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PHYLOGENY AND EMBRYO SAC EVOLUTION IN THE ENDEMIC AUSTRALASIAN PAPILIONOID TRIBES MIRBELIEAE AND BOSSIAEEAE

MICHAEL D. CRISP AND LYN G. COOK

School of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia

Abstract

The Mirbelieae and Bossiaeeae are related tribes restricted to the Australian region. This study analyses the most comprehensive sample to date of DNA sequences (*trn*L intron and ITS) from both tribes. Monophyly of the Mirbelieae + Bossiaeeae with respect to putatively related tribes is supported, albeit weakly. Bossiaeeae is a strongly supported clade but nested within a paraphyletic Mirbelieae. There is evidence for alternative groupings based on embryo sac morphology and development. Bossiaeeae could be expanded to include the *Daviesia* group of Mirbelieae, with which it shares giant antipodals, but stronger evidence is needed for monophyly of this group. Mirbelieae could be reduced to the clade comprising *Isotropis* and the *Mirbelia* group, which share absence of antipodals. The *Mirbelia* group appears to have rapidly diversified into many lineages that do not cluster to form well defined genera. Several genera currently recognised in this group are not supported by the molecular data. To achieve consistency of generic delimitation within the tribes, this group should perhaps be treated as a single genus by expanding *Pultenaea* to include all genera currently recognised in the *Mirbelia* group.

Introduction

The Mirbelieae and Bossiaeeae are two papilionoid tribes endemic to Australia and New Guinea. Most species in these tribes are ericoid shrubs with yellow and red ('egg and bacon') flowers. They are conspicuous, sometimes dominant, understorey members of sclerophyll communities (heathland and eucalypt-dominated woodland and forest), on poor soils of the south-west, south and east coast of Australia. The Bossiaeeae includes 6 genera and about 77 species (Ross and Crisp, in press) and the Mirbelieae includes about 670 species in 25 genera (Crisp *et al.*, in press), although recently four genera were combined into a much enlarged *Gastrolobium* (Chandler *et al.*, 2002).

The definition and relationships of both tribes have been problematic. After a long history of placement of its genera in the Podalyrieae, Mirbelieae was recognised in its present circumscription by Polhill (1981). Bossiaeeae was segregated from Genisteae (Hutchinson, 1964), and included the *Bossiaea* and *Templetonia* groups of Australian genera (Polhill, 1976, 1981). Subsequently, the *Templetonia* group was

* author for correspondence: Mike.Crisp@anu.edu.au

found to group strongly with the neotropical tribe Brongniartieae, according to morphology (Crisp and Weston, 1987), nuclear ribosomal DNA (Crisp *et al.*, 2000; Thompson *et al.*, 2001) and chloroplast DNA (Kajita *et al.*, 2001). When restricted to include only the *Bossiaea* group, the Bossiaeeae appeared to form a clade with the Mirbelieae which was not closely related to either the Brongniartieae or the genistoid tribes (Crisp and Weston, 1987; Crisp *et al.*, 2000; Kajita *et al.*, 2001). Although Mirbelieae + Bossiaeeae was monophyletic according to morphology (Crisp and Weston, 1987) and ITS sequences (Crisp *et al.*, 2000), support for this clade has been weak. Within the *Oxylobium* and *Pultenaea* groups of Mirbelieae, high morphological diversity has been the basis for recognising about 450 species in 20 genera, yet generic delimitation has continued to be problematic (Sands, 1975; Crisp and Weston, 1987, 1995).

