AM 180181		
A. micrantha (E. Mey.) Walp.	Swartberg Mountains	*Schutte 751**	X	AM 180182		
A. monticola A.L. Schutte	Hex River Mountains, Matroosberg	*Schutte 562**	X	AM 180183		
A. muirii (Granby) A.L. Schutte	Touwsberg	*Vlok & Schutte 157**	X	AM 180184		
A. muraltioides (Benth.) A.L. Schutte	Swartberg Mountains	*Vlok & Schutte 354**	X	AM 180185		
A. obtusiloba (Granby) A.L. Schutte	Kamiesberg	*N.A. Helme 3449*, NBG	X	AM 180186		
A. oppositifolia L. Bolus	Kogelberg Nature Reserve	*M. Johns s.n.*, JRAU	X	AM 180187		
A. pageae (L. Bolus) A.L. Schutte	Montagu	*Vlok & Schutte 215**	X	AM 180188		
A. parvifolia (Thunb.) A.L. Schutte	Swartberg Mountains	*Vlok & Schutte 190**	X	AM 180189		
A. phylicoides Eckl. & Zeyh.	Outeniqua Mountains, Saagtandberg	*Vlok & Schutte 18**	X	AM 180190		
A. rostrata A.L. Schutte & B.E. van Wyk	Pearly Beach, Carruthers Hill	*Vlok & Schutte 69**	X	AM 177361		
A. speciosa Schltr.	Bredasdorp, Heuningberg Nature Reserve	*Vlok & Schutte 367**	X	AM 177362		

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Table 2.1 Continued.

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
A. spinosa (Harv.) A.L. Schutte	Matjiesfontein	*Van Wyk 2195**	X	AM 177363		
A. stokoei L. Bolus	Kogelberg Nature Reserve	*Vlok & Schutte 297**	X	AM 177364		
A. tomentosa (Thunb.) Granby	Kogelberg Nature Reserve	*Vlok, Van Wyk & Schutte 92**	X	AM 177365		
A. tortilis (E. Mey.) Benth.	Groot Winterhoek Nature Reserve	*Schutte 599**	X	AM 177366		
A. villosa Schltr.	Laingsburg	*Vlok & Schutte 117**	X	AM 177367		
A. violacea (E. Mey.) Benth.	Outeniqua Mountains, Moordkuils River	*Vlok & Schutte 407**	X	AM 177368		
A. virgata Eckl. & Zeyh.	Fernkloof Nature Reserve	*Boatwright & Magee 65*, JRAU	X	AM 177369		
A. vlokii (A.L. Schutte & B.E. van Wyk) A.L. Schutte	Uniondale, Fortkoppie	*Schutte 744**	X	AM 177370		
A. williamsonii Harv.	Baviaanskloof Mountains	*Euston-Brown s.n.**	X	AM 177372		
Calpurnia **E. Mey.**						
C. aurea (Aiton) Benth.	Living collection, RBG, Kew	*RBG, Kew 1991-1626*, K	AJ 409913[1]	-		
C. glabrata Brummit	Transvaal, Carolina District	*K. Baldwin & M-J Baldwin 8502*, J	X	AM 177372		
C. intrusa (R.Br. in W.T.Aiton) E. Mey.	Meiringspoort	*Schutte s.n.**	X	AM 177373		
C. sericea Harv.	Suikerbosrand Nature Reserve	*Boatwright 86*, JRAU	X	AM 177374		
C. sericea x *C. woodii*	Living collection from Moor Park Nature Reserve	*Beaumont s.n.*, NU	X	X		
C. woodii Schinz.	Estcourt	*Beaumont s.n.*, NU	X	AM 177375		
Cyclopia **Vent.**						
C. alopecuroides A.L. Schutte	Kammanassie Nature Reserve	*Vlok & Schutte 129**	AM 050828	X		
C. alpina A.L. Schutte	Hottentots Holland Nature Reserve	*Vlok & Schutte 250**	AM 050830	X		
C. aurescens Kies	Klein Swartberg Mountains	*AL & BvW 771b*, JRAU	AM 050826	X		
C. bolusii Hofmeyr & Phillips	Swartberg Nature Reserve	*Schutte 826**	X	X		

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Table 2.1 Continued.

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
C. burtonii Hofmeyr & Phillips	Swartberg Pass	*Vlok & Van Wyk 189*, JRAU	AJ 310733[1]	-		
C. falcata (Harv.) Kies	Groot Winterhoek, Voorberg	*AL 598*, JRAU	X	X		
C. galioides (P.J. Bergius) DC.	Cape Point Nature Reserve	*De Lange 13**	AM 050825	X		
C. genistoides (L.) R.Br.	De Hoop Nature Reserve, Potberg	*Boatwright & Magee 53*, JRAU	AM 050819	X		
C. glabra (Hofmeyr & Phillips) A.L. Schutte	Hex River Mountains, Matroosberg	*Schutte 558**	AM 050830	X		
C. intermedia E.Mey.	Mossel Bay	*AL 658*, JRAU	X			
C. longifolia Vogel	Vanstadensrivier Mountains	*Vlok & Schutte 422**	AM 050820	X		
C. maculata (Andrews) Kies	Garcia Forest Station	*Schutte 609-611*, JRAU	AJ 409896[1]	X		
C. meyeriana Walp.	Hottentots Holland Nature Reserve	*Vlok & Schutte 251**	AM 050818	X		
C. plicata Kies	Uniondale, Hoopsberg	*AL 670b*, JRAU	X	X		
C. pubescens Eckl. & Zeyh.	Port Elizabeth	*Schutte 685-689*, JRAU	AJ 409897[1]	X		
C. sessiliflora Eckl. & Zeyh.	Swellendam, Langeberg	*Vlok & Shutte 213**	AM 050831	X		
C. subternata Vogel	Outeniqua Pass	*Boatwright & Magee 35*, JRAU	AM 050821	X		
***Liparia* L.**						
L. angustifolia (Eckl. & Zeyh.) A.L. Schutte	Fernkloof Nature Reserve	*Boatwright & Magee 66*, JRAU	X	AM 177376	X	X
L. bonaespei A.L. Schutte	Hottentots Holland Mountains, Mooredenaarskop	*N.A. Helme & D. Raimondo 3430*, NBG	X	AM 177377	X	X
L. boucheri (E.G.H. Oliv. & Fellingham) A.L. Schutte	Kogelberg Nature Reserve	*M. Johns s.n.*, JRAU	X	AM 177378	X	-
L. calycina (L. Bolus) A.L. Schutte	Hottentots Holland Mountains	*Vlok & Schutte 129**	X	AM 177379	-	-
L. capitata Thunb.	Klein Swartberg	*ALS & BvW 776*, JRAU		AM 177380	X	X
L. confusa A.L. Schutte	Swartberg Mountains	*Vlok & Schutte 502**	X	X	X	X
L. congesta A.L. Schutte	Swartruggens	*Bean 2619**	X	X	-	-
L. genistoides (Lam.) A.L. Schutte	Kammanassie Mountains	*Schutte 752**	X	X	X	X

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Table 2.1 Continued.

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
L. hirsuta Thunb.	Montagu Pass	*Boatwright & Magee 33*, JRAU	X	X	X	X
L. latifolia (Benth.) A.L. Schutte	Franschhoek Mountains	*N.A. Helme 3455*, NBG	X	X	X	X
L. myrtifolia Thunb.	Zeeliesrug	*Van Wyk 2639**	X	X	X	X
L. parva Vogel ex Walp.	Cape Point	*Van Wyk 3149, 3243*, JRAU	AJ 409909[1]	X	X	X
L. racemosa A.L. Schutte	Swartberg Mountains	*Vlok & Schutte 501**	X	X	X	-
L. rafnioides A.L. Schutte	Kogelberg Nature Reserve	*M. Johns s.n.*, JRAU	X	X	X	X
L. splendens (Burm. F.) Bos & De Wit subsp. *comantha* (Eckl. & Zeyh.) Bos & De Wit	Langeberg Mountains	*Vlok & Schutte 211**	X	X	X	X
L. splendens (Burm. F.) Bos & De Wit subsp. *splendens*	Fernkloof Nature Reserve	*Boatwright & Magee 8*, JRAU		X	X	X
L. striata A.L. Schutte	Heidelberg, Verkykerskop	*Schutte 759**	X	X	X	X
L. umbellifera Thunb.	Hex River Mountains, Matroosberg	*Schutte 561**	X	X	X	-
L. vestita Thunb.	Fernkloof Nature Reserve	*Boatwright & Magee 62*, JRAU	X	X	X	X
***Podalyria* Willd.**						
P. argentea (Salisb.) Salisb.	Kogelberg Nature Reserve	*Vlok, Van Wyk & Schutte 4**	X	X	X	-
P. biflora (L.) Lam.	Kortefontein, Langeberg	*Vlok s.n.**	X	X	X	X
P. burchelli DC.	Zuurberg Mountains	*B-E & M. van Wyk 7**	X	X	X	X
P. buxifolia (Retz.) Lam.	Montagu Pass	*Boatwright & Magee 34*, JRAU	X	X	X	-
P. calyprata (Retz.) Willd.	-	*Chase 16091*, K	X	X	-	-
P. canescens E. Mey	Paarl	*Van Wyk 3237*, JRAU	X	X	X	-
P. cordata (Thunb.) R.Br.	Kogelberg Nature Reserve	*Vlok & Schutte 311**	X	X	X	-
P. cuneifolia Vent.	Port Elizabeth	*Van Wyk 2888, 3177*, JRAU	AJ 409904[1]	X	X	-
P. hirsuta (W.T. Aiton) Willd.	Kogelberg Nature Reserve	*Vlok & Schutte 437**	X	X	X	-

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Table 2.1 Continued.

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
P. intermedia Eckl. & Zeyh.	Franschhoek Pass	*Van Wyk 3003*, JRAU	AJ 409899[1]	-	-	-
P. lanceolata (E.Mey) Benth.	Langeberg Mountains, Tradouws Pass	*Vlok & Schutte 76**	X	X	X	-
P. leipoldtii L. Bolus ex A.L. Schutte	Paleisheuwel	*Van Wyk 3128*, JRAU	AJ 409902[1]	X	X	-
P. microphylla E.Mey.	Durbanville, Klipheuwel	*Vlok & Schutte 423**	X	X	X	-
P. myrtillifolia (Retz.) Willd.	Franschhoek Pass	*Van Wyk 2995, 3004*, JRAU	AJ 409901[1]	X	X	-
P. oleaefolia Salisb.	Kogelberg Nature Reserve	*Vlok, Van Wyk & Schutte 76**	X	X	X	X
P. orbicularis (E. Mey.) Eckl. & Zeyh.	Caledon Swartberg	*Vlok & Schutte 428**	X	X	X	X
P. pearsonii E.Phillips	Nieuwoudtville	*Vlok & Schutte 47**	X	X	X	X
P. rotundifolia (P.J. Bergius) A.L. Schutte	Paarlberg	*Vlok & Schutte 441**	X	X	X	X
P. sericea (Andrews) R. Br.	Du Toit's Kloof	*Vlok & Schutte 63b*, JRAU	AJ 409903[1]	X	X	X
P. speciosa Eckl. & Zeyh.	Kogelberg Nature Reserve	*Boatwright & Magee 79*, JRAU	X	X	X	X
P. variabilis A.L. Schutte (ined.)	Riviersonderend Mountains	*Vlok & Schutte 230**	X	X	X	X
P. velutina Burch. Ex Benth.	-	*A.E. van Wyk 337*, PRU	-	X	-	-
***Stirtonanthus* B.E. van Wyk & A.L. Schutte**						
S. chrysanthus (Adamson) B.E. van Wyk & A.L. Schutte	Klein Swartberg	*Van Wyk & Schutte 3297*, JRAU	X	X		
S. insignis (Compton) B.E. van Wyk & A.L. Schutte	Montagu	*Schutte & Van Wyk 721*, JRAU	AJ 409906[1]	X		
S. taylorianus (L. Bolus) B.E. van Wyk & A.L. Schutte	Swartberg Pass	*Van Wyk & Schutte 3248*, JRAU	AJ 409907[1]	X		
***Virgilia* Poir.**						
V. divaricata Adamson	The Craggs	*Van Wyk 879-888*, JRAU	AJ 409910[1]	X		

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Table 2.1 Continued.

Species	Source	Voucher & Herbarium	GenBank accession number			
			ITS	*rbcL*	*trnL-F*	*trnS-trnG*
V. oroboides (P.J. Bergius) T.M. Salter subsp. *ferruginea* B.E. van Wyk	Ruitersbos	*Van Wyk 956, 957*, JRAU	AJ 409911[1]	X		
V. oroboides (P.J. Bergius) T.M. Salter subsp. *oroboides*	Betty's Bay	*Van Wyk 802-806*, JRAU	AJ 409912[1]	X		
***Xiphotheca* Eckl. & Zeyh.**						
X. canescens (Thunb.) A.L. Schutte & B.E. van Wyk	Van Rhyns Pass	*AL 595*, JRAU	X	X		
X. cordifolia A.L. Schutte & B.E. van Wyk	-	*N.A. Helme 2852*, NBG	X	X		
X. elliptica (DC.) A.L.Schutte & B.E. van Wyk	Kogelberg Nature Reserve	*M. Johns s.n.*, JRAU	X	X		
X. fruticosa (L.) A.L Schutte & B.E. van Wyk	Montagu, Pypsteelfontein	*Schutte 673-675*, JRAU	AJ 310726[1]	X		
X. guthriei (L. Bolus) A.L. Schutte & B.E. van Wyk	Viljoenshof	*Vlok & Schutte 4**	X	X		X
X. lanceolata (E.Mey.) Eckl. & Zeyh.	Durbanville, Klipheuwel	*Vlok & Schutte 424**	X	X		X
X. phylicoides A.L. Schutte & B.E. van Wyk	Attaquaskloof Nature Reserve	*Vlok 2500**	X	X		
X. reflexa (Thunb.) A.L. Schutte & B.E. van Wyk	Heidelberg Division, Verkykerskop	*AL 760*, JRAU	X	X		
X. tecta(Thunb.) A.L. Schutte & B.E. van Wyk	Du Toit's Kloof	*Schutte 714, 738*, JRAU	AJ 310727[1]	X		
Sophoreae						
***Cadia* Forssk.**						
C. commersoniana Baill.	Madagascar, Toliara	*Ambri & Arifin W584*, K	X	X		
C. pedicellata Baker	Madagascar, Fianarantsoa	*J.-N. Labat 2423*, K	X	X		
C. pubescens Bojer ex Baker	Madagascar, Antananarivo	*L.J. Dorr, L.C. Barnett & R. Brooks 3279*, K	X	-		
C. purpurea (Ait.) Forssk.	Somalia	*J.J. Beckett 1702*, K	X	X		

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Table 2.2 Primer sequences and references for the gene regions studied.

Region	Primer sequence (5'--3')	Reference
cpDNA		
rbcL	1F (ATG TCA CCA CAA ACA GAA AC)	Olmstead *et al.*, 1992
	724R (TCG CAT GTA CCT GCA GTA GC)	Fay *et al.*, 1997
	636F (GCG TTG GAG AGA TCG TTT GT)	"
	1460R (TCC TTT TAG TAA AAG ATT GGG CCG AG)	Olmstead *et al.*, 1992
trnL-F intergenic spacer	*c* (CGA AAT CGG TAG ACG CTA GG)	Taberlet *et al.*, 1991
	d (GGG GAT AGA GGG ACT TGA AC)	"
	e (GGT TCA AGT CCC TCT ATC CC)	"
	f (ATT TGA ACT GGT GAC ACG AG)	"
trnS- trnG (GCU)	GCC GCT TTA CAC TCA GC	Hamilton, 1999
(UCC)	GAA CGA ATC ACA CTT TTA CCA C	"
ncDNA		
ITS	AB 101 (ACG AAT TCA TGG TCC GGT GAA GTG TT)	Sun *et al.*, 1994
	AB 102 (TAG AAT TCC CCG GTT CGC TCG CCG TT)	"
	ITS-2 (GCT GCG TTC TTC ATC GAT GC)	White *et al.*, 1990
	ITS-3 (GCA TCG ATG AAG AAC GCA GC)	"

Table 2.3 Cycling protocols used for PCR amplifications.

Gene	Premelt	Denaturation	Annealing	Extension	Final extension
cpDNA					
rbcL	94°C (3 min)	94°C(1 min)	48°C (1 min)	72°C (1:30 min)	72°C (7 min)
trnL-F	94°C(3 min)	94°C(1 min)	48°C (1 min)	72°C (1 min)	72°C (7 min)
trnS-trnG	94°C(3 min)	94°C(1 min)	50°C (1 min)	72°C (1:30 min)	72°C (7 min)
ncDNA					
ITS	94°C (1 min)	94°C (1 min)	48°C (1 min)	72°C (3 min)	72°C (7 min)

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Table 2 4 Unamplified taxa for the genes studied.

Regions	**Species**
cpDNA	
rbcL	*Cadia pubescens, Calpurnia aurea, Cyclopia burtonii, Podalyria intermedia*
trnL-F	*Liparia calycina*, *L. congesta*, *Podalyria calyptrata*
trnS-trnG	*Liparia boucheri*, *L. calycina*, *L. congesta*, *L. racemosa*, *L. umbellifera*, *Podalyria argentea*, *P. buxifolia, P. calyptrata, P. canescens, P. cordata, P. cuneifolia, P. hirsuta, P. lanceolata, P. leipoldtii, P. microphylla, P. myrtillifolia*
ncDNA	
ITS	*Podalyria velutina*

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Table 2.5 Sources of plant material used as outgroups in the ITS analysis (*unpublished).

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Argyrolobium* Eckl. & Zeyh.**			
A. harmsianum Schltr. Ex Harms	*Crisp 9042,* CANB	AF 287685	Crisp *et al.*, 2000
A. lunare Druce	*Crisp 9039*, CANB	AF 287686	Crisp *et al.*, 2000
***Aspalathus* Amm.**			
A. longifolia Benth.	*B.-E. van wyk 2799*, JRAU	-	Van der Bank*
A. nivea Thunb.	*B.-E. van Wyk 2938*, JRAU	-	Van der Bank*
***Crotalaria* L.**			
C. capensis Jacq.	*B.-E. & M. van Wyk 1863*, JRAU	-	Van der Bank*
C. lebeckioides Bond	*B.-E. van Wyk 3315*, JRAU	-	Van der Bank*
***Dichilus* DC.**			
D. strictus E. Mey.	*Crisp 9073,* CANB	AJ 287684	Crisp *et al.*, 2000
***Lebeckia* Thunb.**			
L. cytisoides Thunb.	*A.L. Schutte 286*, JRAU	-	Van der Bank*
L. wrightii (Harv.) Bolus	*B.-E. van Wyk 3354*, JRAU	-	Van der Bank*
***Lotononis* (DC.) Eckl & Zeyh.**			
L. alpina (Eckl. & Zeyh.) B.-E. van Wyk	*B.-E. & M. van Wyk 1478*, JRAU	-	Van der Bank*
L. laxa Eckl. & Zeyh.	*Crisp 9075,* CANB	AF 287677	Crisp *et al.*, 2000

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Table 2.5 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Maackia* Rupr. & Maxim.**			
M. amurensis Rupr. & Maxim	Botanical Gardens Göttingen, Germany	Z 72336 & Z 72352	Käss , 1995
***Melolobium* Eckl. & Zeyh.**			
M. adenodes Eckl. & Zeyh.	*Van Wyk 4036*, JRAU	AM 050832	Moteetee, 2003
M. candicans Eckl. & Zeyh.	*Van Wyk 4016*, JRAU	AM 050833	Moteetee, 2003
***Pearsonia* Dümmer**			
P. grandifolia (Bolus) subsp. *latibracteolata* (Dümmer) Polhill	*B.-E. van Wyk 3047*, JRAU	-	Van der Bank*
P. sessilifolia (Harvey) Dümmer	*Crisp 9078*, CANB	AJ 287675	Crisp *et al.*, 2000
***Polhillia* C. H. Stirton**			
P. pallens C.H. Stirton	*B.-E. van Wyk 2128*, JRAU	-	Van der Bank*
***Rafnia* Thunb.**			
R. alata G.J. Campbell & B.-E. van Wyk	*Campbell & van Wyk 8*, JRAU	AJ 744938	Motsi, 2004
R. vlokii G.J. Campbell & B.-E. van Wyk	*Van Wyk 3172*, JRAU	AJ 744937	Motsi, 2004
***Sophora* L.**			
S. tetraphylla J.S. Muell.	*RBG, Kew 1977-1212*, K	AJ 310734	Van der Bank *et al.*, 2002
S. toromiro Skottsb.	*RBG, Kew 1994-2331*, K	AJ 409921	Van der Bank *et al.*, 2002
***Styphnolobium* Scott.**			
S. japonicum Scott.	*RBG, Kew 1972-10834*, K	AJ 409920	Van der Bank *et al.*, 2002

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Table 2.5 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Thermopsis* R.Br.**			
T. divaricarpa Nelson	*Wang, Sun & Yang 191730*	AY 091575	Wang *et al.**
T. montana Torrey & A. Gray	*HbUR/Ktm 101*	AF 384336 & AF 384337	Ainouche *et al.*, 2003

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Table 2.6 Voucher information for outgroups used in the *rbcL* analysis (*unpublished).

Species	Voucher & Herbarium	GenBank accession number *rbcL*	Reference
***Argyrolobium* Eckl. & Zeyh.**			
A. marginatum Bolus	*T. Edwards 471*	Z 95547	Käss & Wink, 1997
A. uniflorum (Decne.) Jaub. & Spach	*El-Shazly 477*	Z 95548	Käss & Wink, 1997
***Aspalathus* L.**			
A. cephalotes Thunb.	*Heidrich 373*	Z 70132	Käss, 1995
***Crotalaria* L.**			
C. capensis Jacq.	*Heidrich 366*	Z 70133	Käss, 1995
C. incana L.	Botanical Gardens Coimbra, Portugal	Z 70134	Käss & Wink, 1995
***Dichilus* DC.**			
D. lebeckioides DC.	*McMurtry 6367*, K	U 74223	Doyle *et al.*, 1997
***Euchresta* Benn.**			
E. japonica Hook.f. ex Regel	*Kato & Kuribayashi 930674*, KYO	AB 127040	Lee *et al.*, 2004
***Lotononis (DC.)* Eckl. & Zeyh.**			
Lotononis galpinii Dümmer	*T. Edwards 480*	Z 95538	Käss & Wink, 1997
***Maackia* Rupr. & Maxim.**			
M. floribunda (Miq.) Takeda	*Kurosaki & Nogamasu 2324*, KYO	AB 127042	Lee *et al.*, 2004
M. tashiroi (Yatabe) Makino	*Deguchi et al. 46910*, KYO	AB 127043	Lee *et al.*, 2004

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Table 2.6 Continued.

Species	**Voucher & Herbarium**	**GenBank accession number *rbcL***	**Reference**
***Melolobium* Eckl. & Zeyh.**			
M. microphyllum (L.f.) Eckl. & Zeyh.	*T. Edwards 470*	Z 95539	Käss & Wink, 1997
M. obcordatum Harv.	*T. Edwards 469*	Z 95540	Käss & Wink, 1997
***Sophora* L.**			
S. microphylla Aiton	*CHR 529930*	AY 725480	Heenen *et al.*, 2004
S. tomentosa L.	*CHR 569752*	AY 725481	Heenen *et al.*, 2004

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Table 2.7 Voucher information and GenBank accession numbers for the genistoid legumes used in the high-level analysis (*unpublished).