All taxa in the Mirbelieae and Bossiaeeae which have been investigated have unusual embryology (Cameron and Prakash, 1990, 1994) in comparison with most other legumes. Typical embryology in the legumes is *Polygonum*-type which produces an 8-nucleate embryo sac (Prakash, 1987). Some taxa in the Mirbelieae and Bossiaeeae have *Polygonum*-type development but produce greatly enlarged antipodal cells, possibly serving a role in nutrition for the embryo (Cameron and Prakash, 1990). Hereafter this embryology is termed the 'giant antipodals' (GA) type. GA-type taxa comprise the Bossiaeeae and the *Daviesia* group (*Daviesia, Erichsenia, Gompholobium, Sphaerolobium* and *Viminaria*) of Mirbelieae (Crisp and Weston, 1995). The remaining taxa within Mirbelieae have a more unusual type of development (Cameron and Prakash, 1994). It resembles *Oenothera*-type (Willemse and van Went, 1984; Reiser and Fischer, 1993) embryology but megagametogenesis involves three mitoses (not two) to produce five nuclei (not four). No antipodals are produced and polarity is reversed during megasporogenesis, so that the micropylar (not chalazal) megaspore is functional and any to all megaspores (not just the chalazal) undergo mitosis to produce partially or fully developed embryo sacs. Cameron and Prakash (1994) called this development *'Mirbelia'* type after one genus in which it is found but here we refer to it by the descriptive term 'no antipodals' (NA) type. There are two variants of the NA type. One is as described above and here is termed the 'five-nucleate embryo sac' (FNES) type. The other NA type, called '*Jacksonia*' type by Cameron and Prakash (1994), produces multiple archesporial cells and embryo sacs, some of which may be aposporous although apomixis has not been confirmed. This form is here termed the 'multiple embryo sac' (MES) type.

Embryology is not known to vary within GA-type genera of the Mirbelieae and Bossiaeeae. However, some NA-type genera (*Dillwynia*, *Jacksonia* and *Mirbelia*) have MES type and FNES type in different species (Cameron and Prakash, 1994). Above genus level, the phylogenetic distribution of embryology types is unclear. Different studies have found each of the GA and NA embryological groups within Mirbelieae and Bossiaeeae to be either monophyletic or paraphyletic. A recent analysis using ITS sequences found the GA and NA embryological groups to be monophyletic sister taxa, whereas Mirbelieae and Bossiaeeae were paraphyletic (Crisp *et al.*, 2000). Giant antipodals also have been reported from two species of *Indigofera* (Cameron and Prakash, 1990, 1994). Given that Indigofereae appears not to group with Mirbelieae/Bossiaeeae (Crisp *et al.*, 2000; Kajita *et al.*, 2001), giant antipodals may not be homologous in these two tribal groups.

This study aimed to test whether the Mirbelieae and Bossiaeeae are monophyletic sister taxa, or whether alternative groupings based on embryo sac type are supported. Within these tribes, relationships among genera were investigated and compared with existing classification. This study differed from previous phylogenetic analyses of these tribes by sampling comprehensively at genus level using DNA sequences from the nuclear and chloroplast genomes.

Materials and methods

Specimens and DNA extraction

To represent the diversity of Mirbelieae and Bossiaeeae, 66 exemplars (Table 1) were sampled from all but one of the 31 genera recognised in the tribes (Crisp *et al.*, in press; Ross and Crisp, in press). The 14 outgroups (Table 1) include exemplars of nine tribes putatively related to the ingroup (Crisp *et al.*, 2000; Hu, 2000; Kajita *et al.*, 2001). These include representatives of the Brongniartieae, genistoid tribes, Indigofereae, 'Hologalegina' (Wojciechowski *et al.*, 2000) and the millettioid/phaseoloid clade (Hu, 2000; Kajita *et al.*, 2001). Based on the results of three of these previous phylogenetic analyses (Crisp *et al.*, 2000; Hu, 2000; Kajita *et al.*, 2001), the trees were rooted using Brongniartieae. Forty seven sequences had previously been generated in our lab and 15 sequences were obtained from GenBank (Benson *et al.*, 1994). To produce the remaining 70, total genomic DNA was extracted from fresh, NaCl-CTAB-stored (Rogstad, 1992), or dry specimens using CTAB/chloroform extraction. Vouchers for all accessions are lodged in CANB or PERTH and cited by collector's name and number in Table 1.