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Acosmium* Schott**			
A. panamense (Benth.) Yakovlev	*Hughes 1308*, FHO	AF 187084	Lavin *et al.*, 2001
***Adenocarpus* DC.**			
A. viscosus (Willd.) Webb & Berth	*Käss 343*	Z 72300 & Z 72301	Käss, 1995
***Aenictophyton* A.T. Lee**			
A. reconditum A.T. Lee	*Fryxell 4500*, CANB	AF 287654	Crisp *et al.*, 2000
***Anagyris* L.**			
A. foetida L.	*Wang, Sun & Yang 49739*	AY 091571	Wang *et al.* *
***Anarthrophyllum* Benth.**			
A. cumingii (Hook. & Arn.) Philippi f.	*AC 23756*, G	AY 609186 & AY 609196	Ainouche & Misset*
***Aotus* Sm.**			
A. carinata Meisn.	*Chappill 6581*	AY 883352	Orthia *et al.*, 2005
A. cordifolia Benth.	*Chappill 6587*	AY 883353	Orthia *et al.*, 2005
***Argyrocytisus* (Maire) Raynaud**			
A. battandieri (Maire) Raynaud	*Wink 397*	Z 95580 & Z95581	Käss & Wink, 1997
***Argyrolobium* Eckl. & Zeyh.**			
A. harveyanum Oliver	*T. Edwards 471*	Z 95582 & Z 95583	Käss & Wink, 1997
A. marginatum Bolus	*T. Edwards 477*	Z 95564 & Z 95565	Käss & Wink, 1997
A. zanonii (Turra) F.W. Ball	*Käss 172*	Z 72274 & Z 72275	Käss, 1995

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Aspalathus* Amm.**			
A. cordata (L.) R. Dahlgr.	*Crisp 9067* , CANB	AF 287681	Crisp *et al.* , 2000
A. corrudifolia P.J. Bergius	*Crisp 9037* , CANB	AF 287682	Crisp *et al.* , 2000
A. linearis L. (N.L. Burm.) R. Dahlgr.	*Van Wyk 3630* , JRAU	AJ 744951	Motsi, 2004
A. subulata Thunb.	*B.-E. van Wyk 1425* , JRAU	-	Motsi, 2004
***Baphia* Afzel. ex Lodd.**			
B. madagascariensis (A.A. Heller) A.A. Heller	*D.J. Du Puy M554* , K	U 59888	Hu *et al* ., 2002
***Baptisia* Vent.**			
B. australis R.Br. var. *aberans* (Larisey) M.G. Mendenh.	*Wang, Sun & Yang 149633*	AY 091572	Wang *et al.* *
B. tinctoria (L.) R.Br.	Botanical Gardens Heidelberg, Germany	Z 72314 & Z 72315	Käss, 1995
***Bolusanthus* Harms**			
B. speciosus (Bolus) Harms	*J.P. 37 et* , H.G.	-	Motsi, 2004
***Bossiaea* Vent.**			
B. lenticularis DC.	*MDC 9289*	AF 518104	Crisp & Cook, 2003
B. linophylla R.Br.	*Crisp 8927* , CANB	AF 287657	Crisp *et al.* , 2000
***Brongniartia* Kunth.**			
B. alamosana Rydb.	*Hu 1120* , DAV	AF 467022	Hu *et al.* , 2002
***Calicotome* Link**			
C. villosa (Poir.) Link	*Käss 175*	Z 72252 & Z 72253	Käss, 1995

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
***Chorizema* Labill.**			
C. aciculare C.A. Gardner	*MDC 9202*	AF 518108	Crisp & Cook, 2003
C. varium Benth. Ex Lindl.	*MDC 8528*	AF 518112	Crisp & Cook, 2003
***Crotalaria* L.**			
C. capensis Jacq.	*Heidrich 366*	Z 72310 & Z 72311	Käss, 1995
C. hyssopifolia Klotzsch	*Singh, Malathum & Murray 1352*	AF 313494	Jourand*
C. lanceolata E. Mey.	*Jourand 165770*	AF 313495	Jourand*
C. lathyroides Guill. & Perr.	*Jourand 165771*	AF 313496	Jourand*
C. ochroleuca G. Don.	*Negri, Webster, Hill & Heyward*	AF 313497	Jourand*
C. pallida Ait.	Botanical Gardens Lome, Togo	Z 72312 & Z 72313	Käss, 1995
C. perrottetii DC.	*Jourand 165773*	AJ 313498	Jourand*
C. podocarpa DC.	*Jourand 48249*	AJ 313500	Jourand*
C. retusa L.	*Jourand 165774*	AJ 313501	Jourand*
C. senegalensis (Pers.) DC.	*Jourand 165775*	AJ 313502	Jourand*
***Cyclolobium* Benth.**			
C. nutans C.T. Rizzini & E.P. Heringer	*Ratter et al. 7431* , E	AF 467041	Hu *et al.* , 2002
***Cytisophyllum* O. Lang**			
C. sessilifolium (L.) O. Lang	Botanical Gardens Hohenheim, Germany	Z 72254 & Z 72255	Käss, 1995
***Daviesia* Sm.**			
D. mimosoides R.Br.	*Crisp 9151*	AY 883356	Orthia *et al.* , 2005
***Diplotropis* Benth.**			

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
D. martiusii Benth.	*Beck, Henner & Jo Cardosa 166*, US	AY 553711	Wojciechowski*
***Echinospartum* (Spach) Rothm.**			
E. boissieri (Spach) Rothm.	*MAF 148150*	AY 609188 & AY 609193	Ainouche & Misset*
***Erinacea* Adans.**			
E. anthyllis Link	Botanical Gardens Tübingen, Germany	Z 72256 & Z 72257	Käss, 1995
***Genista* L.**			
G. teretifolia Willk.	*MAF 162924*	AY 263668	Pardo *et al.*, 2004
G. tournefortii Spach	*MAF 160762*	AY 263669	Pardo *et al.*, 2004
***Goodia* Salisb.**			
G. lotifolia Salisb.	*ANBG 702052*	AF 287655	Crisp *et al.*, 2000
G. medicaginea F. Muell.	*MDC 9274*	AF 518103	Crisp & Cook, 2003
***Hesperolaburnum* Maire**			
H. platycarpum (Maire) Maire	*MA 586956*	AY 263678	Pardo *et al.*, 2004
***Hovea* R.Br.**			
H. elliptica (Sm.) DC.	*Crisp 8924*, CANB	AF 287640	Crisp *et al.*, 2000
***Hypocalyptus* Thunb.**			
H. coluteoides (Lam.) R. Dahlgr.	*Schutte 730*, JRAU	AJ 409917	Van der Bank *et al.*,
H. oxalidifolius (Sims) Baillon	*Schutte 468*, JRAU	AJ 409918	Van der Bank *et al.*,

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
H. sophoroides (P.J. Bergius) Baillon	*Van Wyk 3012*, *3319*, JRAU	AJ 409919	Van der Bank *et al.*,
***Isotropis* Benth.**			
I. foliosa Crisp	*MDC 9121*	AF 518105	Crisp & Cook, 2003
I. forrestii F. Muell.	*Crisp 9261*	AY 883357	Orthia *et al.*, 2005
***Jacksonia* R.Br. Ex Sm.**			
J. alata Benth.	*MDC 8956*	AF 518106	Crisp & Cook, 2003
J. macrocalyx Meisn.	*MDC 9272*	AF 519107	Crisp & Cook, 2003
***Laburnum* Fabr.**			
L. anagyroides Medik.	*MAF 162279*	AY 263679	Pardo *et al.*, 2004
***Lebeckia* Thunb.**			
L. inflata Bolus	*Belle Barker 204*, JRAU	-	Motsi, 2004
L. lotononoides Schltr.	*B.-E. van Wyk 149*, JRAU	-	Motsi, 2004
L. sericea Thunb.	*C.M. van Wyk 2584*, JRAU	-	Motsi, 2004
L. sessilifolia (Eckl. & Zeyh.) Benth.	*Crisp 9041*, CANB	AF 287678	Crisp *et al.*, 2000
***Leptosema* Benth.**			
L. daviesioides (Turcz.) Benth.	*Crisp 9193*	AY 883360	Orthia *et al.*, 2005
***Lotononis* (DC.) Eckl. & Zeyh.**			
L. oxyptera (E. Mey) Benth.	*B.-E. van Wyk 2316*, JRAU	-	Motsi, 2004
L. sericophylla Benth.	*B.-E. van Wyk 1647*, JRAU	-	Motsi, 2004
***Lupinus* L.**			
L. arcticus S. Watson	*Hb, ALTA/95826*	AF 007495	Ainouche & Bayer, 1999

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
L. polyphyllus Lindley	*USDA/504404*	AF 007496	Ainouche & Bayer, 1999
Melolobium **Eckl. & Zeyh.**			
M. canescens Benth.	*Dean 648*, JRAU	AM 050834	Moteetee, 2003
M. calycinum Benth.	*Moteetee 10*, JRAU	AM 050835	Moteetee, 2003
M. humile Eckl. & Zeyh.	*Van Wyk 2351*, JRAU	AM 050836	Moteetee, 2003
M. lampolobum (E. Mey.) Moteetee & B.-E. van Wyk	*Van Wyk 2145*, JRAU	AM 050837	Moteetee, 2003
Mirbelia **Sm.**			
M. longifolia C.A. Gardner	*Crisp 9263*	AY 883361	Orthia *et al*., 2005
M. speciosa DC.	*ANBG 8100876*	AF 518116	Crisp & Cook, 2003
Muelleranthus **Hutch.**			
M. trifoliolatus (F. Muell.) Hutch.	*Lally 743*, CANB	AF 287653	Crisp *et al.*, 2000
Nemcia **Domin**			
N. plicata (Turcz.) Crisp	*Crisp & Cook 150654*	AF 518119	Crisp & Cook*
Oxylobium **Andrews**			
O. cordifolium Andrews	*MDC 9133*	AF 518117	Crisp & Cook, 2003
Petteria **C. Presl**			
P. ramentacea (Sieber) C. Presl	Botanical Gardens Gießen, Germany	Z 72232 & Z 72233	Käss, 1995
Pickeringia **Nutt. ex Torr. & Gray**			

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
P. montana Torrey & A. Gray	*Wang, Sun & Yang 191728*	AY 091568	Wang *et al.* *
***Piptanthus* Sweet**			
P. tomentosus Franchet	*Wang, Sun & Yang 111852*	AY 091570	Wang *et al.* *
***Podolobium* R.Br.**			
P. aciculiferum F. Muell.	*GTC 606*	AF 518118	Crisp & Cook, 2003
***Poecilanthe* Benth.**			
P. falcata (Vell.) Heringer	*De Lima 2*, RJ	AF 467492	Hu *et al*., 2002
***Pultenaea* Sm.**			
P. pedunculata Hook.	*De Kok 756*	AY 883374	Orthia *et al.*, 2005
P. stipularis Sm.	*De Kok 701*	AY 883378	Orthia *et al.*, 2005
***Rafnia* Thunb.**			
R. acuminata (E. Mey.) G.J. Campbell & B.-E. van Wyk	*Campbell & Van Wyk 17*, JRAU	AJ 744942	Motsi, 2004
R. amplexicaulis (L.) Thunb.	*Campbell & Van Wyk 26*, JRAU	AJ 744943	Motsi, 2004
R. crassifolia Harv.	*Campbell & Van Wyk 150*, JRAU	AJ 744939	Motsi, 2004
R. diffusa Thunb.	*Campbell & Van Wyk 44*, JRAU	AJ 744944	Motsi, 2004
R. elliptica Thunb.	*Van Wyk & Van Wyk 615*, JRAU	AJ 744940	Motsi, 2004
R. ovata E. Mey.	*Campbell & Van Wyk 128*, JRAU	AJ 744941	Motsi, 2004
R. perfoliata E. Mey.	*Crisp, Gilmore & Van Wyk 140947*	AF 287679	Crisp *et al.*, 2000
R. schlechteriana Schinz	*Campbell & Van Wyk 33*, JRAU	AJ 744950	Motsi, 2004
R. spicata Thunb.	*Campbell & Van Wyk 141*, JRAU	AJ 744945	Motsi, 2004
***Retama* Raf.**			

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Table 2.7 Continued.

Species	Voucher & Herbarium	GenBank accession number ITS	Reference
R. monosperma (L.) Boiss.	*MAF 162126*	AY 263681	Pardo *et al*., 2004
R. sphaerocarpa (L.) Boiss.	*MAF 160442*	AY 263682	Pardo *et al.*, 2004
Sophora **(L.)**			
S. microphylla (Meyen.)	*RBG, Kew 1969-16092*, K	AJ 409924	Van der Bank *et al*.,
S. prostrata J. Buch.	*RBG, Kew 1988-2824*, K	AJ 409922	Van der Bank *et al*.,
Spartium **L.**			
S. junceum L.	*MAF 159908*	AF 351088	Cubas *et al.*, 2002
Sphaerolobium **Sm.**			
S. minus Labill.	*MDC 9154*	AF 518101	Crisp & Cook, 2003
S. nudiflorum (Meisn.) Benth.	*RB 891*	AF 518102	Crisp & Cook, 2003
Stauracanthus **Link**			
Stauracanthus genistoides (Brot.) Samp. subsp. *genistoides*	*MAF 7908*	AF 384340 & AF 384341	Ainouche *et al*., 2003
Templetonia **R.Br.**			
T. retusa R.Br.	*Crisp 8996*, CANB	AF 287636	Crisp *et al*., 2000
Ulex **L.**			
U. densus Webb	*HbUR/UD 7*	AF 384356 & AF 384357	Ainouche *et al*., 2003
U. parviflorus Pourr.	*LB-UR-Fr/G53*	AF 007470	Ainouche & Bayer, 1999

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CHAPTER 3

MOLECULAR PHYLOGENETICS OF THE TRIBE PODALYRIEAE

3.1 Introduction

3.1.1 General

The tribe Podalyrieae is a group of Papilionoid legumes that, with the exception of *Calpurnia*, are endemic to the CFR of South Africa. It forms part of the Cape floral clades together with amongst others Crotalarieae *pro parte* (*Aspalathus* and *Rafnia*) and Psoraleeae *pro parte* (*Psoralea* L. and *Otholobium* C.H. Stirton). These clades, according to Linder (2003), can be defined as those clades that have had most of their evolutionary history in the CFR and have been there since the Pliocene. Podalyrieae currently contains eight genera: *Amphithalea*, *Calpurnia*, *Cyclopia*, *Liparia*, *Podalyria*, *Stirtonanthus*, *Virgilia* and *Xiphotheca*. All the species are long-lived perennials with notable variation in growth form. They range from tall, upright trees to erect woody shrubs and subshrubs or sprawling shrublets. A variety of leaf types can be found in the tribe varying from imparipinnately compound leaves in *Calpurnia* and *Virgilia*, to trifoliate leaves in *Cyclopia* and simple leaves in *Amphithalea*, *Liparia*, *Podalyria*, *Stirtonanthus* and *Xiphotheca*. The structure of the inflorescence is a useful taxonomic character at both inter- and infrageneric level. They are either subterminal, axillary racemes or panicles in *Calpurnia* and *Virgilia,* or axillary, subterminal inflorescences in the rest of the tribe. The flowers are normally firmly textured and adapted for pollination by xylocopid bees (Schutte and Van Wyk, 1998a). Whitehead *et al.* (1987) state that flowers pollinated by bees tend to be blue or yellow with nectar guides and a sweet odour. According to Van Wyk (1993), no correlation exists between sugar ratios of nectar and the types of pollinators that are attracted by members of the tribe. Nectar in Podalyrieae seems to be sucrose rich and unspecialised for pollination. The high sucrose levels support a notion of a 'long-tongued bee syndrome', i.e. most species are pollinated by xylocopid bees, but the correlation and implied co-evolution is not completely convincing (Van Wyk, 1993).

The two fire-survival strategies, i.e. reseeders and resprouters, can be observed in Podalyrieae. Resprouting and reseeding taxa differ in their habitat specificity, population densities, relative regional abundance and seed germination tempo, as there is a tendency of reseeding species to germinate more rapidly than resprouting species (Schutte *et al.*, 1995). In legumes, resprouting versus reseeding is often an important distinguishing character in morphologically similar taxa, but this character is often difficult to include in taxonomic studies, seeing that fire survival strategy is not visible on herbarium specimens.

The genistoid alliance was described by Polhill (1976; 1981) as a group of predominantly southern Hemisphere tribes suspected of being closely related. These include the northern Hemisphere Genisteae, Euchresteae; Thermopsideae and some Sophoreae; the South African Crotalarieae, Podalyrieae and Hypocalypteae; the Australian Bossiaeeae and Mirbelieae and the neotropical Brongniartieae. Molecular studies show

Podalyrieae to be sister to a clade consisting of Crotalarieae and Genisteae (Figure 3.1). These together with Euchresteae, Sophoreae (*Maackia* and some species of *Sophora*) and Thermopsidae form the 'core' genistoids (Käss and Wink, 1995; 1996; 1997; Doyle *et al.*, 2000; Crisp *et al.*, 2000; Wojciechowski, 2003, Wojciechowski *et al.*, 2004). These results are in agreement with earlier work done by Van Wyk and Schutte (1995a) that incorporated morphological and chemical evidence. The species in these tribes are mainly centred in Africa and Eurasia and include many genera from temperate and subtropical regions, e.g. *Aspalathus*, *Genista*, *Podalyria* and *Thermopsis* (Wojciechowski, 2003).

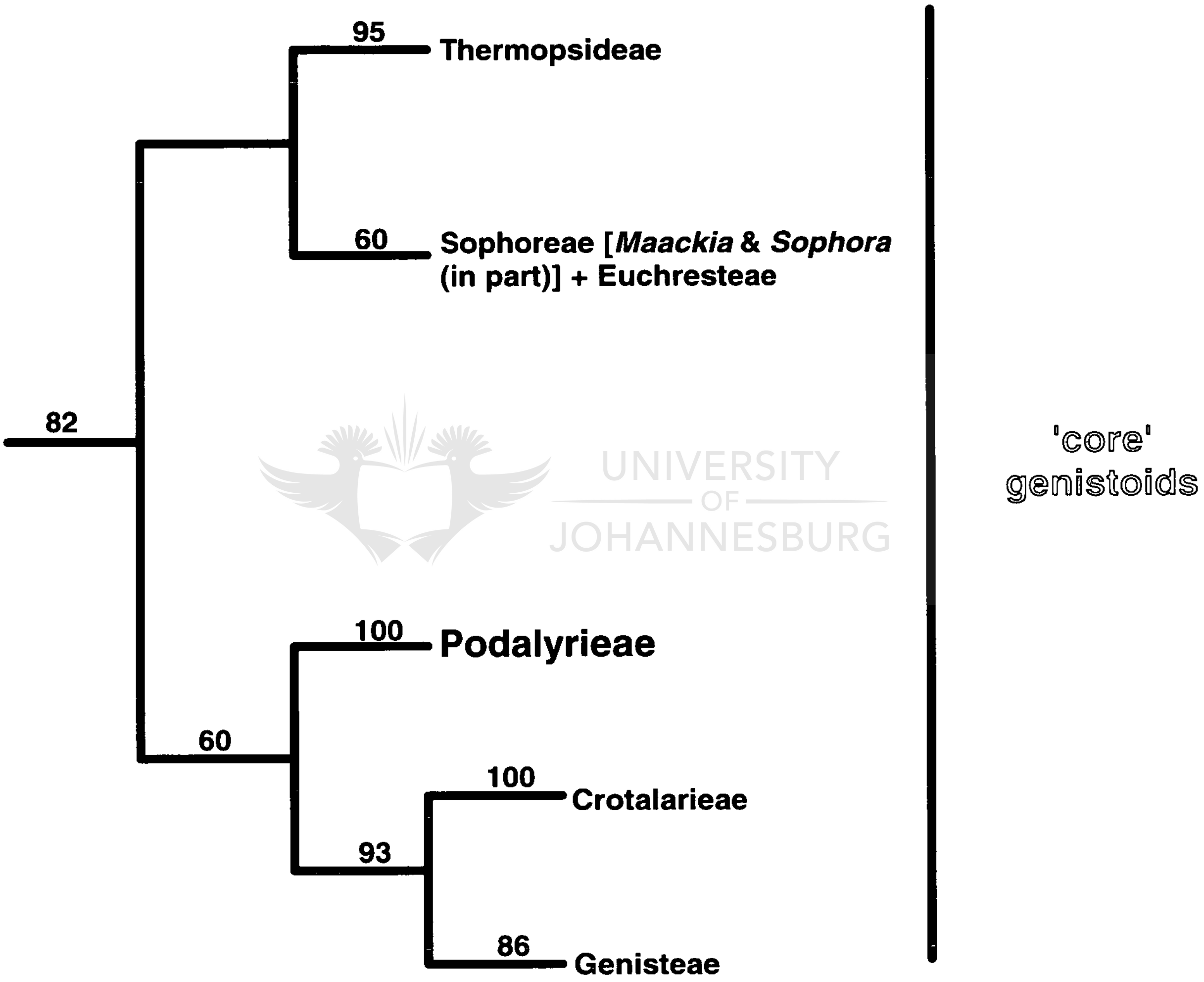

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Figure 3.1 Relationships within the 'core' genistoids (from Crisp *et al.*, 2000).

Schutte and Van Wyk (1998a) in their study of the tribes Liparieae and Podalyrieae proposed many important changes at both the tribal and generic level. They suggested an amalgamation of the two tribes and consequently placed Liparieae into synonymy under Podalyrieae. *Hypocalyptus* was excluded from the tribe and now constitutes the monotypic tribe Hypocalypteae and is probably more closely related to the Australian Mirbelieae and

Bossiaeeae (Schutte and Van Wyk, 1998b). The paraphyletic genus *Priestleya* DC. was dissolved and its members split between *Liparia* and *Xiphotheca* (Schutte and Van Wyk, 1993; 1994). *Coelidium* Vogel ex Walp. was moved into synonymy with *Amphithalea* (Schutte, 1995a), *Stirtonanthus* described as a new genus (Van Wyk and Schutte 1994; 1995b) and *Calpurnia* transferred to Podalyrieae (Van Wyk and Schutte, 1995a). The tribe was divided into two subtribes (Figure 3.2): Podalyriinae (consisting of *Calpurnia*, *Cyclopia*, *Liparia*, *Podalyria*, *Stirtonanthus* and *Virgilia*) and Xiphothecinae (consisting of *Amphithalea* and *Xiphotheca*). Members of the Xiphothecinae typically have a non-intrusive calyx base, obtuse keel petal, reduced number of ovules and a thickened lobe on the abaxial surface of the wing petals. Podalyriinae have an intrusive calyx base and rostrate keel petal (Schutte and Van Wyk, 1998a).

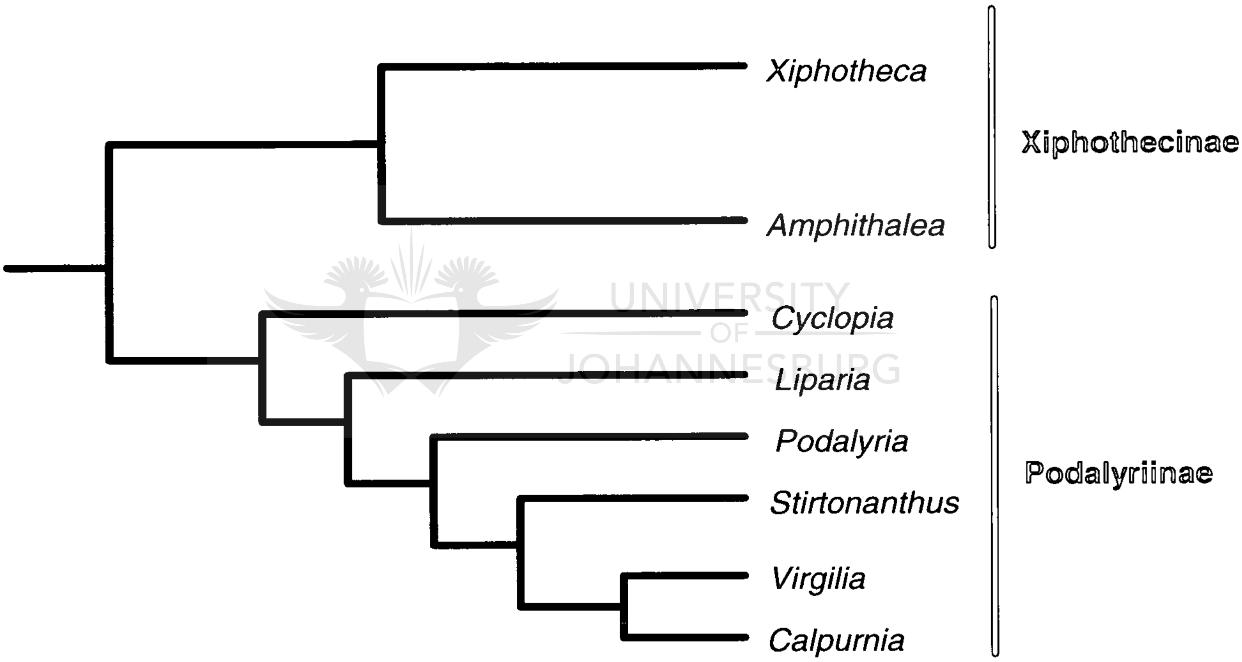

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Figure 3.2 Cladogram of relationships within Podalyrieae (from Schutte and Van Wyk, 1998a).

Van der Bank *et al.* (2002) in a study of Podalyrieae, combining ITS sequence data with morphological and chemical data, confirmed the monophyly of Liparieae and Podalyrieae, but found the subtribe Podalyriinae to be non-monophyletic, with *Cyclopia* forming a grouping sister to the rest of the tribe. They suggested that a broader concept of Podalyrieae be accepted to include *Cyclopia*, rather than erecting another subtribe to accommodate the genus (Figure 3.3).