PCR and Sequencing

The ITS region (incorporating ITS-1, 5.8S rRNA and ITS-2) was amplified using primers P1L and P2R (Crisp *et al.*, 1999). The chloroplast *trn*L (UAA) Group IC3 intron and the *trn*L (UAA) 3' exon were amplified using primers c and f (Taberlet *et al.*, 1991) and, for some specimens, internal primer trn540R (Crisp *et al.*, 1999). Each 25 µl PCR reaction contained 5 pmol each primer, 3mM $MgCl_2$, 2.5 µl 10xPCR buffer (Perkin Elmer), 0.2 mM each dNTP and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer).

Sequence Editing, Alignment and Partitions

Sequences were aligned by eye and account taken of predicted secondary structures for the *trn*L intron (Damberger and Gutell, 1994). The secondary structure for highly variable regions within this partition was estimated using Mfold (Mathews *et al.*, 1999; Zuker *et al.*, 1999) and it was further partitioned to reflect each of the nine helices, with stems and loops partitioned independently, and single stranded regions between helices.

Phylogenetic Analysis

Tree estimation was performed by maximum parsimony (MP), Neighbor-Joining (NJ) and maximum likelihood (ML) using PAUP* (v4.0b6) (Swofford, 2001). The two data partitions were initially analysed separately because they represent different genomes. Base composition differences among taxa (non-stationarity) were tested using the χ^2 test as implemented in PAUP* for each of the partitions. A partition homogeneity test, as implemented in PAUP*, with uninformative characters excluded (see Lee, 2001), was performed to assess congruence between the *trn*L intron and ITS data. Subsequently the two data sets were combined.

Heuristic MP searches comprised 100 random addition sequence starting trees saving only 10 trees followed by searching from the resulting trees with MAXTREES set to 20,000. This strategy was run several times and the resulting trees compared. Bootstrap (BS) tests (Felsenstein, 1985) were conducted using 1000 replicates, each with 10 random addition starting sequences saving no more than 100 MP trees.

A reduced set of 20 terminals with known embryo sac type was used for ML tree estimation. ModelTest (Posada and Crandall, 1998) was used to determine the model that best fitted the data (GTR + I + G) and to estimate parameter values for input to PAUP*.