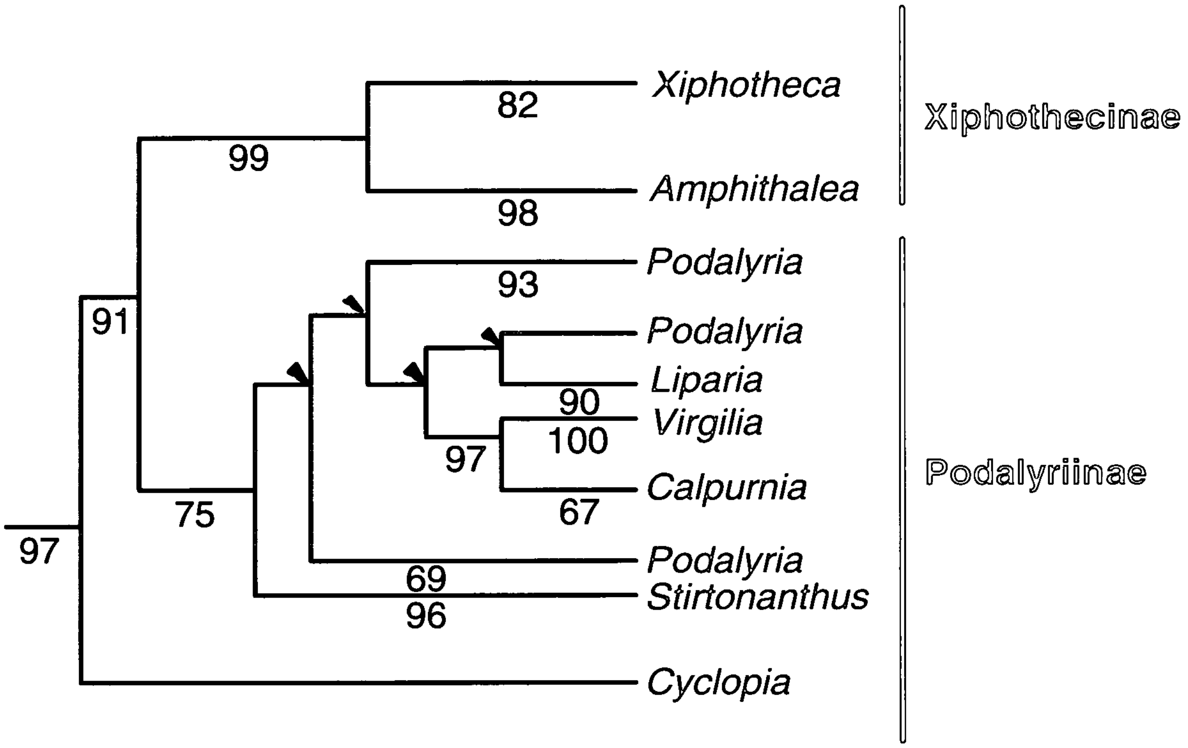

Figure 3.3 One of the 68 most parsimonious trees from the combined analysis of the ITS region, morphological and chemical data (from Van der Bank *et al.*, 2002).

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3.1.2 Genera in Podalyrieae

3.1.2.1 *Amphithalea*

The genus consists of 42 species endemic to the Cape Province of South Africa. Schutte (1995a) found the genera *Amphithalea* (then consisting of 21 species) and *Coelidium* (which also consisted of 21 species) to be congeneric and reduced *Coelidium* into synonymy under *Amphithalea.*

The members of the genus are typically shrubs or shrublets with mostly simple, opposite leaves varying from linear to lanceolate or ovate, with flat or strongly recurved or incurved margins. Petioles are generally absent and the stipules are greatly reduced. The flowers are purple, mauve or pink and found in axillary one or two flowered inflorescences. They occur from the Kamiesberg near Garies in the north-western Cape through the Cape Peninsula, extending as far as Grahamstown in the east as indicated in Figure 3.4 (Schutte, 1995a). Red data list information and fire survival strategies are provided for *Amphithalea* in Table 3.1 and for all the genera to follow in Tables 3.2--3.8. This information was obtained from Schutte (1995a), Schutte *et al.* (1995), Hilton-Taylor (1996) and Schutte (pers. comm.).

3.1.2.2 *Calpurnia*

Calpurnia consists of seven species (one of which is presumably extinct) and a putative hybrid between *C. sericea* and *C. woodii*. The species are narrow endemics of South Africa with one species extending into Ethiopia, *C. aurea* subsp. *aurea*, and southern India, *C. aurea* subsp. *indica* (Figure 3.5). All the members of the genus are slender trees or shrubs with imparipinnately compound leaves that are pulvinate and petiolate. Their stipules are small and appear triangular to subulate. The inflorescences are racemose to paniculate; either terminal or axillary with bright golden to yellow flowers. The fruits are linear, compressed, one to six-seeded and dehiscent (Beaumont *et al.*, 1999).

The genus was originally placed in the tribe Sophoreae, but based on the intrusive calyx base in some species, hairs on the stamens, the accumulation of carboxylic acid esters of quinolizidine alkaloids and chromosome base number of x = 9 (all characters shared with genera of Podalyrieae), it was transferred to the tribe Podalyrieae (Van Wyk and Schutte, 1995a). This transfer was later confirmed by Van der Bank *et al.* (2002) where *Calpurnia* grouped with *Virgilia* with high support (97BP) as is illustrated in Figure 3.3.

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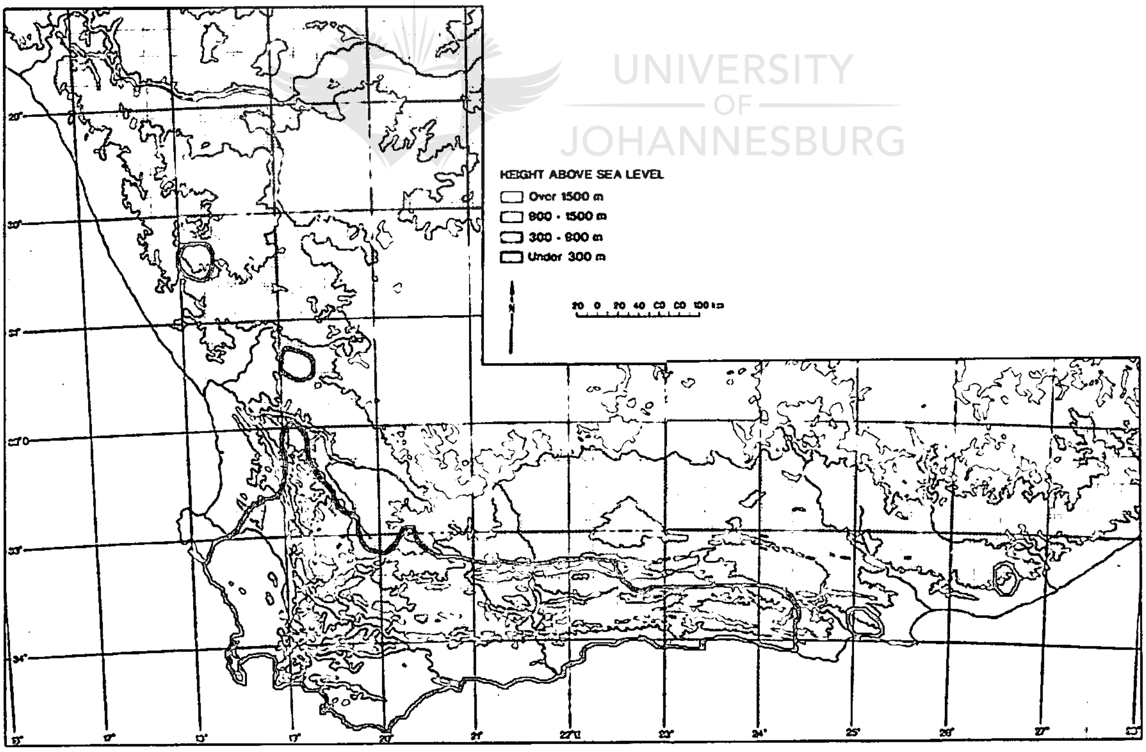

Figure 3.4 Known geographical distribution of *Amphithalea* (from Schutte, 1995a).

Table 3.1 Fire-survival strategy and Red Data List information of *Amphithalea* (Red indicates species not included in this study).

Species	**Reseeder**	**Resprouter**	**Unknown**	**Status**
A. alba	X			Vulnerable
A. axillaris	X			Rare
A. biovulata		X		Vulnerable
A. bodkinii Dummer			X	Localised
A. bowiei (Benth.) A.L. Schutte		X		Rare & localised
A. bullata (Benth.) A.L. Schutte		X		Rare
A. cedarbergensis (Granby) A.L. Schutte		X		Restricted to Cedarberg Mts
A. ciliaris		X		Limited distribution
A. concava Granby			X	Rare & localised
A. cuneifolia	X			Limited distribution
A. cymbifolia (C.A. Sm.) A.L. Schutte			X	Rare
A. dahlgrenii		X		Rare
A. ericifolia		X		Rare
A. esterhuyseniae (Granby) A.L. Schutte			X	Rare
A. flava		X		Rare
A. fourcadei	X			Rare
A. imbricata	X			Rare
A. intermedia		X		Limited distribution
A. micrantha		X		Limited distribution
A. minima (Granby) A.L. Schutte			X	Rare
A. monticola		X		Limited distribution
A. muirii		X		Limited distribution
A. muraltioides	X			Limited distribution
A. obtusiloba	X			Rare
A. oppositifolia	X			Endangered
A. pageae	X			Rare
A. parvifolia	X			Limited distribution
A. perplexa Eckl & Zeyh.			X	Widespread
A. phylicoides		X		Limited distribution
A. purpurea (Granby) A.L. Schutte			X	Rare
A. rostrata		X		Endangered
A. sericea Schltr.			X	Vulnerable
A. speciosa		X		Endangered
A. spinosa	X			Limited distribution
A. stokoei	X			Endangered
A. tomentosa		X		Vulnerable
A. tortilis		X		Limited distribution
A. villosa		X		Limited distribution
A. violacea		X		Limited distribution
A. virgata		X		Endangered
A. vlokii		X		Rare
A. williamsonii		X		Limited distribution

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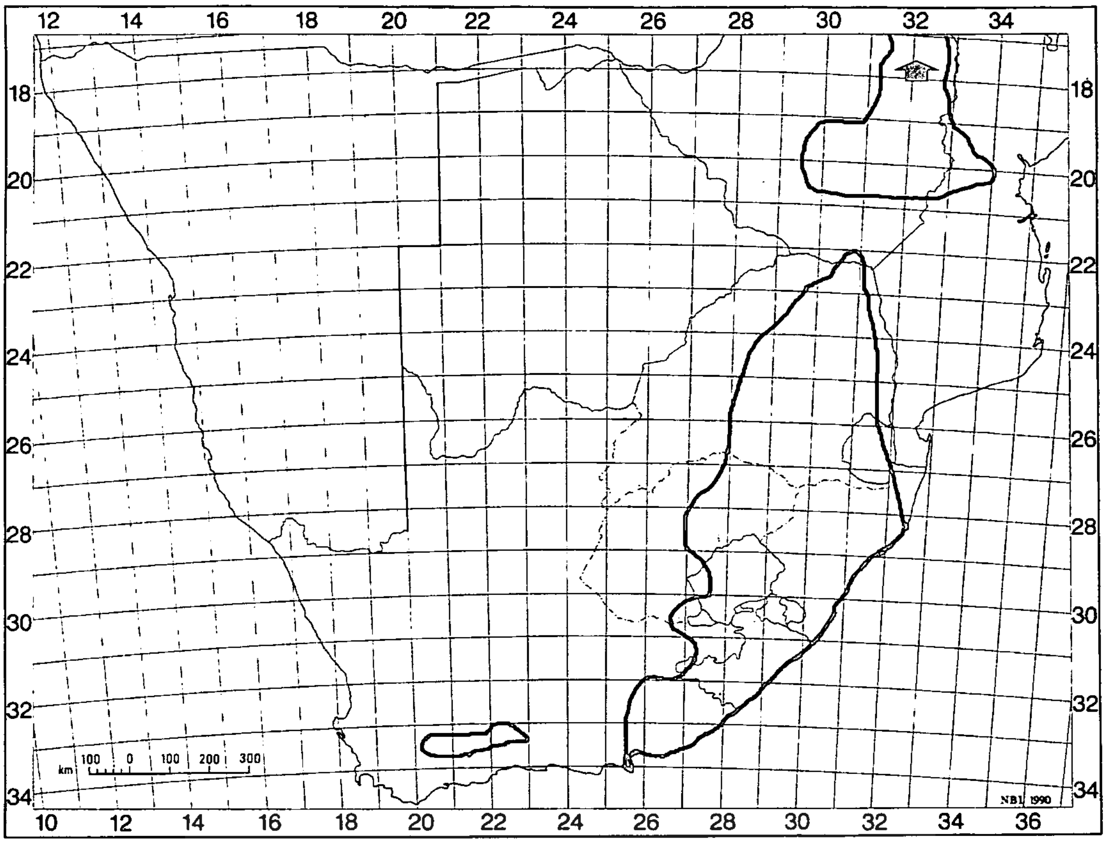

Figure 3.5 Known geographical distribution of *Calpurnia* (from Schutte, 1995a).

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Table 3.2 Fire-survival strategy and Red Data List information of *Calpurnia* (Red indicates species not included in this study).

Species	Reseeder	Resprouter	Unknown	Status
C. aurea	X			Not threatened
C. floribunda Harv.			X	Not threatened
C. glabrata			X	Not threatened
C. intrusa	X			Not threatened
C. reflexus A.J. Beaumont			X	Extinct
C. sericea	X			Not threatened
C.sericea x woodii			X	Not threatened
C. woodii		X		Rare

3.1.2.3 *Cyclopia*

Cyclopia consists of 23 species that are endemic to the CFR (Figure 3.6). The habits of the species are diverse and vary from tall, erect tree-like shrubs to woody, virgate subshrubs or small sprawling shrublets. The leaves are digitately trifoliate and petiolate with stipules present, but fused with the petiole. The leaflets show pinnate venation with prominent, decurrent leaf bases as found in *Liparia*. The inflorescences are single flowered with the flowers situated in the axils of the upper leaves. Their flowers are yellow with a rigid texture and sweet scent. Distinct grooves are found on the standard petal that act as nectar guides for xylocopid bees. The pods are coriaceous with beaked distal ends and are laterally compressed in most species, whilst inflated in others (Schutte, 1997b). No alkaloids are found in members of *Cyclopia*, making the genus distinct in Podalyrieae (Van Wyk and Schutte, 1995a).

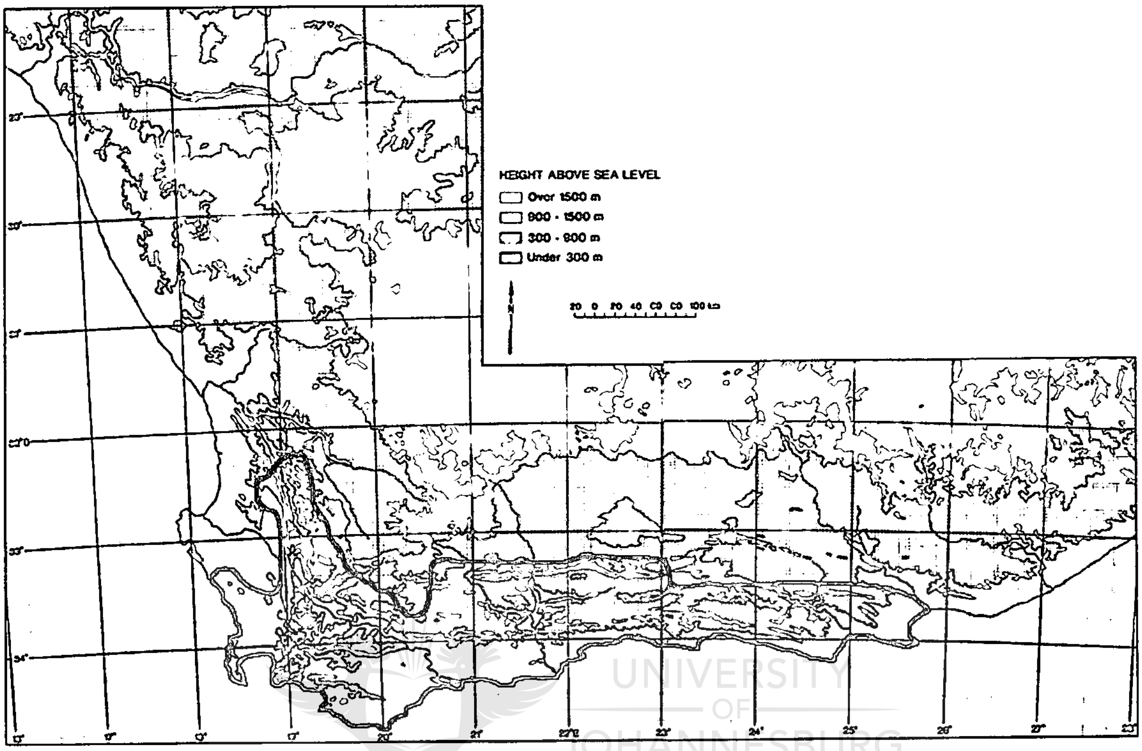

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Figure 3.6 Known geographical distribution of *Cyclopia* (from Schutte, 1995a).

Table 3.3 Fire-survival strategy and Red Data List information of *Cyclopia* (*Reseeders capable of resprouting and red indicates species not included in this study).

Species	**Reseeder**	**Resprouter**	**Unknown**	**Status**
C. alopecuroides	X			Limited distribution
C. alpina		X		Rare
C. aurescens		X		Rare
C. bolusii		X		Rare & localised
C. bowieana Harv.	X			Not threatened (limited distribution)
C. burtonii	X	*		Rare
C. buxifolia (Burm. f.) Kies		X		Widespread
C. falcata		X		Widespread
C. filiformis Kies			X	Extinct
C. galioides		X		Limited distribution
C. genistoides		X		Widespread
C. glabra		X		Rare
C. intermedia		X		Widespread
C. latifolia DC.			X	Endangered
C. laxiflora Benth.			X	Extinct

Species	Reseeder	Resprouter	Unknown	Status
C. longifolia	X			Endangered
C. maculata	X			Sporadic distribution
C. meyeriana	X			Widespread
C. plicata	X			Rare
C. pubescens	X			Endangered
C. sessiliflora		X		Limited distribution
C. squamosa A.L. Schutte			X	Rare
C. subternata	X			Widespread

3.1.2.4 *Liparia*

After a reevaluation of the generic delimitations of *Liparia* and *Priestleya*, Schutte and Van Wyk (1994) found the 12 species remaining in *Priestleya* to be congeneric with *Liparia.* These were consequently placed into synonymy under *Liparia* and after the description of five new species (Schutte, 1995b) the genus is composed of 20 species, all of which are endemic to the CFR (Figure 3.7).

All the species in *Liparia* are long-lived perennials, varying from erect woody shrubs to virgate, multi-stemmed shrubs or small rounded subshrubs and sprawling shrublets. The leaves are alternate, simple and sessile with distinctly pulvinate and decurrent leaf bases. The venation pattern is very distinctive with three or more veins arising from the leaf base, whereas other genera in the tribe show pinnate venation. Stipules are present, although sometimes reduced. The inflorescences are axillary, simple racemes with an apical extension of the inflorescence axis. The flowers are mostly bright yellow with one species having bright orange-red flowers (*L. splendens*) and two others lemon yellow flowers (*L. boucheri* and *L. parva*). The changes in inflorescence and floral structure found in *Liparia* can be ascribed to adaptation to various pollinators, e.g. *L. splendens* for sunbird pollination (Schutte, 1997c).

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3.1.2.5 *Podalyria*

Some uncertainty as to the correct number of species and nomenclature of the genus still exists. Schutte (1995a) recorded 19 species and four that are insufficiently known. The species are distributed from north of Nieuwoudtville and extend southwards to the Cape Peninsula and eastwards up to the Transkei and southern Kwazulu-Natal (Figure 3.8). The plants are usually woody shrubs or subshrubs with alternate leaves that are simple and petiolate, ranging from linear to cordate with stipules usually present. The inflorescences are one to several flowered racemes with purple, pink or white firmly textured flowers. The pods are coriaceous and inflated with three to several seeds in each pod (Schutte, 1995a).

Podalyria accumulates large amounts of quinolizidine alkaloids and alkaloid esters are derived from angelic- or tiglic acid, rather than carboxylic acid (Van Wyk *et al.*, 1992).

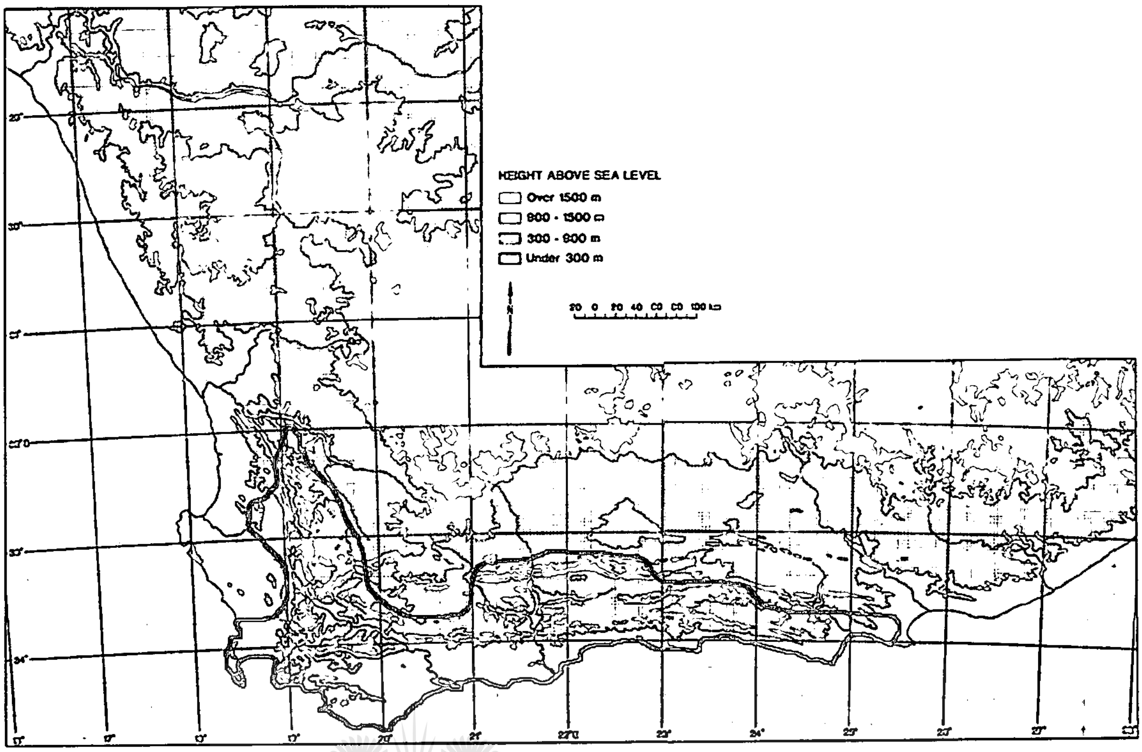

Figure 3.7 Known geographical distribution of *Liparia* (from Schutte, 1995a).

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Table 3.4 Fire-survival strategy and Red Data List information of *Liparia* (*Reseeders capable of resprouting and red indicates species not included in this study).

Species	**Reseeder**	**Resprouter**	**Unknown**	**Status**
L. angustifolia	X			Endangered
L. bonaespei	X			Rare
L. boucheri	X			Rare
L. calycina	X			Rare
L. capitata		X		Limited distribution
L. confusa		X		Limited distribution
L. congesta	X			Rare
L. genistoides	X			Rare
L. graminifolia L.			X	Extinct
L. hirsuta	X	*		Widespread
L. laevigata (L.) Thunb.	X			Rare
L. latifolia		X		Limited distribution
L. myrtifolia	X			Not threatened
L. parva		X		Rare
L. racemosa	X			Rare
L. rafnioides	X			Rare
L. splendens		X		Rare
L. striata		X		Endangered
L. umbellifera	X			Widespread (Montane)
L. vestita		X		Limited distribution

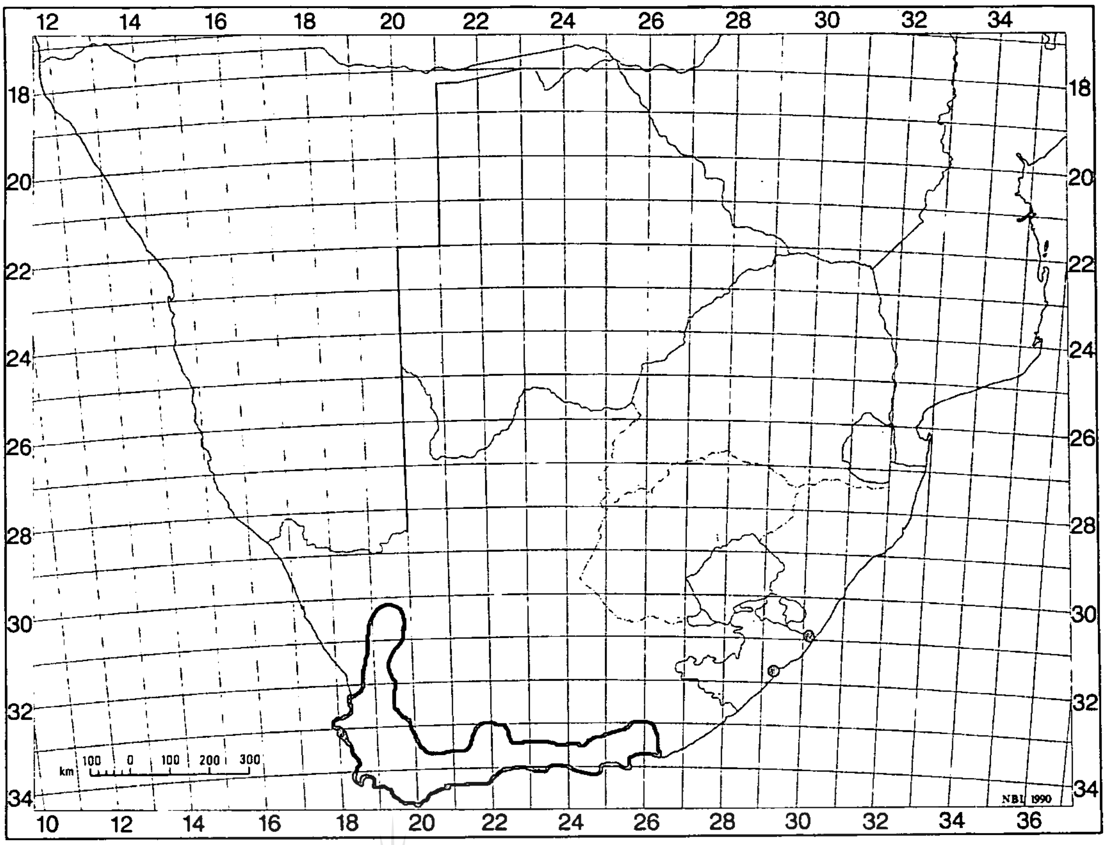

Figure 3.8 Known geographical distribution of *Podalyria* (from Schutte, 1995a).

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Table 3.5 Fire-survival strategy and Red Data List information of *Podalyria* (*Reseeders capable of resprouting and red indicates species not included in this study).

Species	Reseeder	Resprouter	Unknown	Status
P. argentea		X		Rare
P. biflora		X		Widespread
P. burchellii		X		Not threatened
P. buxifolia		X		Widespread (Montane)
P. calyptrata	X	*		Widespread
P. cordata		X		Rare
P. cuneifolia	X	*		Widespread
P. hirsuta		X		Unknown
P. intermedia	X			Rare
P. lanceolata	X			Rare
P. leipoldtii		X		Localised
P. microphylla	X			Highly localised (Extinct?)
P. myrtillifolia		X		Widespread
P. oleaefolia		X		Limited distribution
P. orbicularis		X		Rare
P. pearsonii		X		Rare
P. reticulata Harv.		X		Rare
P. rotundifolia		X		Widespread (Montane)

Species	Reseeder	Resprouter	Unknown	Status
P. sericea	X			Vulnerable
P. variabilis		X		Unknown
P. velutina			X	Indeterminate

3.1.2.6 *Stirtonanthus*

Stirtonia was described by Van Wyk and Schutte (1994) to accommodate three yellow-flowered species of *Podalyria*. It differed mainly in the decussate inflorescences, yellow flower colour, non-fleshy rim-aril of the seeds and difference in the combination of quinolizidine alkaloids found in these plants. However, the name *Stirtonia* was found to be illegitimate and replaced with *Stirtonanthus* (Van Wyk and Schutte, 1995b). *Stirtonanthus* consists of three species, known from only a few isolated localities in the south-western and southern Cape (Figure 3.9). They vary from single to multi-stemmed shrubs or small trees with simple, alternate leaves that are obovate to orbicular in shape. Paired stipules are present and the inflorescences are axillary peduncles with two, four or six decussate flowers (Van Wyk and Schutte, 1994).

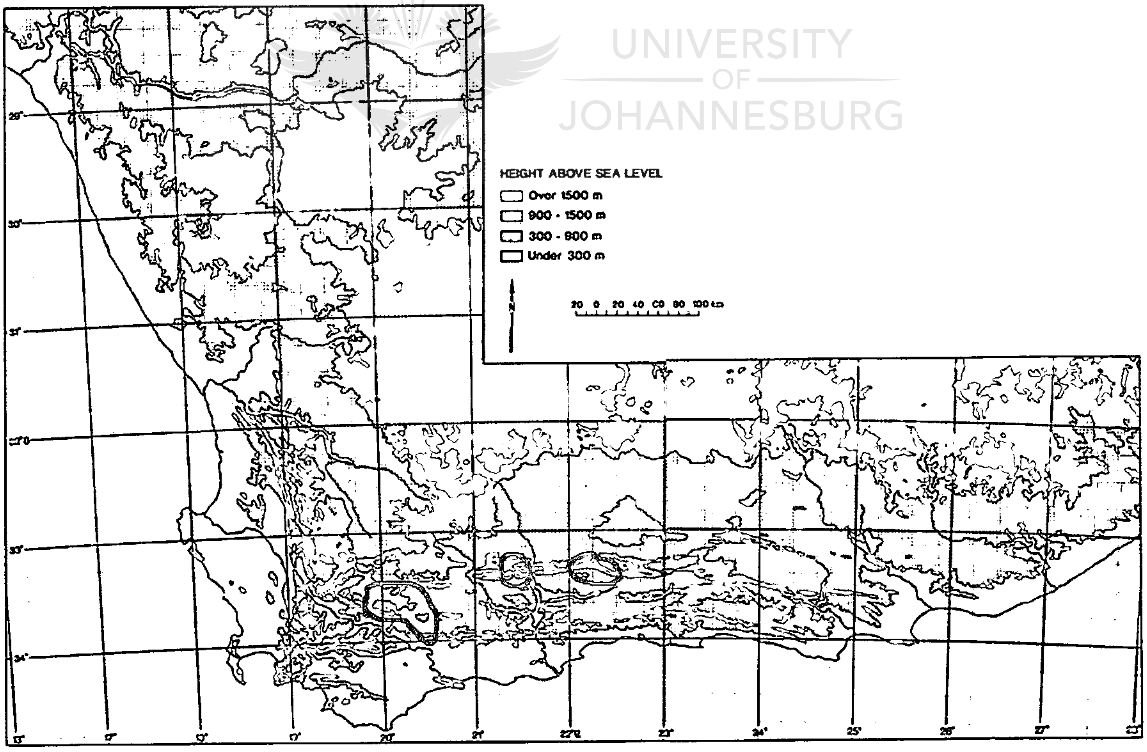

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Figure 3.9 Known geographical distribution of *Stirtonanthus* (from Schutte, 1995a).

Table 3.6 Fire-survival strategy and Red Data List information of *Stirtonanthus*.

Species	Reseeder	Resprouter	Unknown	Status
S. chrysanthus	X			Rare
S. insignis		X		Vulnerable
S. taylorianus	X			Rare

3.1.2.7 *Virgilia*

Virgilia consists of two species, one of which has two subspecies: *V. oroboides* subsp. *oroboides* and *V. oroboides* subsp. *ferruginea*. It has a limited distribution with the species occurring on moist sites from the Cape Peninsula to Port Elizabeth (Figure 3.10). The species are typically small trees between four to 15 m tall with a single or branched main stem. Leaves are imparipinnately compound with subsessile, pulvinate pinnae that are linear to narrowly ovate in shape and opposite or alternate. Stipules are usually present and can be either caducous or persistent. The inflorescences are axillary and subterminal racemes (rarely panicles) with three to 16 flowers, which vary from rose-violet, violet-purple, pink or white with a sweet scent. Pods are linear, dehiscent, two-valved and slightly compressed between the seeds (Van Wyk, 1986).

Van Wyk (1986) noted that various characters in the genus are geographically correlated along an east-west gradient and that these are of great taxonomic value to distinguish between the species and subspecies. By means of starch-gel electrophoresis, Van der Bank *et al.* (1996) found the differences in allozyme variation between the taxa mostly quantitative and also indicated an east-west gradient of character variation. They suggested that the genus could be a product of recent speciation with introgressive hybridisation leading to the geographical and ecological patterns of character variation.

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3.1.2.8 *Xiphotheca*

During their study of the relationships of the genera of Podalyrieae and Liparieae, Schutte and Van Wyk (1993) found the genus *Priestleya* to be paraphyletic. The name *Xiphotheca* was thereafter reinstated for *Priestleya* sect. *Aneisothea* based on the inflorescence structure, non-intrusive calyx (except for *X. cordifolia*), obtuse and pocketed keel petals, leaf shape and size and evidence from alkaloids (anabasine as major alkaloid).

Xiphotheca is composed of nine species, all of which are endemic to the fynbos regions of South Africa (Figure 3.11). The species vary in habit from single-stemmed, tree-like shrubs to many stemmed, virgate shrubs or straggling shrublets. Leaves are simple and petiolate with pinnate venation and stipules are present throughout the genus, although reduced in size. The inflorescences consist of yellow flowers arranged in simple, axillary racemes, which are two-flowered and decussate. The name *Xiphotheca*, meaning sword-like container, accurately describes the pods which are laterally compressed, sessile and constricted between the seeds (Schutte, 1997a).

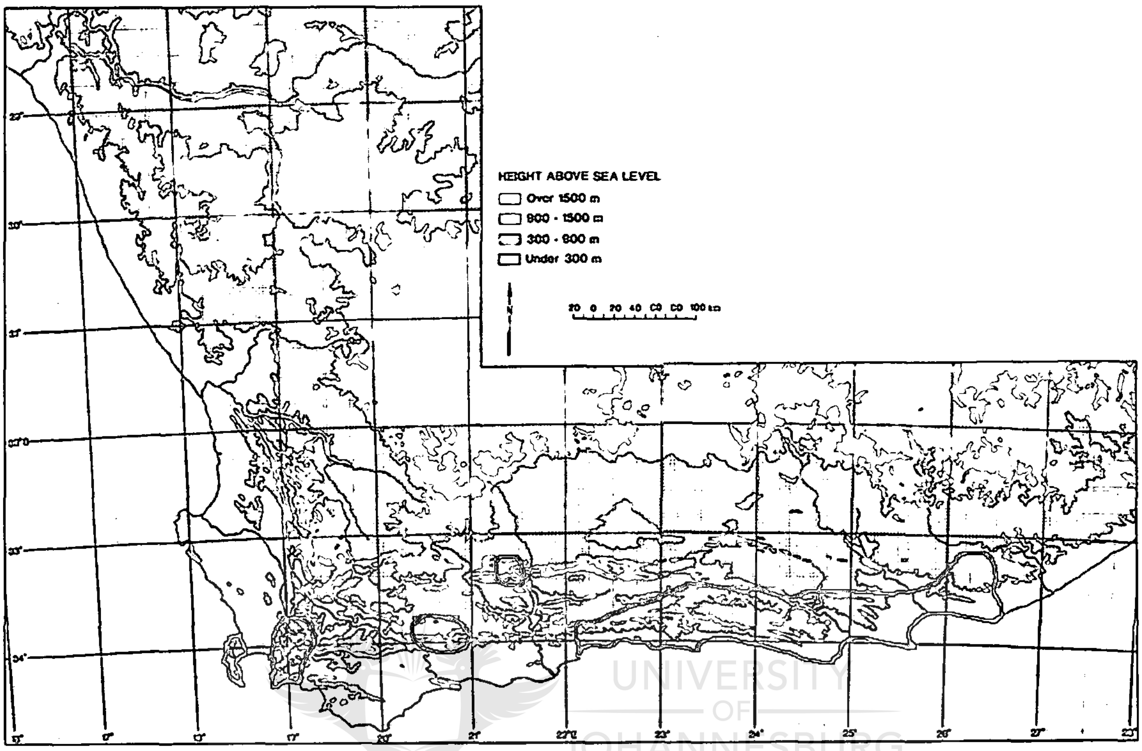

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Figure 3.10 Known geographical distribution of *Virgilia* (from Schutte, 1995a).

Table 3.7 Fire-survival strategy and Red Data List information of *Virgilia.*

Species	**Reseeder**	**Resprouter**	**Unknown**	**Status**
V. divaricata	X			Limited distribution
V. oroboides subsp. *ferruginea*	X			Limited distribution
V. oroboides subsp. *oroboides*	X			Limited distribution

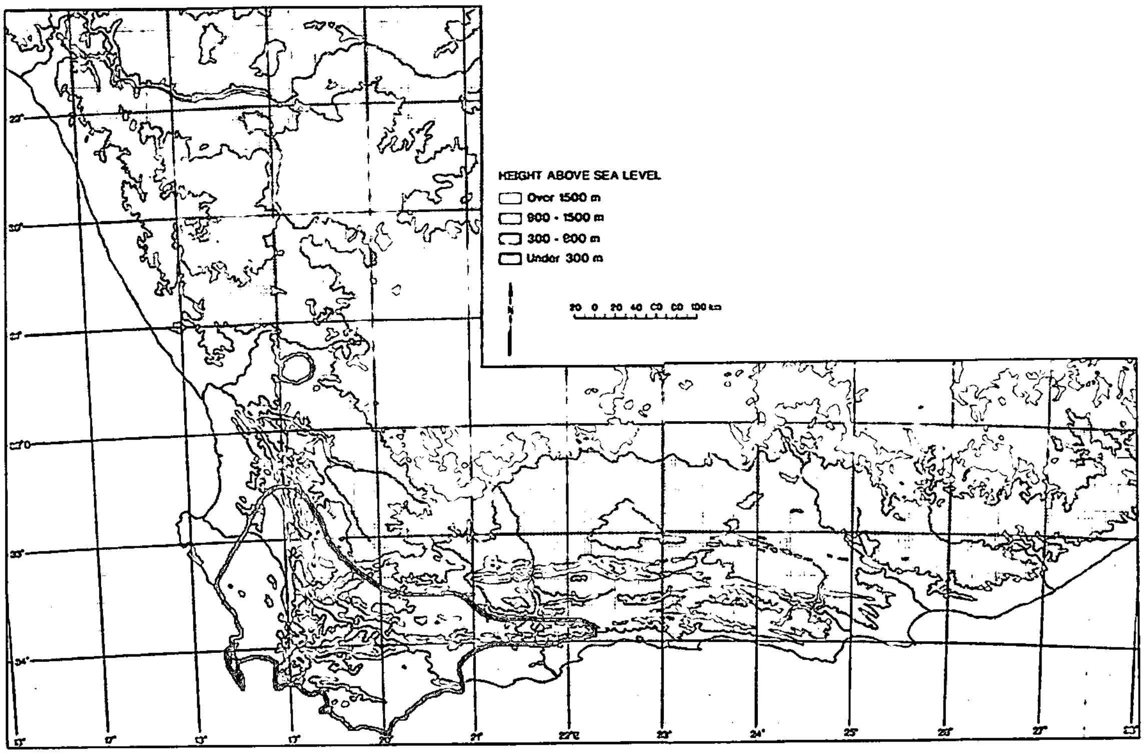

Figure 3.11 Known geographical distribution of *Xiphotheca* (from Schutte, 1995a).

Table 3.8 Fire-survival strategy and Red Data List information of *Xiphotheca*.

Species	**Reseeder**	**Resprouter**	**Unknown**	**Status**
X. canescens	X			Vulnerable
X. cordifolia	X			Limited distribution
X. elliptica		X		Not threatened
X. fruticosa	X			Not threatened
X. guthriei	X			Endangered
X. lanceolata	X			Endangered
X. phylicoides		X		Vulnerable
X. reflexa		X		Vulnerable
X. tecta		X		Not threatened

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3.1.3 Aims of the chapter

This chapter aims to present:

1. The results of the species-level analysis using *rbcL* and ITS (MP and ML analyses).
2. A comparison of the rates of molecular evolution between reseeders and resprouters.
3. A date for the root node of Podalyrieae (high-level analysis).

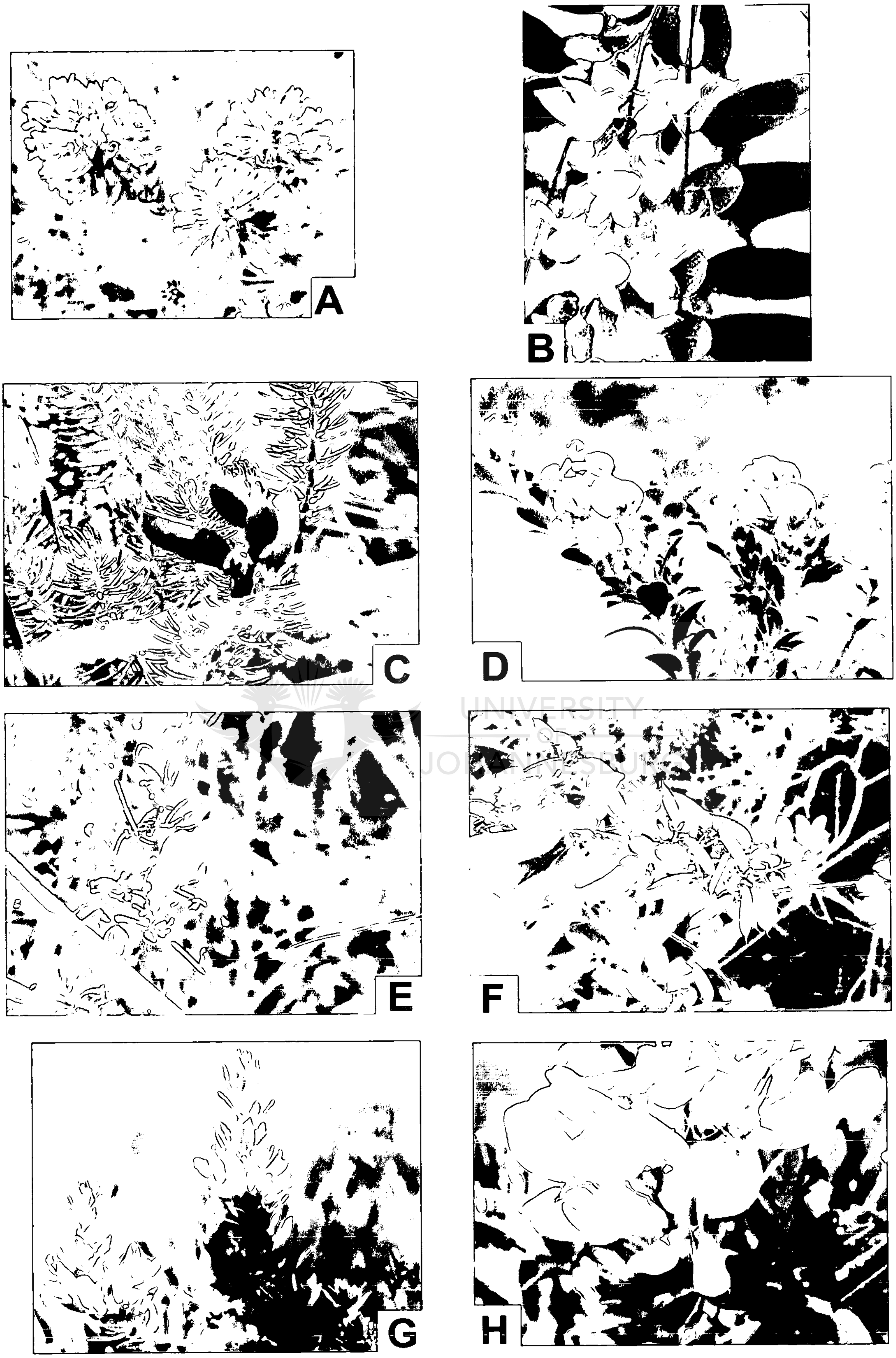

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Figure 3.12 Representatives of Podalyrieae. A: *Amphithalea virgata* (from www.fernkloof.com); B: *Calpurnia aurea* (from www.plantweb.co.za); C: *Cyclopia genistoides*; D: *Liparia hirsuta*; E: *Podalyria buxifolia*; F: *Stirtonanthus taylorianus*; G: *Xiphotheca guthriei* (Mark Johns); H: *Virgilia divaricata*.

3.2 Results

3.2.1 Species-level phylogeny

3.2.1.1 Statistics

A summary of the statistics for each analysis is provided in Table 3.9.

3.2.1.2 Separate molecular analysis

Plastid results

The *rbcL* matrix included 1415 sites of which 1183 were constant, 232 (16.4%) variable and 154 (10.9%) potentially informative. 173 equally most parsimonious trees of 447 steps with a CI of 0.62 and an RI of 0.83 were obtained (Table 3.9). The *rbcL* region had the highest transitions (ts): transversions (tv) ratio (Table 3.10).

Nuclear results

The analysis of the ITS region consisted of 734 characters of which 359 were constant, 375 (51.1%) variable and 234 (31.9%) potentially informative. 6650 equally most parsimonious trees of 891 steps, a CI of 0.61 and an RI of 0.83 were obtained (Table 3.9).

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3.2.1.3 Combined molecular analysis (Total evidence)

A comparison between the bootstrap consensus trees of the *rbcL* and ITS data is presented in Figure 3.13. These datasets were combined directly as no strongly supported incongruent patterns exist between them. Fitch analysis produced 140 equally most parsimonious trees (TL=1176; CI=0.61; RI=0.83). Successive weighting resulted in 950 trees (Figure 3.14; CI=0.61; RI=0.83). The matrix included 2148 characters of which 1618 were constant, 530 (24.7%) variable and 323 (15.0%) potentially parsimony informative (Table 3.9).

The overall resolution was low in the resulting trees, but several major clades could be identified within the tribe. The first major clade contains the genera *Amphithalea* and *Xiphotheca*. A relationship between these genera receives low support (53BP, 54SW). Sister to this clade is a grouping containing *Liparia*, *Podalyria* and *Stirtonanthus*. *Podalyria* is weakly supported to be monophyletic (63BP, 65SW) and groups with *Liparia* with low support (56BP, 59SW). *Liparia* is paraphyletic, with *L. calycina* not included in the *Liparia* clade. *Stirtonanthus* is sister to this grouping and weakly supported to be monophyletic (69BP, 71SW). The next

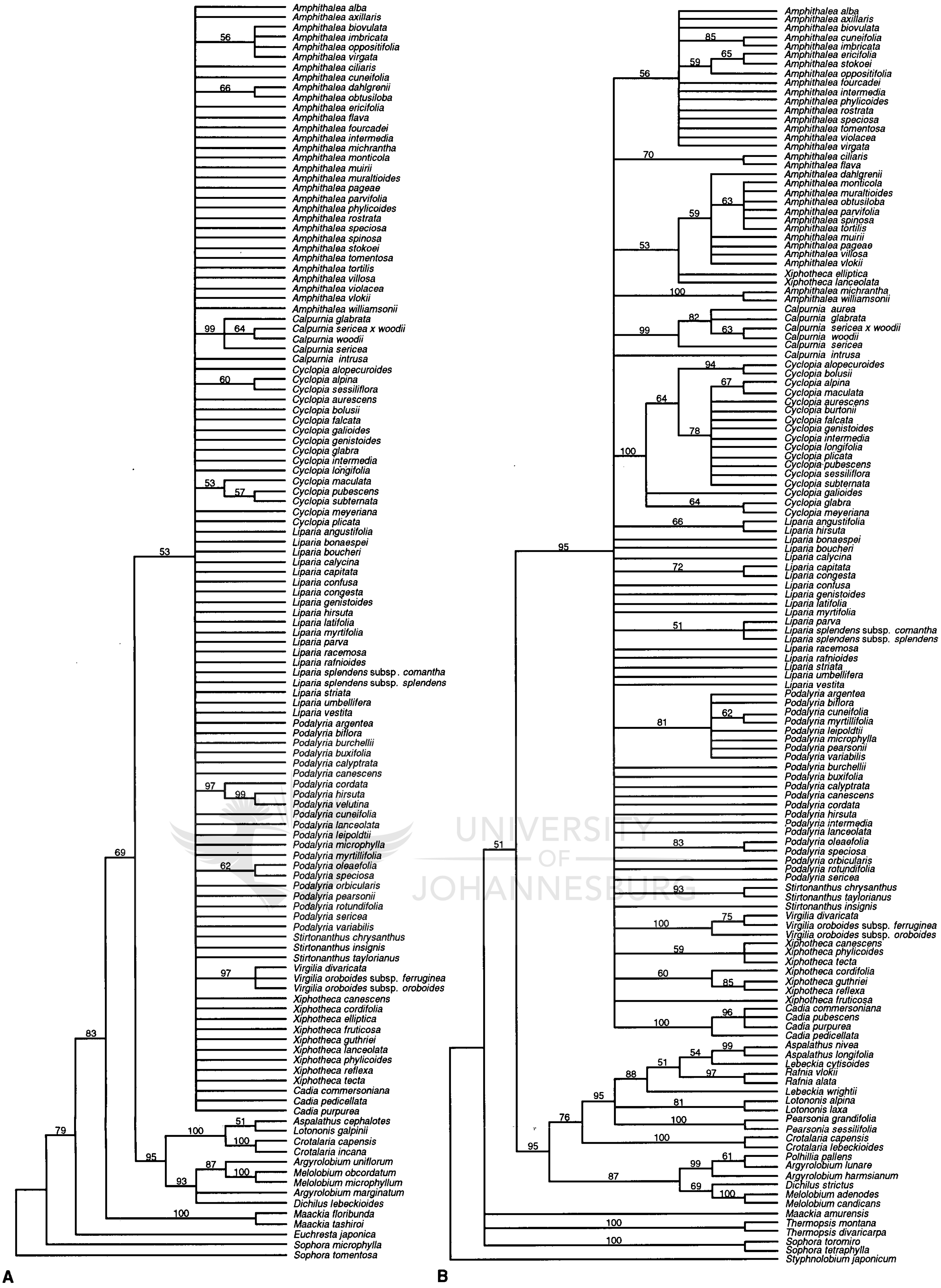

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Figure 3.13 Comparison between the bootstrap consensus trees of A: the *rbcL* analysis and B: the ITS analysis. Bootstrap percentages over 50% are shown above each branch.