TABLE 1. Taxa sampled

Tribe	Genus	Species	Collector's code[a]	GenBank Accession No. *trn*L-F	GenBank Accession No. ITS
Outgroup taxa					
Brongniartieae	*Templetonia* R.Br.	*T. sulcata* Benth.	MDC9057b	AF518122	AF287635[b]
	Hovea R.Br.	*H. elliptica* (Sm.) DC.	MDC8924	AF518123	AF287640[b]
Fabeae	*Vicia* L.	*V. faba* L.		X51471[c]	X17535[c]
Crotalarieae	*Aspalathus* L.	*A. cordata* (L.) R.Dahlgren	MDC9037	AF518124	AF287681[b]
Podalyrieae	*Virgilia* Poir.	*V. oroboides* (P.J.Bergius) T.M.Salter	MDC9036	AF518125	AF287669[b]
Hypocalypteae	*Hypocalyptus* Thunb.	*H. sophoroides* (P.J.Bergius) Baill.	MDC9063	AF518126	AF287643[b]
Indigofereae	*Indigofera* L.	*I. australis* Willd.	MDC9095	AF518164	AF518098
		I. hilaris Eckl. & Zeyh.		AF274367[c]	AF274694[c]
	Vaughania S.Moore	*V. pseudocompressa* Du Puy, Labat & Schrire		AF274373[c]	AF274701[c]
Phaseoleae	*Glycine* Willd.	*G. clandestina* Wendl.	KH308	AF518127	U60534[c]
Galegeae	*Swainsona* Salisb.	*S. pterostylis* (DC.) Bakh.f.		AF126999[c]	U56007/8[c]
	Glycyrrhiza L.	*G. lepidota* Pursh		AF124238[c]	U50578/9[c]
Wisterieae	*Wisteria* Nutt.	*W. frutescens* (L.) Poir.		AF124239[c]	U55997/8[c]
Millettieae	*Tephrosia* Pers.	*T. tenella* A.Gray		AF124240[c]	U50754/5[c]
Ingroup taxa					
Bossiaeeae	*Aenictophyton* A.T.Lee	*A. reconditum* A.T.Lee	PF4500	AF518144	AF287654[b]
	Bossiaea Vent.	*B. lenticularis* Sieber ex DC.	MDC9289	AF518140	AF518104
		B. linophylla R.Br.	MDC8927	AF518141	AF287657[b]
	Goodia Salisb.	*G. lotifolia* Salisb.	ANBG702052	AF518138	AF287655[b]
		G. medicaginea F.Muell.	MDC9274	AF518139	AF518103
	Muelleranthus Hutch.	*M. trifoliolatus* (F.Muell.) Hutch.	TL743	AF518143	AF287653[b]
	Platylobium Sm.	*P. formosum* Sm.	ANBG732901	AF518142	AF287651[b]
	Ptychosema Benth.	*P. anomalum* F.Muell.	ML560	AF518165	AF287652[b]
Mirbelieae	*Almaleea* Crisp & P.H.Weston	*A. cambagei* (Maiden & E.Betche) Crisp & P.H.Weston	MDC9055	AF113775[b]	AF113758[b]
	Aotus Sm.	*A.* sp. 'Esperance'	MDC9197	AF518163	AF518121
	Callistachys Vent.	*C. lanceolata* Vent.	GTC474	AY015072[b]	AY015189[b]
	Chorizema Labill.	*C. aciculare* (DC.) C.A.Gardner	MDC9202	AF518149	AF518108
		C. carinatum (Meisn.) J.M.Taylor & Crisp	MDC9237	AF518150	AF518109

TABLE 1. continued

Tribe	Genus	Species	Collector's code[a]	GenBank Accession No. *trn*L-F	GenBank Accession No. ITS
		C. genistoides (Meisn.) C.A.Gardner	MDC9026	AF518151	AF287649[b]
		C. parviflorum Benth.	MDC9116	AF518152	AF518110
		C. rhombeum R.Br.	MDC9230	AF518153	AF518111
		C. varium Benth.	MDC8528	AF518154	AF518112
	Daviesia Sm.	*D. elliptica* Crisp	MDC9051	AF518130	AF518099
		D. pachyloma Turcz.	MDC9025	AF518131	AF287662[b]
		D. ulicifolia Andrews	MDC9115	AF518132	AF518100
	Dillwynia Sm.	*D. parvifolia* R.Br. ex Sims	JMT360	AF113777[b]	AF113759[b]
		D. phylicoides Cunn.	MDC9049	AF113778[b]	AF113760[b]
	Erichsenia Hemsl.	*E. uncinata* Hemsl.	MDC8524	AF518133	AF287663[b]
	Euchilopsis F.Muell.	*E. linearis* (Benth.) F.Muell.	MDC8535	AF113779[b]	AF113761[b]
	Eutaxia R.Br.	*E. microphylla* (R.Br.) J.M. Black	MDC8918	AF113780[b]	AF113762[b]
	Gastrolobium R.Br.	*G. bilobum* R.Br.	MDC8485	AY015073[b]	AY015190[b]
		G. bracteolosum (F.Muell.) G.T. Chandler & Crisp	MDC8982	AY015063[b]	AY015180[b]
		G. celsianum (Lem.) G.T.Chandler & Crisp	MDC9009	AY015064[b]	AY015181[b]
		G. formosum (Kippist ex Lindl.) G.T. Chandler & Crisp	MDC8933	AY015085[b]	AY015202[b]
		G. latifolium (R.Br.) G.T.Chandler & Crisp	GTC365	AY015065[b]	AY015182[b]
		G. leakeanum Drumm.	MDC8949	AY015091[b]	AY015208[b]
		G. ebracteolatum G.T.Chandler & Crisp	MDC8471	AY015102[b]	AY015219[b]
		G. plicatum Turcz.	MDC9014	AF518161	AF518119
	Gompholobium Sm.	*G. obcordatum* Turcz.	MDC9031	AF518128	AF287659[b]
		G. villosum (Meisn.) Crisp	MDC8951	AF518129	AF287658[b]
	Isotropis Benth.	*I. cuneifolia* (Sm.) Benth. ex Heynh.	MDC8917	AY015083[b]	AF287647[b]
		I. foliosa Crisp	MDC9121	AF518145	AF518105
	Jacksonia Sm.	*J. alata* Benth.	MDC8956	AF518146	AF518106
		J. horrida DC.	MDC8934	AY015084[b]	AF287645[b]
		J. macrocalyx Meisn.	MDC9272	AF518147	AF518107
	Latrobea Meisn.	*L. hirtella* (Turcz.) Benth.	MDC8478	AF113781[b]	AF113763[b]
	Leptosema Benth.	*L. aphyllum* (Hook.) Crisp	MDC9019	AF518148	AF287646[b]