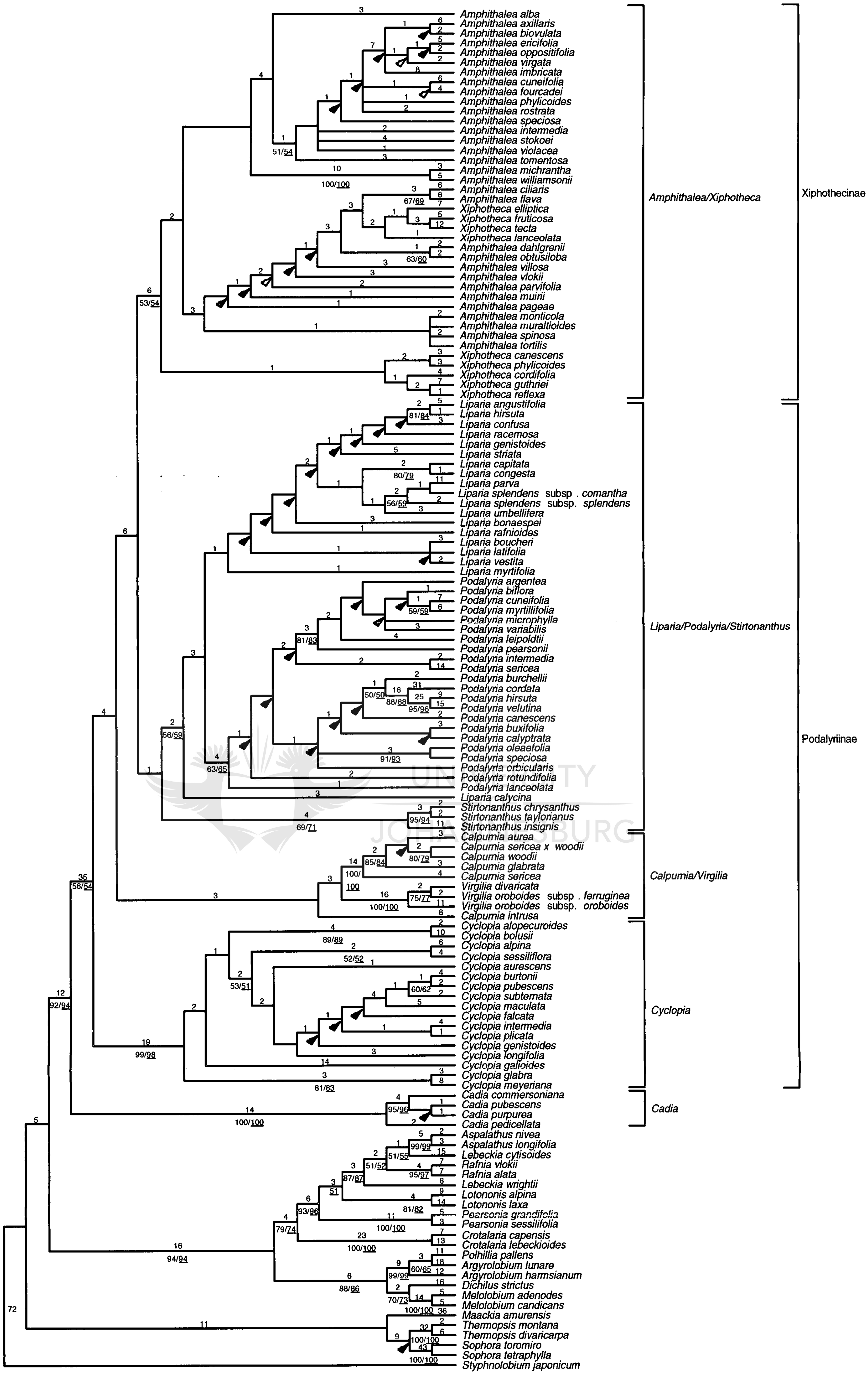

Figure 3.14 One of the equally parsimonious trees from the combined molecular analysis of *rbcL* and ITS. Numbers above the branches are Fitch lengths (DELTRAN optimisation) and those below are bootstrap percentages over 50% (SW bootstrap results underlined). Solid arrows indicate groups not present in the Fitch strict consensus tree and open arrows indicate groups not present in the both the SW and Fitch strict consensus trees (CI=0.61; RI=0.83; TL=1176).

clade contains the genera *Calpurnia* and *Virgilia*. *Calpurnia* is paraphyletic, while *Virgilia* is strongly supported to be monophyletic (100BP, 100SW). The members of *Cyclopia* form the following clade and the monophyly of the genus receives high support (99BP, 98SW). *Cadia* is well supported to be part of, or sister to Podalyrieae (92BP, 94SW). The monophyly of *Cadia* receives high support (100BP, 100SW), but resolution within the genus is low.

On the specific level, a few supported groupings are found. *Amphithalea michrantha* and *A. williamsonii* group together with strong support (100BP, 100SW) and the sister groupings between *A. ciliaris* and *A. flava*, and *A. dahlgrenii* and *A. obtusiloba* receive low support (67BP, 69SW; 63BP, 60SW). In *Liparia*, groupings with moderate support include: *L. angustifolia* with *L. hirsuta* (81BP, 84SW); *L. capitata* with *L. congesta* (80BP, 79SW); and with low support include: *L. parva* with *L. splendens* (56BP, 59SW). *Podalyria hirsuta* and *P. velutina* form a well supported grouping (95BP, 96SW), with *P cordata* and *P. burchellii* successively sister (88BP, 88SW; 50BP, 50SW). *P. oleaefolia* and *P. speciosa* group together with high support (91BP, 93SW). *Stirtonanthus chrysanthus* and *S. taylorianus* are strongly supported as a sister pair (95BP, 94SW). The resolution on the specific level was better within *Calpurnia* and *Virgilia*. The hybrid *C. sericea x woodii* groups with one of the parent species, *C. woodii* (80BP, 79SW). The *Calpurnia* clade receives high support, but *C. intrusa* is excluded from this grouping. In *Virgilia*, *V. divaricata* groups with *V. oroboides* subsp. *ferruginea* (75BP, 77SW). Groupings receiving moderate to high support in *Cyclopia* are *C. alopecuroides* with *C. bolusii* (89BP, 89SW), and *C. glabra* with *C. meyeriana* (81BP, 83SW).

3.2.1.4 Maximum likelihood (Bayesian) analysis

The topology of the majority rule consensus tree from the Bayesian analysis differed slightly from the Fitch tree and therefore is presented separately (Figure 3.16). *Amphithalea* and *Xiphotheca* are again strongly supported to be closely related (PP 0.95) but group with *Stirtonanthus*, although support for this is low (PP 0.49). *Liparia* and *Podalyria* form a strongly supported clade (PP 1.0). *Liparia* is again paraphyletic, with *L. calycina and L. umbellifera* not included in the *Liparia* clade. *Virgilia* and *Calpurnia* form separate well supported clades (PP 1.0 for *Virgilia*; PP 0.79 for *Calpurnia*). These groupings differ from the Fitch tree where they group together, although this grouping lacks bootstrap support. In this analysis *Calpurnia* is supported to be monophyletic, with *C. intrusa* included in the *Calpurnia* clade (PP 0.79). *Cyclopia* forms the next grouping and its monophyly receives high support (PP 1.0). *Cadia* is well supported to be monophyletic (PP 1.0) and part of or sister to Podalyrieae (PP 1.0).

On the specific level, the relationships were similar to those indicated in the Fitch tree, with either higher or lower support. Reseeders and resprouters are indicated on the majority rule consensus tree as this is the phylogeny that was used in the CAIC analysis.

3.2.2 Comparison between the rates of molecular evolution in reseeders and resprouters

The raw data from the CAIC output files are supplied in Appendix 1 (Table A1.1 and A1.2). The analysis resulted in a total of 33 contrasts for Podalyrieae and 15 for *Protea* (Table 3.11). In Podalyrieae, 21 of the contrasts are positive contrasts and 12 negative contrasts. The results for *Protea* gave 10 positive and 5 negative contrasts. A positive contrast indicates that the character state with the higher integer (i.e. reseeders scored as 1) has higher branch length values (Table A1.1 and A1.2, column 3). More positive contrasts are indicative of higher rates of molecular evolution in reseeders as more pairs of contrasts are present where the occurrence of longer branch lengths leads to a reseeder (Figure 3.15). A negative contrast in a continuous variable means that among the taxa being contrasted, higher branch length values are found in taxa having the categorical trait (survival strategy) in a lower state (i.e. resprouters scored as 0), thus indicating that the branch length leading to a reseeder is shorter than in a resprouter (Figure 3.15). Contrasts are phylogenetically independent as long as the lines linking compared species never meet or cross.

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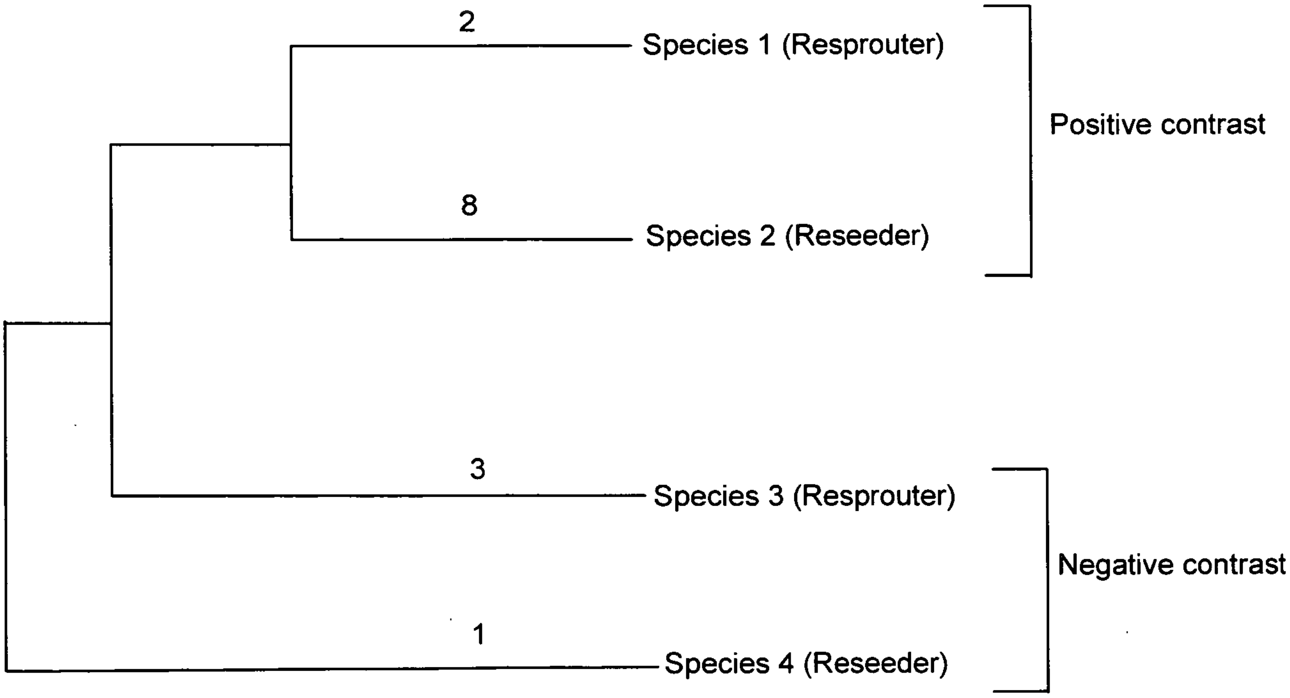

Figure 3.15 A schematic illustration of a positive and a negative contrast. Numbers above the branches are branch lengths. See text for explanation.

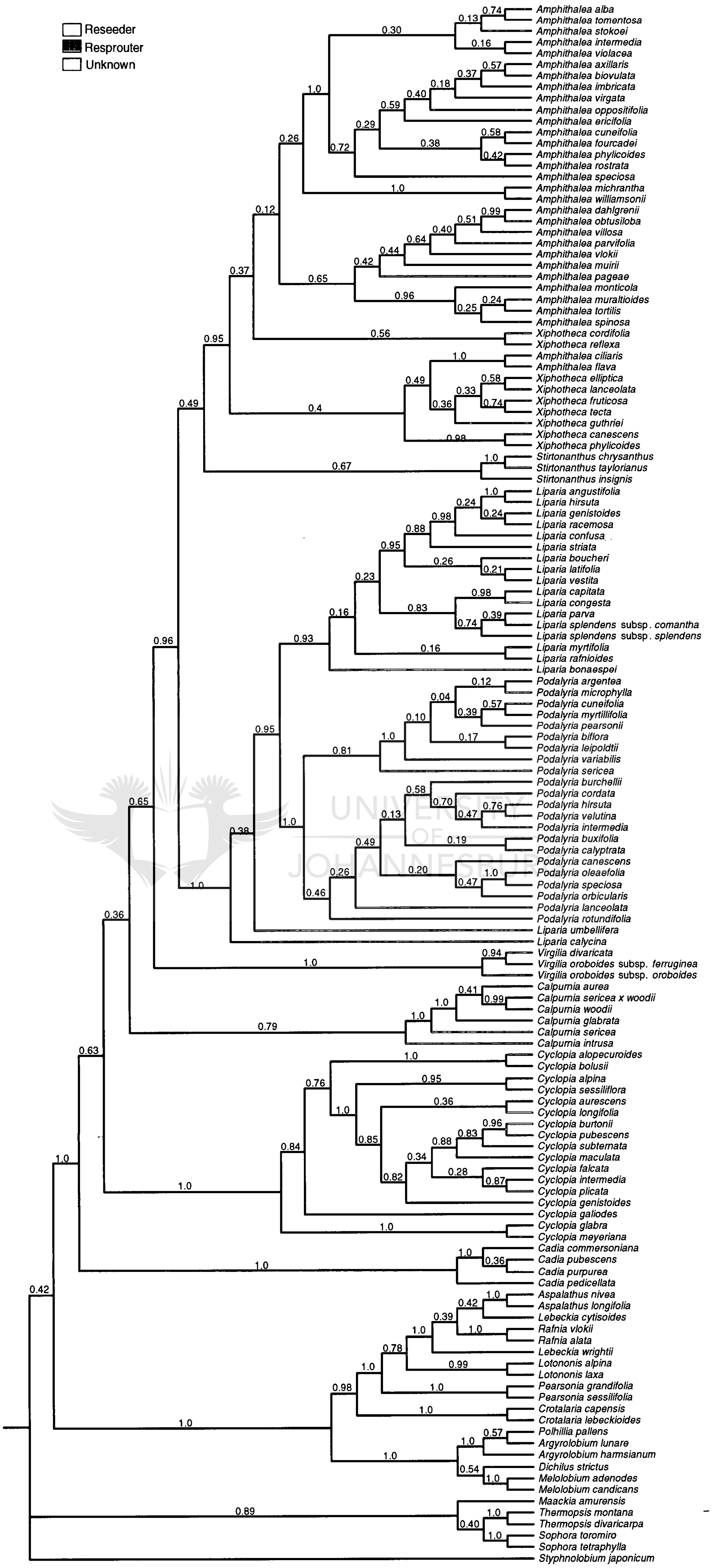

Figure 3.16 Bayesian analysis of combined *rbcL* and ITS dataset. Majority rule consensus tree with PP shown above the branches. Reseeders and resprouters are indicated by coloured lines.

3.2.3 High-level analysis

3.2.3.1 Statistics

A summary of the statistics for the ITS analysis is provided in Table 3.12.

3.2.3.2 Age estimation of the root node of Podalyrieae

The analysis of the ITS region consisted of 745 characters of which 233 were constant, 512 variable and 425 potentially informative (Table 3.12). The high-level analysis resulted in 260 equally most parsimonious trees of 3122 steps, a CI of 0.33 and an RI of 0.77 (Figure 3.17). The ultrametric tree produced by the NPRS procedure is presented in Figure 3.18. *Diplotropis* was used to calibrate the tree in absolute time and a date for the root node of Podalyrieae is estimated at 28.55 MYA. To assess the confidence levels for this date it is necessary to calculate the variance in age estimates due to possible sampling bias. This is done by using the bootstrap resampling method, which recalculates the date 100 times using the MP branch lengths from bootstrapping the DNA regions. The resulting bootstrap distribution of age estimates gave a mean age estimate of 29.91 MYA with a standard error of ±1.094 MYA.

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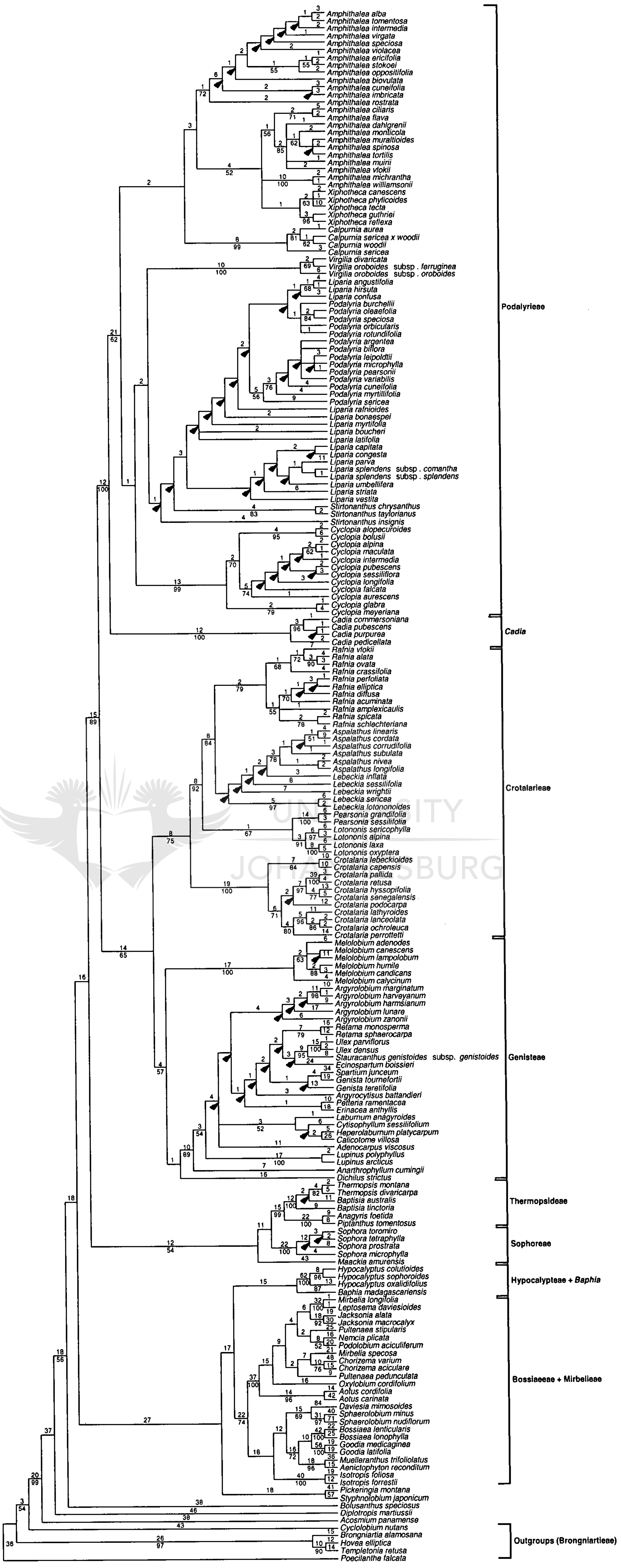

Figure 3.17 One of the equally parsimonious trees from the high-level ITS analysis. Numbers above the branches are Fitch lengths (DELTRAN optimisation) and those below are bootstrap percentages above 50%. Solid arrows indicate groups not present in the Fitch strict consensus trees (CI=0.33; RI=0.77; TL=3122).

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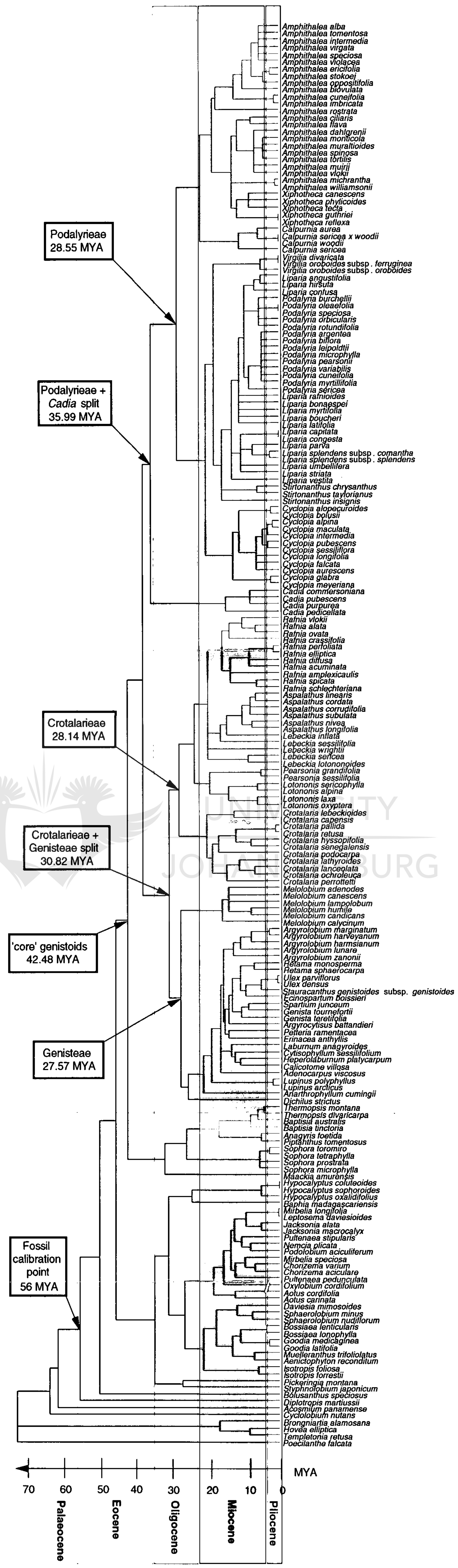

Figure 3.18 The ultrametric tree based on ITS produced by non-parametric rate smoothing (NPRS). A time scale in million years ago (MYA) indicates the divergence times of the various nodes.

Table 3.9 Statistics from PAUP analyses of the separate and combined datasets.

	rbcL	ITS	Combined
No. of included positions in matrix	1415	734	2148
No. of variable sites	232	375	530
	16.40%	51.09%	24.67%
No. of parsimony-informative sites	154	234	323
	10.88%	31.88%	15.04%
No. of trees (Fitch)	173	6650	140
No. of steps (Tree length)	447	891	1176
CI	0.62	0.61	0.61
RI	0.83	0.83	0.83
Average no. of changes per variable sites (no. of steps/ no. of variable sites)	1.9	2.3	2.2
No. of trees (SW)			950
No. of steps (Tree length)			882.25718/1176
CI (SW)			0.61
RI (SW)			0.83

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Table 3.10 Number of steps, CI and RI values for transitions and transversions of each gene region based on separate analysis.

	rbcL		ITS	
	ts	tv	ts	tv
No. of steps	292	155	538	353
CI	0.63	0.60	0.59	0.64
RI	0.82	0.84	0.83	0.83
ts:tv	1.88		1.52	

Table 3.11 Number of positive and negative contrasts generated by CAIC for Podalyrieae and *Protea.*

	Number of positive contrasts	**Number of negative contrasts**	**Total number of contrasts**
Podalyrieae	21	12	33
Protea	10	5	15

Table 3.12 Statistics obtained from PAUP for the ITS matrix used in the high-level analysis.

	ITS region
No. of included positions in matrix	745
No. of constant sites	233
No. of variable sites	512
No. of parsimony-informative sites	425
No. of trees (Fitch)	260
No. of steps (Tree length)	3122
CI	0.33
RI	0.77
Average no. of character changes per variable site (no. of steps/ no. of variable sites)	6.1

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3.3 Discussion

3.3.1 Utility of ITS

The trees produced by the separate analysis of ITS and *rbcL* showed no clear incongruences and thus permitted combination of the two datasets. ITS had a higher number of variable sites (51.09%; Table 3.9) than *rbcL* (16.40%; Table 3.9) and about one and a half times the number of parsimony-informative sites. It thus provided a more resolved phylogeny and shows that faster evolving nuclear genes are important in phylogenetic reconstruction and may be highly informative at lower taxonomic levels, as was the case in Podalyrieae. Soltis and Soltis (1998) mention that ITS is valuable for phylogenetic reconstruction in angiosperms and can be used especially when comparing species and closely related genera. The greater evolutionary rate in nuclear genes ensures more efficient sequencing, since more variation is detected per unit of sequence than in organellar genes. The combination of data from multiple genes and character sets leads to improved performance of phylogenetic analyses so that computer run times are shorter, permitting a more rigorous analysis in a given time, with the resulting phylogenetic trees being more highly resolved and supported.

3.3.2 Relationships within Podalyrieae

The relationship between *Amphithalea* and *Xiphotheca*, although weakly supported, corresponds to the findings of Schutte and Van Wyk (1998a) and Van der Bank *et al.* (2002). These two genera constitute the subtribe Xiphothecinae and share several morphological characters: a non-intrusive calyx base; obtuse keel petal; reduced number of ovules; and wing petals with a thickened lobe on the abaxial surface as is shown in Figure 3.19 (Schutte and Van Wyk, 1998a).

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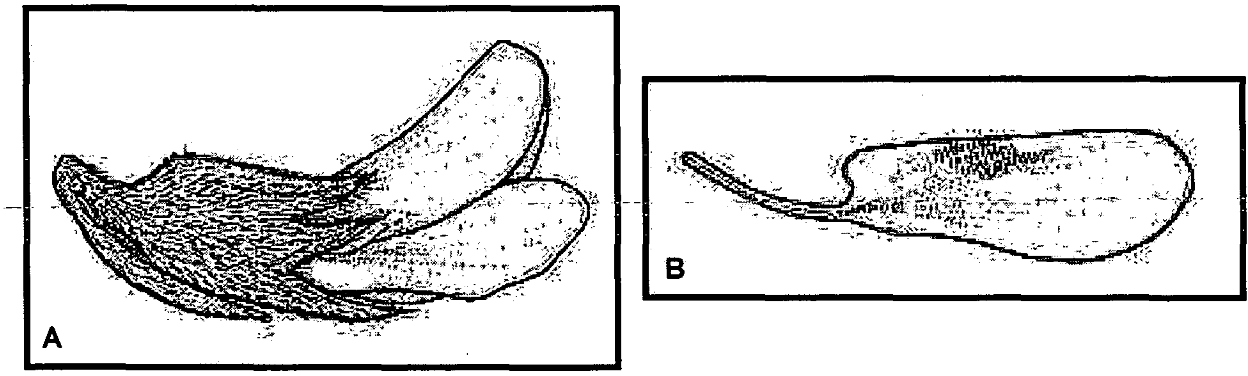

Figure 3.19 Flower and wing petal of *Xiphotheca fruticosa*. A: The flower has a non-intrusive calyx base and obtuse keel petal. B: A thickened lobe is found on the abaxial surface of the wing petal (from Schutte, 1995a).

The resolution within the *Amphithalea/Xiphotheca* clade was low in both the MP and ML analyses, but a few sister pairs were noted. *Amphithalea michrantha* and *A. williamsonii* were strongly supported to be closely related. This relationship was noted by Schutte (1995a), which states that *A. williamsonii* differs from *A. michrantha* only in its densely sericeous calyx and pink standard and wing petals. *Amphithalea ciliaris* and *A. flava* are also closely related. The latter differs from *A. ciliaris* in having bright yellow flowers, instead of white flowers with a brown keel tip. No clear relationship exists between *A. dahlgrenii* and *A. obtusiloba. Amphithalea dahlgrenii* is probably more closely related to *A. esterhuysiniae*, which was not included in the study due to the difficulty of obtaining material, seeing that it is a rare endemic of the Hex River Mountains (Table 3.1). Schutte (1995a) mentions that the distinction between these two species is doubtful, but that *A. dahlgrenii* has tomentose calyx lobes, as opposed to the glabrous calyx lobes of *A. esterhuyseniae*, and shorter bracts than the latter.

The subtribe Podalyriinae is clearly not monophyletic. This confirms the results obtained by Van der Bank *et* al. (2002) where *Cyclopia* formed a grouping sister to the rest of Podalyrieae. The six genera that constitute the subtribe have an intrusive calyx base and rostrate (beaked) keel petal as indicated in Figure 3.20 (Schutte and Van Wyk, 1998a).

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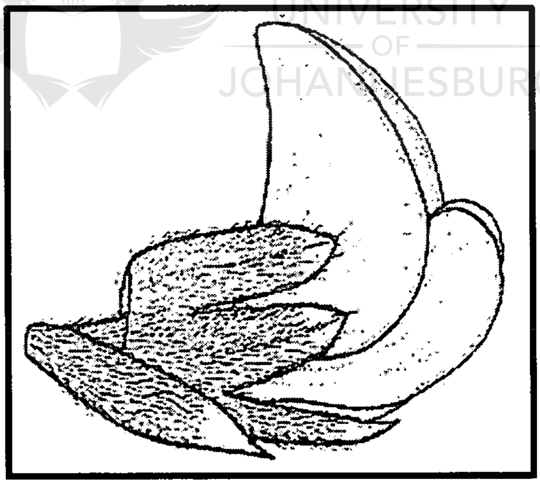

Figure 3.20 Flower of *Liparia congesta* showing the intrusive calyx base and rostrate keel petal that are characteristics of the subtribe Podalyriinae (from Schutte, 1995a).

In this study, three groupings were recognised within Podalyriinae. The first of these is a clade containing *Liparia*, *Podalyria* and *Stirtonanthus*. This grouping is sister to *Amphithalea* and *Xiphotheca*, but it receives low support in the Fitch tree and *Stirtonanthus* is not represented as part of this grouping in the ML analysis. A further analysis on this group using additional plastid genes was performed and is discussed in detail in Chapter 4.

The second group consists of *Calpurnia* and *Virgilia*. These genera were both originally placed in Sophoreae (Van Wyk, 1986; Beaumont *et al.*, 1999). They share several characters, including imparipinnately compound leaves and the major alkaloids virgiline and its carboxylic acid ester (Van Wyk and Schutte, 1995a; Schutte and Van Wyk, 1998a). This grouping is not present in the majority rule consensus tree of the Bayesian analysis where the genera form separate, well supported lineages. *Calpurnia intrusa* is not included in the *Calpurnia* clade on the Fitch tree. This is probably due to the low resolution across the tree, seeing that the genus is monophyletic in the ML analysis. The hybrid between *C. sericea* and *C. woodii* was described by Beaumont *et al.* (1999). Both putative parent species of this hybrid were included in the study and a possible relationship with only *C. woodii* was found. A clear explanation for this is not apparent, but sampling material from the parent species at the hybrid locality might prove valuable. In *Virgilia*, *V. divaricata* and *V. oroboides* subsp. *ferruginea* group together with strong support. Van Wyk (1986) suggested that *V. oroboides* subsp. *ferruginea* probably originated as a hybrid between *V. divaricata* and V. *oroboides* subsp. *oroboides*. This and the fact that it is more or less geographically isolated from *V. oroboides* subsp. *oroboides* could explain the close relationship with *V. divaricata*. Van der Bank *et al.* (1996) also suggest that divergence followed by introgression could account for the similarity in the taxa. They speculate that there could have been an initial divergence in two species, *V. divaricata* and *V. oroboides*, with introgression resulting in a morphologically intermediate *V. oroboides* subsp. *ferruginea*.

Cyclopia is strongly supported to be monophyletic forms the third grouping in Podalyriinae. The genus is unique in Podalyrieae, as it is the only member that has trifoliate leaves and a total absence of alkaloids. It has been suggested that *Cyclopia* shares a close relationship with *Liparia* and *Podalyria*, but that is clearly not the case in this study (Schutte and Van Wyk, 1998a). *Cyclopia alopecuroides* and *C. bolusii* seem to be closely related. Schutte (1997b) suggested a close relationship between *C. aurescens* and *C. bolusii*, but this is not reflected in the results. Similarities between *C. alopecuroides* and *C. bolusii* include the inflated pods that have hairy margins found in both species (Schutte, 1997b). A close relationship exists between *C. glabra* and *C. meyeriana*. *Cyclopia glabra* differs from *C. meyeriana* in that it is multi-stemmed with a glabrous calyx and inner surface of the bracts (Schutte, 1997b).

While the monophyly of the tribe is only weakly supported (56BP, 54SW, PP 0.63) an interesting result is the high support for a close relationship between *Cadia* and Podalyrieae. This result is not surprising and has been suggested by previous authors, e.g. Schutte and Van

Wyk (1998a), and Doyle *et al.* (2000). Chapter 5 presents a more detailed discussion on the relationship between *Cadia* and Podalyrieae.

3.3.3 Comparison between the rates of molecular evolution in reseeders and resprouters

It is evident that the number of positive contrasts is higher in both Podalyrieae and *Protea* (Table 3.11). This shows that the rate of molecular evolution (branch lengths) is higher in reseeders, which one might expect due to the higher number of reproductive cycles and genetic diversification found in reseeders, seeing that DNA replicates more often in these individuals and more seed will fix different kinds of mutations (different sets of alleles).

In this study, the rates of molecular evolution were found to be higher in reseeding species of *Protea* than in the resprouters. Due to the higher rate of molecular evolution in reseeders, more individuals are produced that can accumulate more variation (DNA substitutions), which could result in higher adaptation and speciation rates in these individuals. It may be possible that this could affect the diversification rates in reseeders, as was shown by Reeves (2001), who used a maximum likelihood estimate for the diversification rate of *Protea* under a constant speciation rate. She mentions that in reseeding fynbos species of *Protea*, the diversification rate is higher than in the resprouting lineages, but states that it is evidently possible to attain similar or even higher speciation rates as a resprouter outside of the Cape. Schutte *et al.* (1995) also suggest that the rate of speciation and diversification is more rapid in reseeding species than in resprouters. This is due to the temporal isolation that inhibits gene flow in reseeders, as opposed to the interbreeding populations of the resprouters. Wells (1969) argues that reseeders have shorter generation times than resprouters and that they are subject to selection pressures acting on each discrete generation of seedlings. Therefore reseeders are more prone to diversification than resprouters.

In the genus *Erica*, Verdaguer and Ojeda (2005) suggest that resprouting is ancestral to the reseeder life strategy and they suggest that the marked species diversity and narrow endemism in this genus could be associated with the seeder habit. In *Aspalathus* however, Van der Bank *et al.* (1999) demonstrated through morphological and genetic analyses, that reseeding could be a pleisiomorphic character state with resprouting developing as a fire-survival strategy. They suggest that switches between the two strategies are possible, e.g. in *Cyclopia*, *Podalyria* and *Hypocalyptus*, and that it must still be demonstrated whether the change from reseeding to resprouting was a single evolutionary event or convergence in different populations of *Aspalathus*.

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3.3.4 Age estimation of the root node of Podalyrieae

The use of molecular sequence data for making inferences about the ages of lineages and clade diversification has become more frequent in recent years and is discussed by Linder (2003) and Wojciechowski (2003). Several studies have been done subsequently to determine the ages of well known Cape plant groups, e.g. Richardson *et al.* (2001) dated the major proliferation of *Phylica* at 7-8 MYA; Reeves (2001) dated the radiation of *Protea* at 25 MYA; the divergence of the sister pair *Ferraria* and *Moraea* was dated at 25 MYA (Goldblatt *et al.*, 2002); *Muraltia* started its radiation at 20.7 MYA (Forest, unpublished); the age of *Aspalathus* was estimated at 22 MYA (Edwards, unpublished). Linder (2005) discusses the evolution of diversity in the Cape flora and mentions that the greatest diversity and most recent radiations in southern Africa are found in the more arid western parts of the subcontinent. The largely gradual transformation in climate that has taken place throughout the evolutionary history of South Africa means that there was no single, obvious trigger for the radiation of the Cape flora and this subsequently accounts for the great spread in the dates of initiation of the radiation of various lineages.

Lavin *et al.* (2005) in an evolutionary rates analysis of legumes found that in legumes a rapid diversification of lineages took place in the Tertiary, soon after the family's origin about 60 MYA. In their study, *Diplotropis* was also used to fix the age of the genistoid crown clade at 56 MYA. In this study, the root node of Podalyrieae was dated at 28.55 MYA. This date indicates that Podalyrieae started its diversification in the late Oligocene during the Tertiary. Linder (2003) suggests that two environmental changes in the Tertiary could have triggered the radiations that took place, namely fluctuations in sea-level and climatic changes. At the end of the Oligocene there was a general improvement in the climate of the Cape. The Miocene was marked by high sea-levels, with only ephemeral ice-sheets on Antarctica. Climatic gradients from the equator to the poles became steeper in the Middle Miocene and seasonal aridity became more pronounced in the late Miocene after the glaciation of the northern Hemisphere that led to a symmetrical zonal climate. The South Atlantic high pressure cell became fixed in a position that blocks summer precipitation in the fynbos region (Hendey, 1983; Deacon *et al.*, 1992; Hallam, 1994; Linder, 2003) and with the inception of the Mediterranean type climate during the Pliocene, the climate in South Africa stabilised. It is during this period (approximately 5 MYA) and the late Miocene (approximately 10 MYA) that the major radiation in Podalyrieae took place.

Linder (2005) suggests that several selective forces could be proposed that drove speciation in the Cape flora. Among these are pollinator, edaphic and climatic specialisation.

He comments that although it is possible to investigate the evolution of plant species diversity the generality of the interpretations are limited by four problems:

1. Methods of molecular dating are still flawed and it is not known how large the errors in the estimations are.
2. The poor fossil record of the Cape flora means that we have no independent verification of the suggested paleohistory.
3. The number of clades investigated is still quite small and it is possible that the sample was highly skewed.
4. The methods of inferring the ecologies and distributions of ancestral taxa are still crude.

Although the Cape flora is an excellent place to investigate the generation of exceptionally high species richness, determining the factors that appear to greatly influence the speciation in the CFR is not simple and it is likely that several factors operate simultaneously (Linder, 2003).

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CHAPTER 4

GENERIC RELATIONSHIPS BETWEEN *LIPARIA*, *PODALYRIA* AND *STIRTONANTHUS*

4.1 Introduction

4.1.1 General

In chapter 3 the morphological characters, geographical distribution and Red Data List status of the genera *Liparia*, *Podalyria* and *Stirtonanthus* are discussed. Due to the close relationship noted between the genera (Figure 3.14), additional plastid genes were sequenced to improve the resolution between these groups. This was done for the following reasons:

Firstly, the monophyly of *Podalyria* has been questionable since Van der Bank *et al.* (2002) found the genus to be paraphyletic. They mention that there is little clear evidence, molecular and otherwise, for the status of the monophyly of *Podalyria*. Further study is thus still necessary to resolve this matter and it is addressed in this chapter.

Secondly, *Liparia* is the only genus in Podalyrieae that contains species that are pollinated by pollinators other than xylocopid bees (Schutte and Van Wyk, 1998a). The flowers in Podalyrieae are normally adapted to pollination by xylocopid bees and as a consequence are quite firmly textured (Schutte and Van Wyk, 1998a). The species with alternative pollination vectors seem to mimic members of Proteaceae and display structural changes in both the inflorescences and flowers. The inflorescences are either congested and decussate, or pendant, proteoid heads (Figure 4.1 B, D and E). The flowers are firmly textured and have long, forwardly directed beaks for pollination by birds (e.g. *L. splendens*) or possibly rodents (e.g. *L. parva*) as opposed to the short and upwardly directed keel tip of the bee pollinated species (Schutte, 1997c). The inflorescences of *L. parva* are borne at ground level and the pale colour and yeast odour of the flowers suggest that small mammals might pollinate them (Johnson, 1992), an occurrence that is unique in Fabaceae (Schutte and Van Wyk, 1994). It would thus be interesting to determine whether these adaptations to pollination are reflected in the phylogenetic relationships of *Liparia* and to build a species-level phylogeny for the genus that could be used in studies concerning the pollination biology of the genus.

Thirdly, the monophyly of *Liparia* needs to be assessed, as it has been questioned both in chapter 3 as well as by previous authors.

4.1.2 Aims of the chapter:

This chapter aims to:

1. Present a species-level phylogeny for *Liparia*, *Podalyria* and *Stirtonanthus*, based on ITS, *rbcL*, *trnL-F* and *trnS-trnG*.
2. Assess the monophyly of *Liparia* and *Podalyria*.
3. Investigate whether shifts in pollinators are reflected in phylogenetic relationships.

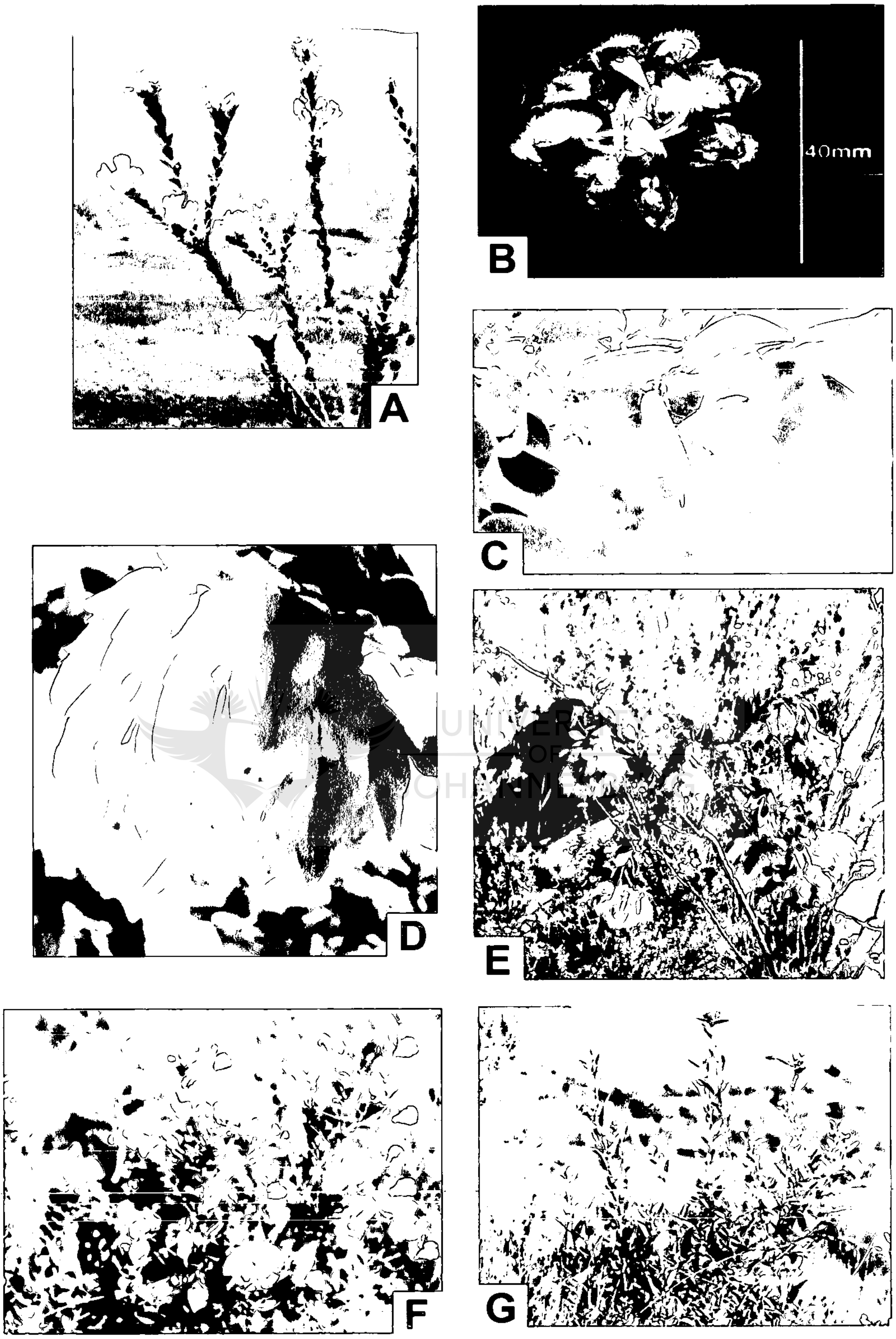

Figure 4.1 Representatives of *Liparia*, *Podalyria* and *Stirtonanthus*. A: *Liparia bonaespei* (Nick Helme); B: *Liparia parva* (from www.plantweb.co.za); C: *Stirtonanthus taylorianus*; D: *Liparia splendens*; E: *Liparia splendens*; F: *Podalyria biflora* (Mark Johns); G: *Podalyria burchellii*.

4.2 Results

4.2.1 Species-level phylogeny

4.2.1.1 Statistics

Statistics for each analysis is provided in Table 4.1.

4.2.1.2 Separate molecular analysis

Molecular evolution

The aligned ITS matrix was the shortest in length, but contained the most parsimony-informative sites (4.6%, Table 4.1) and had a transitions (ts): transversions (tv) ratio of 1.4 (Table 4.2). Among the plastid genes, *trnS-trnG* had the highest number of variable (8.2%) and parsimony-informative (3.7%) sites, but the lowest ts:tv ratio (0.83). The *trnL-F* matrix contained the lowest number of variable sites (6.97), whilst the *rbcL* matrix had the highest ts:tv ratio (1.82). *Liparia* possessed a 29-base pair (bp) deletion at position 235--264 in *trnL-F* and a 36-bp deletion at position 172--208 in the *trnS-trnG* matrix.

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Plastid results

Visual inspection of the separate *rbcL*, *trnL-F* and *trnS-trnG* matrices demonstrated no strongly supported incongruent patterns and these datasets were combined directly. Due to the low resolution in the separate plastid gene trees only the combined plastid analysis is discussed. The parsimony analysis of the combined matrix included 3295 characters of which 3048 were constant, 247 (7.5%) variable and 102 (3.1%) potentially informative. A total of 404 equally most parsimonious trees of 311 steps, a CI of 0.83 and an RI of 0.84 were obtained (Table 4.1).

Nuclear results

Analysis of the ITS region included 675 characters with 575 constant, 100 (14.8%) variable and 31 (4.6%) potentially informative characters. Fitch analysis produced 3428 most parsimonious trees with a length of 125, a CI of 0.86 and an RI of 0.86 (Table 4.1).

4.2.1.3 Combined molecular analysis (Total evidence)

The bootstrap consensus trees from the combined plastid and ITS analyses are presented in Figure 4.2. The trees showed no strongly supported incongruent groupings and the datasets were combined directly. The combined matrix included 3970 characters of which 3623 were constant, 347 (8.7%) variable and 133 (3.4%) potentially informative. Analysis produced 995 equally most parsimonious trees (TL=450; CI= 0.82; RI= 0.82). Successive weighting resulted in 470 trees (Figure 4.3; CI=0.82; RI=0.82, see Table 4.1).

A relationship between *Liparia* and *Podalyria* is strongly supported (87BP, 88SW), as opposed to the low support it received in the combined ITS and *rbcL* analysis (Figure 3.14). The position of *Stirtonanthus* as sister to this clade receives low support (65BP, 63 SW).

Liparia is paraphyletic with *L. calycina* not included in the *Liparia* clade. Maximum likelihood analysis of ITS and *rbcL* (Figure 3.16) indicated that both *L. calycina* and *L. umbellifera* are excluded from the *Liparia* grouping, but that was not the case in this analysis. Groupings within *Liparia* include: *L. angustifolia* with *L. hirsuta* (85BP, 84SW); *L parva* with *L. splendens* (81BP, 79SW); *L. capitata* with *L. congesta* (60BP, 61SW).

Podalyria receives low support to be monophyletic (71BP, 72SW). A grouping of nine species, the *P.argentea* clade, receives low support (64BP, 61SW). *Podalyria cordata* and *P. hirsuta* group together with high support (92BP, 91SW), as do *P. oleaefolia* and *P. speciosa* (99BP, 99SW).

The *Stirtonanthus* clade in this analysis was unresolved, although in the ITS and *rbcL* analysis (Figure 3.14) a relationship between *S. chrysanthus* and *S. taylorianus* is suggested (95BP, 94SW).

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4.2.2 Maximum likelihood (Bayesian) analysis

Due to slight differences in the topologies of the majority rule consensus tree (Figure 4.4) and the Fitch tree (Figure 4.3), the former is included separately. The grouping of *Liparia* and *Podalyria* is again well supported (PP 1.0), with the sister relationship of *Stirtonanthus* also receiving high support (PP 1.0) as opposed to the low support in the Fitch analysis. *Liparia calycina* is again not included in the *Liparia* clade. Some of the groupings on the specific level differ from the Fitch analysis, although these generally receive low support. The groupings between *L. angustifolia* and *L. hirsuta* (PP 1.0), and *L. parva* and *L. splendens* (PP 1.0) receive high support. *Podalyria* is strongly supported to be monophyletic (PP 1.0) and the *P. argentea* clade receives high support (PP 1.0). Strongly supported sister pairs are: *P. oleaefolia* and *P. speciosa* (PP 1.0); *P. cordata* and *P. hirsuta* (PP 1.0). *Stirtonanthus chrysanthus* and *S. taylorianus* group together with high support (PP 0.9).

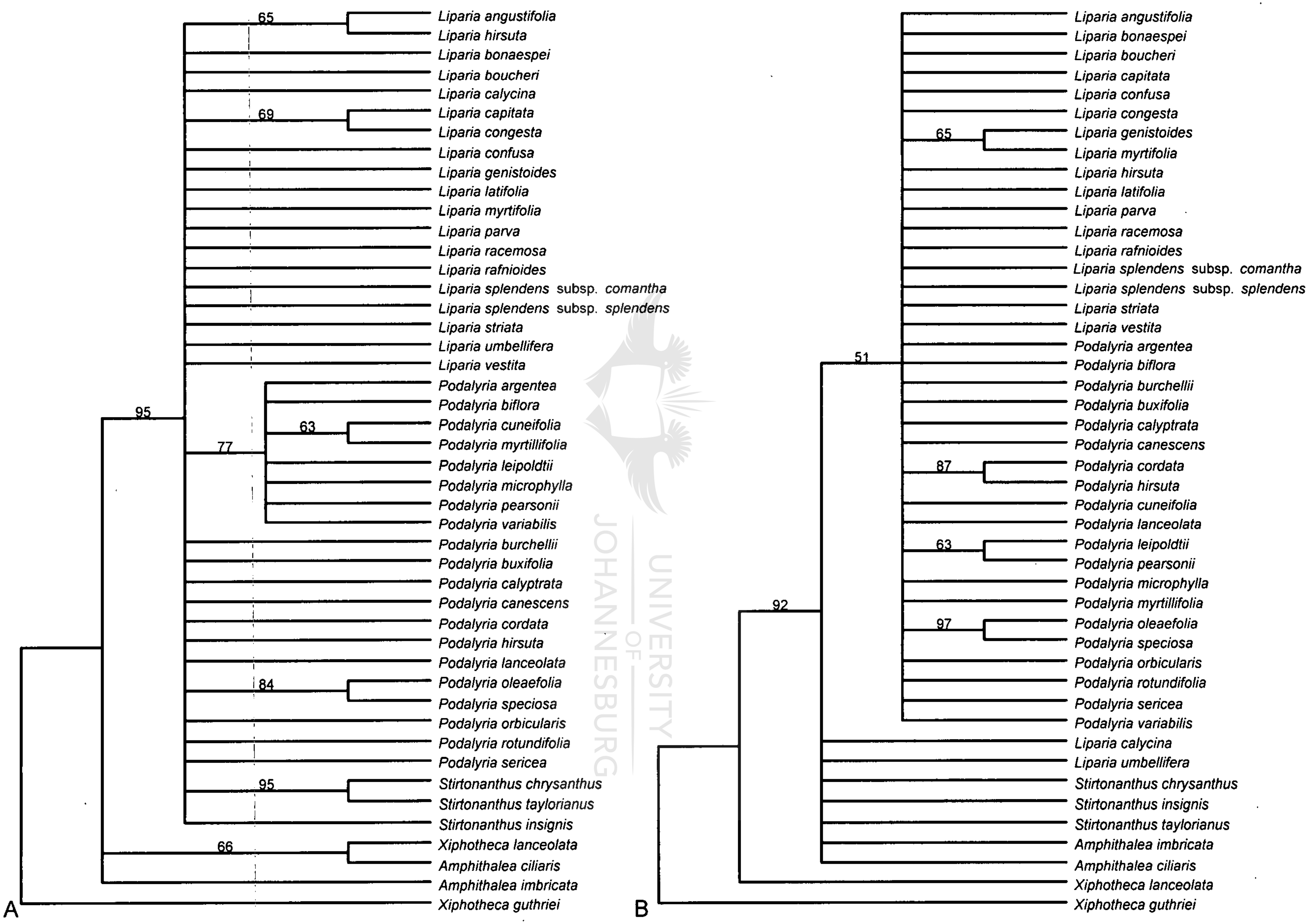

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Figure 4.2 Comparison between the bootstrap consensus trees of A: the ITS analysis and B: the combined plastid analysis. Bootstrap percentages above 50% are shown above each branch.

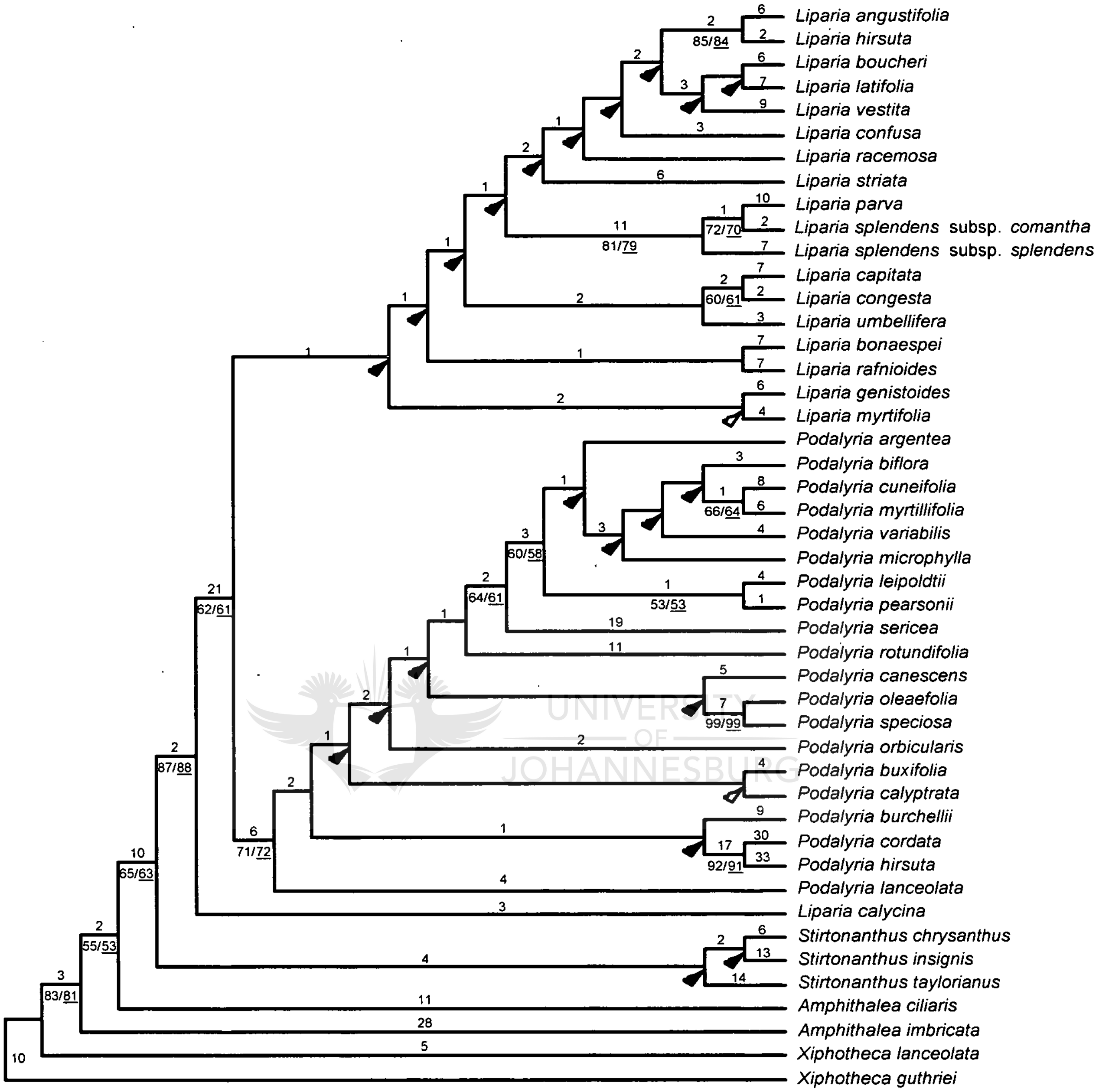

Figure 4.3 One of the equally parsimonious trees from the combined molecular dataset. Numbers above the branches are Fitch lengths (DELTRAN optimisation) and those below the branches are Fitch bootstrap percentages over 50% (SW bootstrap results are underlined). Solid arrows indicate groups not present in the Fitch strict consensus tree and open arrows indicate groups not present in both the SW and Fitch consensus trees (CI=0.82; RI=0.82; TL=450).

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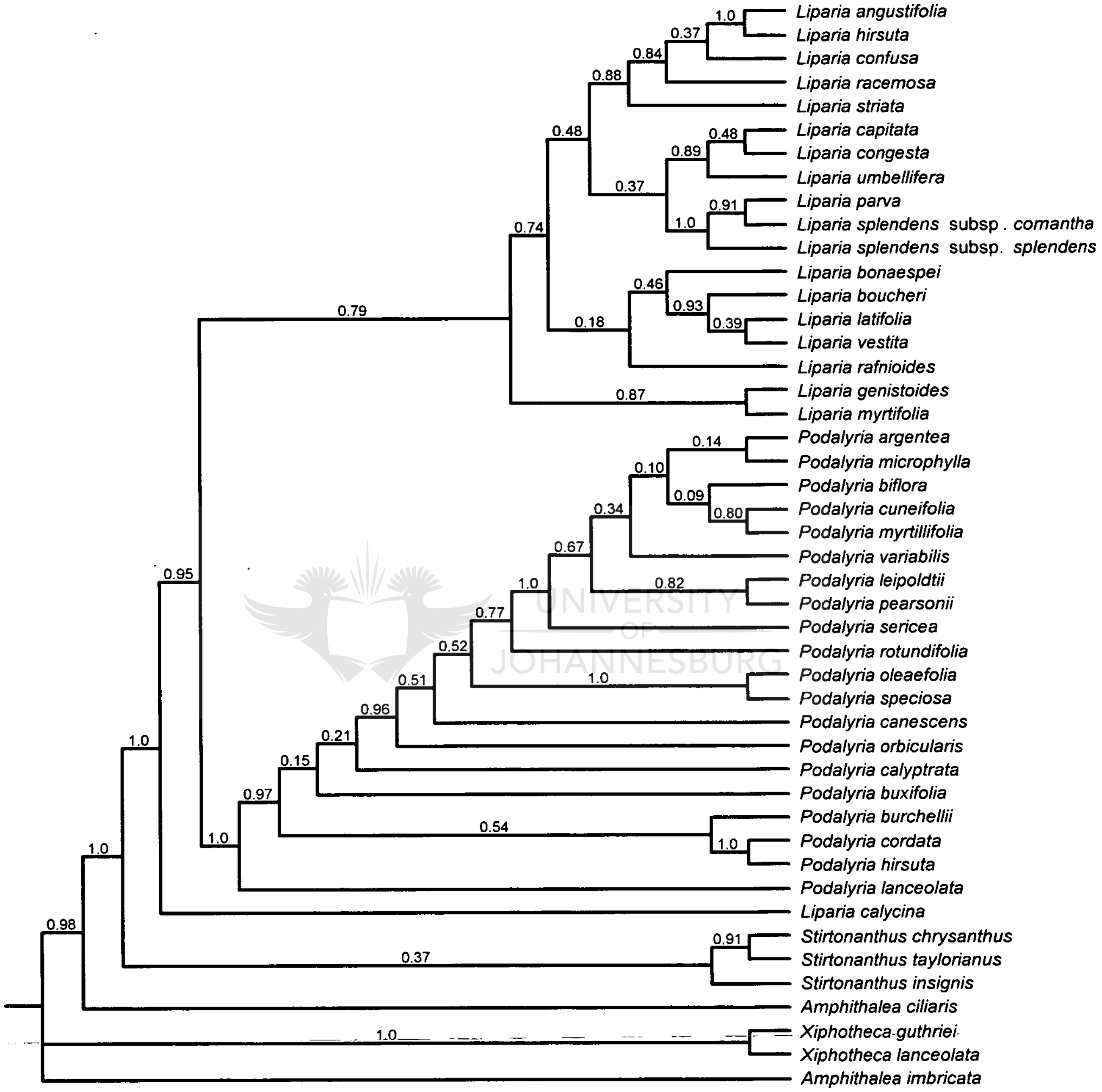

Figure 4.4 Bayesian analysis of the combined ITS and plastid dataset. Majority rule consensus tree with PP shown above the branches.

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Table 4.1 Statistics from PAUP analyses for the separate and combined datasets.

	rbcL	***trnL-F***	***trnS-trnG***	**ITS**	**Combined plastid**	**Combined molecular**
No. of included positions in matrix	1414	1091	790	675	3295	3970
No. of variable sites	107	76	65	69	145	214
	7.56%	6.97%	8.22%	10.22%	4.40%	5.39%
No. of parsimony-informative sites	42	32	29	31	102	133
	2.97%	2.93%	3.67%	4.59%	3.10%	3.35%
No. of trees (Fitch)	1553	914	4194	3428	404	995
No. of steps (Tree length)	138	86	73	125	311	450
CI	0.82	0.92	0.92	0.86	0.83	0.82
RI	0.85	0.92	0.93	0.86	0.84	0.82
Average no. of changes per variable sites (no. of steps/ no. of variable sites)	1.29	1.13	1.12	1.81	2.14	2.1
No. of trees (SW)						470
No. of steps (Tree length)						367.19168/450
CI (SW)						0.82
RI (SW)						0.82

Table 4.2 Number of steps, CI and RI values for transitions and transversions for each gene region based on separate analysis.

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	rbcL		***trnL-F***		***trnS-trnG***		**ITS**	
	ts	tv	ts	tv	ts	tv	ts	tv
No. of steps	89	49	45	41	33	40	73	52
CI	0.87	0.73	0.89	0.95	0.88	0.95	0.85	0.87
RI	0.85	0.85	0.88	0.96	0.88	0.97	0.84	0.89
ts:tv	1.82		1.1		0.83		1.4	

4.3 Discussion

4.3.1 Molecular evolution

When comparing ITS to the plastid regions used, it showed much more variation and contained the most parsimony-informative sites (4.6%, Table 4.1). In combination with the plastid genes, a total of 133 (3.35%, Table 4.1) informative sites were present. ITS proved to be very useful in this study, especially in combination with the plastid genes.

4.3.2 The *Liparia/Podalyria* clade

It is clear that a close relationship exists between *Liparia* and *Podalyria* due to the high support in both the Fitch and Bayesian analyses. The genera share the intrusive calyx base and rostrate keel petal that is characteristic of Podalyriinae and both have few-flowered, racemose inflorescences (Schutte and Van Wyk, 1998a). Schutte (1997b) described a close relationship between *Cyclopia* and *Liparia*, due to the presence of prominent, decurrent leaf bases and sterile bracts at the base of the inflorescences in both genera. From this study (Chapter 3) it is evident that *Cyclopia* retains an isolated position within Podalyrieae forming the earliest diverging lineage in the tribe and does not seem closely allied to *Liparia*. Crisp *et al.* (2000) mention a similar close relationship between *Liparia* and *Podalyria*. In their analysis the genera grouped together with moderate bootstrap support and it thus seems likely that they are sister taxa.

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4.3.3 Monophyly of *Liparia*

All the analyses performed indicate that *Liparia* is not monophyletic, with *L. calycina* not included in the *Liparia* clade (*L. umbellifera* not included in the ML analysis; Figure 3.16). Schutte (1995a) described a close relationship between *L. calycina* and *L. vestita* and the two species share a sympatric distribution (Figure 4.5). *Liparia vestita* in this case is embedded in the *Liparia* clade in a position close to *L. boucheri* and *L. latifolia* (Figures 4.3 and 4.4).

Liparia is easily distinguished from other genera in the tribe by the sessile leaves, unusual leaf venation pattern, the presence of a terminal rachis extension on the inflorescences and the unique combination of alkaloids found in the genus (Schutte, 1997c). Crisp *et al.* (2000) also commented on the possible paraphyly of *Liparia*, with some species of *Liparia* grouping with *Cyclopia* and others with *Podalyria* in their analysis. They ascribed this result to the amalgamation of *Liparia* and *Priestleya* and recommended that this should be tested with a larger sampling from both genera. In this study *Liparia* is almost complete, lacking only *L. graminifolia*, which is presumably extinct, and *L. laevigata*, which due to a low yield of DNA during extraction could not be amplified for any of the genes. The amalgamation of the two genera does not seem to be the problem, seeing that the rest of *Liparia* group together (with moderate support in the ML analysis). It might be wise to recollect material from other populations of *L. calycina* to eliminate possible sequencing error before making a definite conclusion.

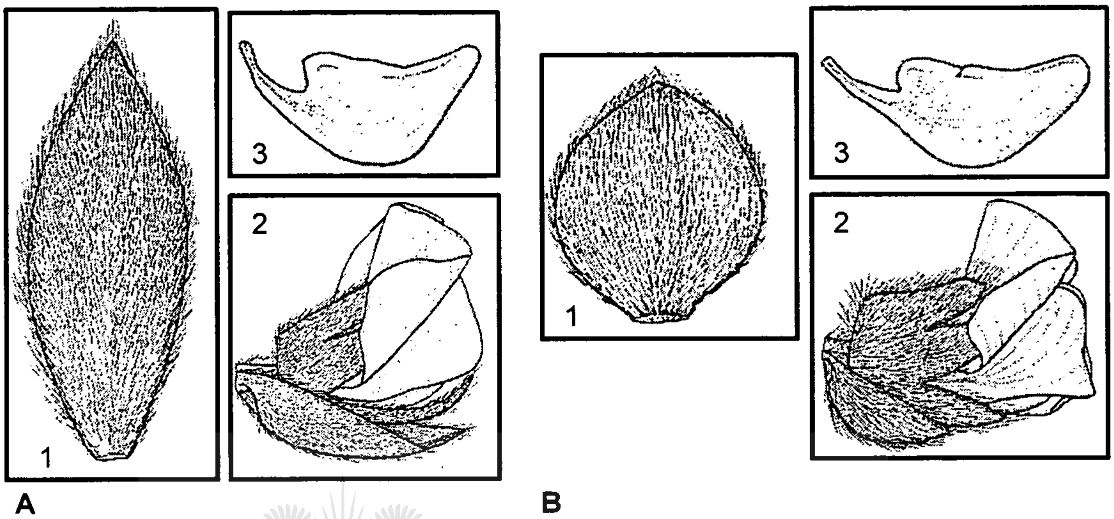

Figure 4.5 The similarity between A: *Liparia calycina* and B: *L. vestita* (1: Leaf; 2: Flower; 3: Wing petal). The bracts are almost as long as the calyx in *L. calycina*, whereas they are only half the length in *L. vestita* (from Schutte, 1995a).

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No apparent morphological characters confirm the grouping between *L. angustifolia* and *L. hirsuta*, but the two are strongly supported to be closely related. *Liparia capitata* and *L. congesta* both have the carinal lobe of the calyx longer than the other lobes, but no apparent close relationship has previously been suggested. *Liparia congesta* occurs on sandy soils, as opposed to the rocky sandstone preferred by *L. capitata* (Schutte, 1995a, 1997c).

4.3.4 Monophyly of *Podalyria*

The support for *Podalyria* to be monophyletic, although low in the MP analysis, differs from the result obtained by Van der Bank *et al.* (2002) where *Podalyria* was paraphyletic with at least three groupings. According to Schutte (1995a) there is no single autapomorphy for the genus, but it has a unique combination of characters, namely simple, distinctly petiolate leaves (shared with *Stirtonanthus* and *Xiphotheca*), pink, purple or white flowers (shared with *Virgilia* and *Amphithalea*), few-flowered racemose inflorescences (shared with some species of *Liparia*) and a characteristic combination of alkaloids. In a chemical study of the genus no less than 16 different alkaloids were found. *Stirtonanthus* (then part of *Podalyria*) differed in accumulating virgiline, 13α-hydrolupanine and two totally different esters (Van Wyk *et al.*, 1992). From this study it is evident that *Podalyria* is most likely monophyletic.

The *P. argentea* clade, consisting of *P. argentea*, *P. biflora*, *P. cuneifolia*, *P. leipoldtii, P. microphylla*, *P. myrtillifolia*, *P. pearsonii*, *P. sericea* and *P. variabilis* (*P. intermedia* is also included in this clade in the ITS and *rbcL* analysis done in chapter 3, but was not included in this analysis due to sequencing difficulty) corresponds more or less to the *P. biflora* group, described by Schutte (1995a). The leaf apices are reflexed in this group, except for *P. intermedia*.

In Figure 3.14, *P. hirsuta* and *P. velutina* form a well-supported grouping, with *P. cordata* as sister. Due to sequencing difficulty, *P velutina* could not be included in the current analysis and is not represented in Figure 4.3. Instead, *P. cordata* and *P. hirsuta* group together with high support. It is difficult to comment on this grouping, as *P. hirsuta* did not form part of the treatment of *Podalyria* done by Schutte (1995a) and thus the morphological characters of this species are not described. From the results of the current study, it seems likely that a close relationship exists between *P. cordata*, *P. hirsuta* and *P. velutina.* Schutte (1995a) describes a close relationship between *P. burchellii* and *P. velutina* and states that they might even be the same species, but in this analysis *P. burchellii* groups with the clade containing *P. velutina* only with low support in the *rbcL* and ITS analysis (Figures 3.14 and 4.3). The high support for *P. oleaefolia* and *P. speciosa* is expected, seeing that *P. speciosa* is now considered a synonym of *P. oleaefolia.*

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4.3.5 Are changes in pollination syndromes reflected in the phylogenetics of *Liparia*?

Two of the species suspected of alternative pollination vectors, *L. parva* and *L. splendens*, form a moderately supported clade in the MP analysis (Figure 4.3) and a strongly supported clade in the ML analysis (Figure 4.4). Both these species have unique floral characteristics. The inflorescences appear as head-like structures subtended by large sterile bracts at the base, mimicking in appearance those of *Protea* (Schutte, 1997c). It is clear that the changes associated with the shift in pollinator from xylocopid bees to either birds or mammals are derived characters in these species, rather than parallel evolution of unrelated traits in the two lineages and thus shifts in pollination are reflected in the phylogenetic relationships of *Liparia*.

Bird pollination in South Africa seems to be strongly associated with plant communities on nutrient poor soils (e.g. fynbos) where it is relatively inexpensive to produce copious amounts of nectar, that is preferred by birds, using the excess carbon unavailable for growth (Rebelo, 1987). Deacon *et al.* (1992) suggest that unfavourable conditions in fynbos (e.g. high wind velocities, frequent mists and rain) inhibit the abundance of insects and could also trigger the shift to alternative pollination vectors. The shift to mammal pollination, especially in Proteaceae, normally occurs in small isolated populations that may have been neglected by birds that favour species forming large monospecific stands (Wiens *et al.*, 1983). Another explanation for the shift to mammal pollinators is that inflorescences hidden beneath foliage might be less visible to harmful insects and would thus suffer less insect damage to flowers (Rebelo and Breytenbach, 1987). What triggered the shift in pollinators in *Liparia* is not clear, but further research into the pollination biology of *Liparia* is underway and promises to deliver very interesting results and insights into the strange floral morphology found in *L. parva* and *L. splendens* especially (Midgley pers. comm.).

4.3.6 Position of *Stirtonanthus*

Stirtonanthus is weakly supported to be sister to the *Liparia/Podalyria* clade. A strange result is the low resolution obtained within the genus in this analysis. In Figure 3.14, a close relationship between *S. chrysanthus* and *S. taylorianus* is suggested. These species are probably closely related and have inflated pods, as opposed to the compressed pods of *S. insignis*, and are both reseeders (Van Wyk and Schutte, 1994). The placement of *Podalyria* in closer relation to *Liparia* is quite unexpected, seeing that *Stirtonanthus* once formed part of *Podalyria*, but the overall placement of *Stirtonanthus* remains unclear, being strongly supported as sister to the *Liparia/Podalyria* grouping in the ML analysis (Figure 4.4) and weakly supported in the MP analysis (Figure 4.3). A definite conclusion on the placement of *Stirtonanthus* is not evident, although it seems likely that it is included in the *Liparia/Podalyria/Stirtonanthus* clade and sister to *Liparia* and *Podalyria*.

CHAPTER 5

AFFINITY OF THE GENUS *CADIA* (SOPHOREAE) WITH THE TRIBE PODALYRIEAE

5.1 Introduction

5.1.1 General

Cadia is an anomalous genus of Papilionoid legumes that consists of seven species. The most widespread of the species is *Cadia purpurea*, occurring in East and North-East Africa and Arabia, while the other six species are endemic to Madagascar (Du Puy *et al.*, 2002). The placement of *Cadia* has been uncertain for some time. It appears to have an affinity with the tribe Podalyrieae, but has been placed in the non-monophyletic tribe Sophoreae by authors like Polhill (1981) and Schutte and Van Wyk (1998a). Recent molecular evidence by Doyle *et al.* (2000) confirms the close relationship between *Cadia* and Podalyrieae based on *rbcL* and non-molecular evidence, e.g. floral development, embryology, chromosome numbers. *Cadia* was supported to be a member of Podalyrieae and the authors suggested that the placement of *Cadia* be reconsidered.

Cadia spp. have tufted, imparipinnate leaves and axillary, racemose inflorescences with pendulous actinomorphic, pink to purple flowers (Du Puy *et al.*, 2002). The floral characteristics of *Cadia* are probably the most confusing, their radial floral symmetry and unstable petal aestivation are unusual within Papilionoideae (Tucker, 1987, 2002, 2003). Pennington *et al.* (2000) in their evaluation of floral evolution of the basal Papilionoideae, based on sequence data from the chloroplast intron *trnL*, interpret the shift from the typical zygomorphic, papilionoid flower in *Cadia* as a reversal, due to unusual pollination biology and the need to attract different pollinators, with *Cadia* being presumably bird pollinated. Tucker (2002) however suggests that this change could alternatively be due to the neotenous nature of the flowers of these groups, i.e. to retain the juvenile state of radial symmetry. The change to a zygomorphic flower is brought about late in floral development and those that appear radial at anthesis lack the final events that would express a zygomorphic flower. She concludes that in terms of floral development, *Cadia* conforms to the consistently unidirectional organogenesis found in other Sophoreae and is in agreement with the majority of other Papilionoid legumes from various tribes.

The chemical composition of *Cadia* shares compounds found within members of the Podalyrieae, again suggesting a close relationship. It contains carboxylic acid esters of quinolizidine alkaloids, also found in *Calpurnia*, *Stirtonanthus*, *Virgilia* and some species of *Liparia* (Van Wyk, 2003; Wink, 2003; Wink and Mohammed, 2003). The isoflavone 3'-hydroxydaidzein, a major seed flavonoid of the Podalyrieae, is also found in *Cadia purpurea* (De Nysschen *et al.*, 1998).

5.1.2 Aims of the chapter

The results of both the species-level (*rbcL* and ITS) and high-level (ITS) phylogenies where used to determine possible relationships between *Cadia* and Podalyrieae.

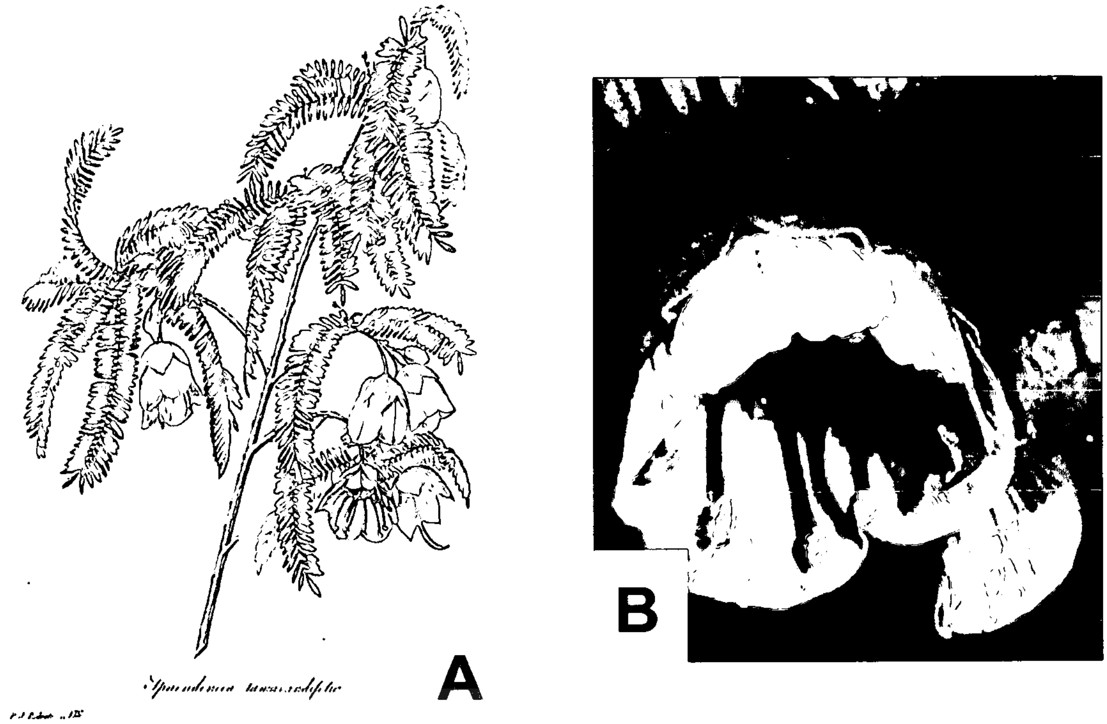

Figure 5.1 The unusual floral morphology of *Cadia purpurea*. A: From www.audubonart.com; B: From www.humanflowerproject.com.

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5.2 Results

5.2.1 Species-level phylogeny

5.2.1.1 Statistics

The statistics for the combined analysis of ITS and *rbcL* is presented in Table 3.9.

5.2.1.2 Combined molecular analysis (Total evidence)

A discussion of the statistics of the combined analysis can be found in section 3.2.1.3. Figure 5.2 presents a shortened version of Figure 3.14. From this it is clear that *Cadia* is closely affiliated to Podalyrieae, as it groups with the tribe with high support (92BP, 94SW). The genus is strongly supported to be monophyletic (100BP, 100SW) and *C. purpurea* groups with two of the Madagascan species, *C. commersoniana* and *C. pubescens* (95BP, 96SW).

5.2.2 High-level analysis

5.2.2.1 Statistics

The statistics for the high-level analysis is presented in Table 3.12.

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5.2.2.2 High-level ITS phylogeny

Figure 5.3 presents a shortened version of Figure 3.17 from which the position of *Cadia* and Podalyrieae in relation to the other genistoid tribes can be seen. *Cadia* groups with Podalyrieae with high support (100BP) and is strongly supported to be monophyletic (100BP). Sister to the Podalyrieae/*Cadia* grouping are the tribes Crotalarieae and Genisteae (89BP). Sophoreae (in part) and Thermopsidae group together with low support (54BP) and are sister to the above mentioned tribes. These tribes constitute the 'core' genistoids and retain an isolated, monophyletic position within the genistoid legumes.

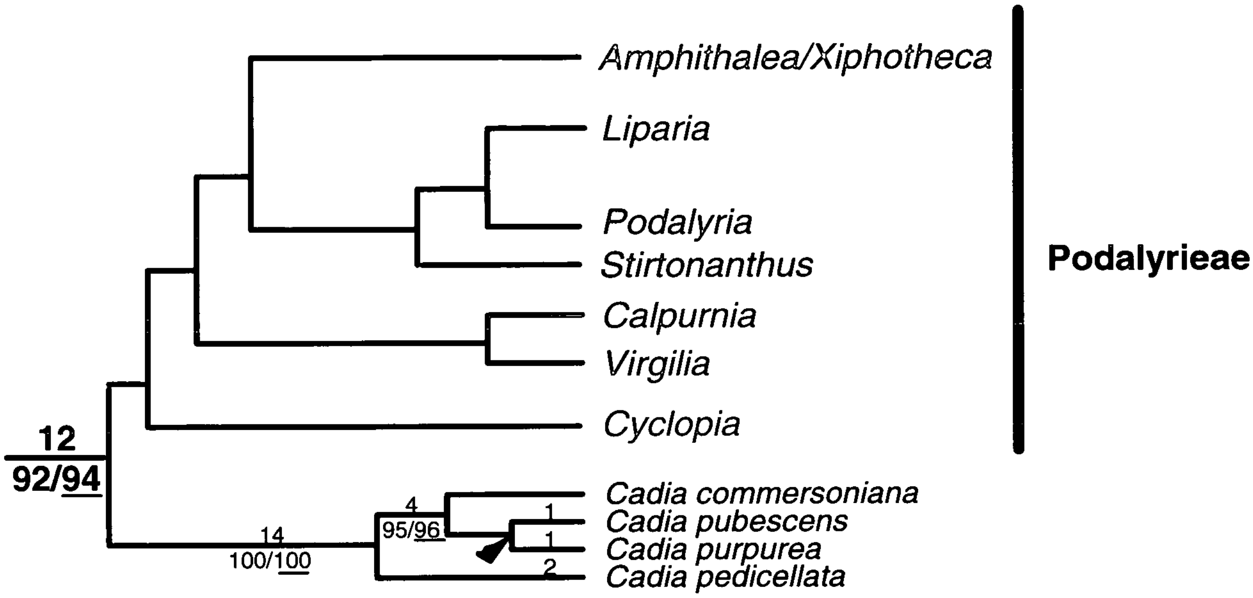

Figure 5.2 A shortened version of one of the most parsimonious trees from the combined *rbcL* and ITS analysis (Figure 3.14). The numbers above the branches are Fitch lengths (DELTRAN optimisation) and those below are bootstrap percentages above 50% (SW bootstrap results underlined). Solid arrows indicate groups not present in the Fitch strict consensus tree (CI=0.61; RI=0.83; TL=1176).

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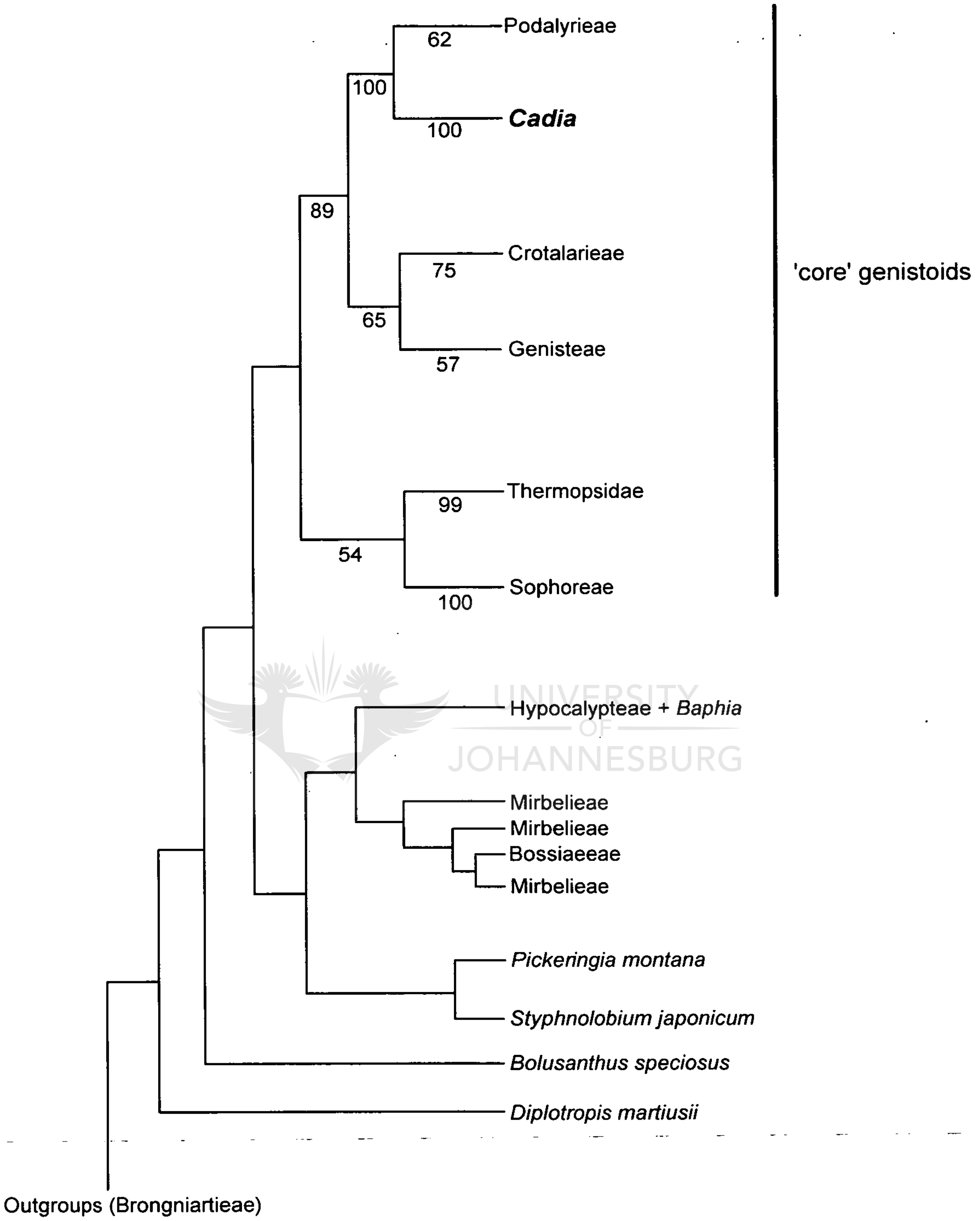

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Figure 5.3 A shortened version of one of the equally parsimonious trees produced by the high-level ITS analysis (Figure 3.17) indicating the position of *Cadia* and Podalyrieae in relation to the other genistoid tribes. The numbers below the branches are bootstrap percentages above 50% (CI=0.33; RI=0.77; TL=3122).

5.3 Discussion

As suggested by several authors, a close relationship seems to exist between *Cadia* and Podalyrieae. Although Schutte and van Wyk (1998a) excluded *Cadia* from Podalyrieae, they mention that it is not impossible that studies involving chemistry or DNA might place *Cadia* in a position close to the tribe. From this study it is evident that the position of *Cadia* is in need of reconsideration, as was mentioned by Doyle *et al.* (2000).

It is clear that the genus is monophyletic and that the widely distributed *Cadia purpurea* is closely related to the Madagascan species. A close relationship seems to exist between *C. commersoniana*, *C. pubescens* and *C. purpurea*. *Cadia commersoniana* and *C. pubescens* both have broad, leafy bracts on the inflorescence and can be distinguished by the strongly pubescent leaves and stems of *C. pubescens*, together with the smaller number of leaflets found in this species. *C. pedicellata* has a similar distribution to *C. pubescens*, but is much less pubescent and has narrow, non-leafy bracts on the inflorescence (Du Puy *et al.*, 2002).

Cadia shares several of its characters with members of Podalyrieae: imparipinnately compound leaves as in *Calpurnia* and *Virgilia*, axillary racemose inflorescences as in most Podalyrieae, similar alkaloids and seed flavonoids and a similar growth form to some Podalyrieae. The radial floral symmetry could therefore be unique to the genus, i.e. autapomorphies, as was the interpretation by Pennington *et al.* (2000) and Tucker (2002). Molecular data from this and previous studies (Van der Bank *et al.*, 2002) do not support the subtribal concepts as was proposed by Schutte and Van Wyk (1998a), due to the paraphyly of the subtribe Podalyriinae. It would therefore be possible, after careful consideration, to transfer *Cadia* to Podalyrieae if a broader tribal concept, possibly without subtribal classification, is proposed. Alternatively a separate subtribe could be erected to accommodate the genus, but this would have to be done for all the clades in Podalyriinae and would not be taxonomically viable. In light of this a transfer of *Cadia* to Podalyrieae is proposed.

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CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

6.1 Conclusions

The tribe Podalyrieae has been the focus of previous morphological (Schutte, 1995a) and molecular studies (Van der Bank *et al.*, 2002), but to date no detailed species-level phylogeny exists for the tribe. The starting point in this study was therefore to reconstruct an almost complete species-level phylogeny for Podalyrieae (based on *rbcL* and ITS sequence data), which was then used to study the phylogenetic and some evolutionary aspects of the tribe.

In Chapter 3, four major clades were noted in the analysis of ITS and *rbcL*: the *Amphithalea/Xiphotheca* clade (corresponding to the subtribe Xiphothecinae), the *Liparia/Podalyria/Stirtonanthus* clade, the *Calpurnia/Virgilia* clade and the *Cyclopia* clade. The current classification of Podalyrieae divides the tribe into two subtribes, Podalyriinae and Xiphothecinae. It is evident that the molecular results in this study do not fit the subtribal concepts in the tribe, due to the paraphyletic nature of the subtribe Podalyriinae. To accommodate these groupings a broader concept of Podalyrieae, without subtribal classification is suggested, as this would be more practical than erecting numerous subtribes (corresponding to each of the separate groupings).

The genus *Cadia* was included in this study to evaluate whether it shares a close relationship with Podalyrieae. In Chapter 5 the molecular analyses (both species and high-level) indicated a close relationship between *Cadia* and Podalyrieae. Due to the various characters *Cadia* shares with members of the tribe together with molecular evidence, it is suggested that the genus be moved either to a position closer to Podalyrieae or included in the tribe.

Also in Chapter 3, two evolutionary aspects of Podalyrieae were studied. Firstly, the rates of molecular evolution between reseeders and resprouters were compared to determine whether reseeders have higher rates of molecular evolution than resprouters. By means of independent contrasts, it was shown that the rates of molecular evolution were indeed higher in the reseeders in Podalyrieae and *Protea* when compared to the resprouters. This confirms the hypothesis of Reeves (2001) that diversification rates are higher in reseeding species of *Protea* and reflects the higher evolutionary rates of the reseeders, due to the larger number of reproductive cycles in these individuals that leads to a greater genetic diversity. Secondly, ITS sequence data was used in a high-level analysis to determine a date for the root node of Podalyrieae. This date was estimated at 28.55 MYA and indicates that a major radiation occurred in the tribe during the Pliocene and does concord with the inception of Mediterranean type climates in the Cape.

In Chapter 4, a species-level phylogeny for *Liparia*, *Podalyria* and *Stirtonanthus* was reconstructed (based on ITS, *rbcL*, *trnL-F* and *trnS-trnG*) to investigate the relationship between these genera, asses the monophyly of especially *Liparia* and *Podalyria* and to test whether a shift in pollination vector is a derived character in *Liparia*. *Liparia* and *Podalyria* are closely related, with *Stirtonanthus* as a possible sister taxon. With the inclusion of more species, *Podalyria* was found to be monophyletic (contrasting to reports by Van der Bank *et al*., 2002). Even with the inclusion of almost all the species, *Liparia* seems to be paraphyletic (as found by Crisp *et al*., 2000). The two species with alternative pollination vectors grouped together as sister taxa, suggesting that the shift from bee pollination, as found in the rest of the genus, to bird or mammal pollination appears to be a derived character and not the result of convergent evolution. Pollination studies on *Liparia* will provide important insights into the interesting pollination biology of the genus.

6.2 Future research

Future studies on Podalyrieae should focus on improving the resolution within the genera and strengthening the 'backbone' of the tree. ITS was very valuable in this study and Small *et al*. (2004) state that one of the primary advantages of nuclear genes for phylogenetic analysis is the elevated rate of sequence evolution relative to organellar genes. In some plant groups, like Podalyrieae, sequences from non-coding plastid and nuclear regions (ITS) provide low resolution of relationships. An alternative to the standard regions that are sequenced in phylogenetic studies are low-copy nuclear genes [e.g. Alcohol dehydrogenase (ADH); Granule-bound starch synthase (GBSSI or Waxy); Floricaula/Leafy (FLO or LFY)] which might provide more robust estimates of phylogeny. These can be used since there may be rare copies of the ITS in an individual that are not amplified due to selective amplification of only one copy. This selective amplification together with concerted evolution could obscure processes such as hybridisation in the history of an organism. It is clear that low-copy nuclear regions are becoming more important in phylogenetic analyses and might be an interesting future step in the phylogenetic reconstruction of Podalyrieae (Crawford and Mort, 2004; Mort and Crawford, 2004).

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APPENDIX

Appendix 1

Table A1.1 Contrasts produced by CAIC for Podalyrieae.

Code	Survival Strategy*	Branch lengths*	Std Dev	Height	Subtaxa	nodal Survival Strategy	nodal Branchlengths	Residuals
CAAAAAAAAAAAAAAAAAAAAAABAAAAAAA	1	2.705	2	-9	2	-9	79.6174	1.84744
CAAAAAAAAAAAAAAAAAAAAAABAAAA	1	1.57689	2.3094	-9	2	-9	73.79402	0.98292
CAAAAAAAAAAAAAAAAAAAAAABABA	1	0.80543	2.23607	-9	2	-9	73.2638	0.39172
CAAAAAAAAAAAAAAAAAAAAAAA	1	-0.92	2	-9	2	-9	71.3074	-0.93055
CAAAAAAAAAAAAAAAAAAABBBB	1	2.793	2	-9	2	-9	73.4754	1.91488
CAAAAAAAAAAAAAAAAAAABBA	1	0.3875	2	-9	2	-9	70.5909	0.07145
CAAAAAAAAAAAAAAAAAAAAA	1	0.3228	2.70416	-9	2	-9	70.53266	0.02186
CAAAAAAAAAAAAAAAAAAAB	1	-0.02983	3	-9	2	-9	73.77406	-0.24837
CAAAAAAAAAAAAAAAAABA	1	-0.09101	2.23607	-9	2	-9	65.4188	-0.29525
CAAAAAAAAAAAAAAAAAA	1	-1.7515	2.82843	-9	2	-9	64.9039	-1.56776
CAAAAAAAAAAAAAAAA	1	1.06349	2.82843	-9	2	-9	63.2464	0.58948
CAAAABAABAAABAAAA	1	1.9755	2	-9	2	-9	57.3085	1.2884
CAAAAAAAAAAAAAA	1	-0.46516	2.44949	-9	2	-9	61.7368	-0.58198
CAAAABAAAAAAAAA	1	-0.01625	2.28709	-9	2	-9	50.2958	-0.23796
CAAAABAABAAABAA	1	-0.49235	2.44949	-9	2	-9	54.1	-0.60282
CAAAABAABBAAABA	1	2.4105	2	-9	2	-9	82.9655	1.62175
CAAAAAAAAAAAAB	1	0.3975	2	-9	2	-9	61.0605	0.07911
CAAAABAAAAAAAB	1	0.184	2	-9	2	-9	49.72	-0.08451
CAAAABAABBAABA	1	0.12835	2.23607	-9	2	-9	54.9072	-0.12715
CAAAABAAAAABB	1	1.7255	2	-9	2	-9	56.2305	1.09681
CAABAAABBBAAA	1	1.43478	2.28709	-9	2	-9	61.19468	0.87402
CAABAAABBBAB	1	0.1705	2	-9	2	-9	62.5975	-0.09485
CAAAAAAAAAA	1	-2.84781	2.44949	-9	2	-9	58.10322	-2.4079
CAAAABAAAAA	1	0.73824	2.73861	-9	2	-9	50.77473	0.34023
CAAAABAABA	1	1.38468	2.28869	-9	2	-9	55.13798	0.83562
CAAAABAABB	1	-1.3047	2.31158	-9	2	-9	52.12984	-1.22536

Table A1.1 Continued.

Code	Survival Strategy*	Branch lengths*	Std Dev	Height	Subtaxa	nodal Survival Strategy	nodal Branchlengths	Residuals
CAABAAABBB	1	0.09298	2.82843	-9	2	-9	59.16525	-0.15425
CAAAABAAA	1	-1.18859	2.86356	-9	2	-9	52.21436	-1.13638
CAAAAAAA	1	-2.16044	2.3094	-9	2	-9	52.904	-1.88115
CAABAAAA	1	0.591	2	-9	2	-9	65.082	0.2274
CAAAAA	1	-0.83738	2.64575	-9	2	-9	50.385	-0.86723
CAABA	1	0.15051	2.73523	-9	2	-9	63.1809	-0.11017
CABAA	1	0.77782	2.82843	-9	2	-9	48.333	0.37056

*Columns 2 and 3 indicate that when a contrast is made by CAIC, a positive contrast indicates that longer branch lengths were present in reseeders, as opposed to a negative contrasts where longer branch lengths were found in resprouters.

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Table A1.2 Contrasts produced by CAIC for *Protea*.

Code	Survival Strategy*	Branch lengths*	Std Dev	Height	Subtaxa	nodal Survival Strategy	nodal Branch length	Residuals
AAAABBAAAA	1	0.066	2	-9	2	-9	7.312	0.16932
AAAAABAAB	1	0.2025	2	-9	2	-9	6.0665	0.17413
AAAAAAA	1	0.20501	2.55402	-9	2	-9	5.68424	0.17421
AAAAABB	1	-0.074	2	-9	2	-9	7.12	0.16439
AAAABA	1	1.74771	2.23607	-9	2	-9	7.6122	0.22855
BAAAAA	1	0.62299	2.28808	-9	2	-9	5.43755	0.18893
BAAAAB	1	-0.105	2	-9	2	-9	7.075	0.1633
BAAAB	1	0.01143	2.44949	-9	2	-9	6.66267	0.1674
CAABA	1	-0.1365	2	-9	2	-9	5.2135	0.16219
AAAA	1	0.37422	3.60518	-9	2	-9	6.41818	0.18017
AABB	1	-0.18228	2.44949	-9	2	-9	3.71375	0.16057
BAAB	1	0.378	2	-9	2	-9	5.226	0.18031
CA	1	0.40516	2.3094	-9	2	-9	3.85287	0.18126
B	1	-106.74984	2.68985	-9	2	-9	219.69965	-3.59258
@Root	1	32.1099	2.83971	-9	2	-9	51.40384	1.29786

*See discussion under Table A1.1.

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