TABLE 1. continued

Tribe	Genus	Species	Collector's code[a]	GenBank Accession No. *trn*L-F	GenBank Accession No. ITS
	Mirbelia Sm.	*M. confertiflora* Pedley	MDC9050	AF518155	AF518113
		M. depressa E.Pritz.	MDC9020	AY015086[b]	AY015203[b]
		M. dilatata R.Br.	MDC8491	AY015087[b]	AY015204[b]
		M. microphylla (Turcz.) Benth.	AM22	AF518156	AF518114
		M. rubiifolia (Andrews) G.Don	ANBG8406509	AF518157	AF518115
		M. speciosa Sieber ex DC.	ANBG8100876	AF518158	AF518116
	Otion Crisp & P.H.Weston *ined.*	*O. microphyllum* (Benth.) Crisp & P.H.Weston *ined.*	MDC8970	AF113782[b]	AF113764[b]
	Oxylobium Andrews	*O. cordifolium* Andrews	MDC9133	AF518159	AF518117
		O. ellipticum (Vent.) R.Br.	MDC9092	AF113784[b]	AF113766[b]
		O. robustum Joy Thomps.	ANBG8700488	AY015104[b]	AY015221[b]
	Phyllota (DC.) Benth.	*P. phylicoides* (Sieber ex DC.) Benth.	MDC9048	AF113785[b]	AF113767[b]
	Podolobium R.Br.	*P. aciculiferum* F.Muell.	GTC606	AF518160	AF518118
		P. alpestre (F.Muell.) Crisp & Weston	ANBG9219976	AY015106[b]	AY015223[b]
		P. ilicifolium (Andrews) Crisp & Weston	MDC8392	AY015107[b]	AY015224[b]
		P. scandens (Sm.) DC.	MDC9128	AY015109[b]	AY015226[b]
	Pultenaea Sm.	*P. arida* E.Pritz.	JC6272	AF518162	AF518120
		P. daphnoides Wendl.	GAUBA22264	AF113786[b]	AF113768[b]
		P. dentata Labill.	MDC9053	AF113787[b]	AF113769[b]
		P. ericifolia Benth.	MDC8451	AF113788[b]	AF113770[b]
	Sphaerolobium Sm.	*S. medium* R.Br.	MDC8942	AF518136	AF287660[b]
		S. minus Labill.	MDC9154	AF518135	AF518101
		S. nudiflorum (Meisn.) Benth.	RB891	AF518137	AF518102
	Urodon Turcz.	*U. dasyphyllus* Turcz.	MDC8523	AF113792[b]	AF113774[b]
	Viminaria Sm.	*V. juncea* (Schrad.) Hoffmanns.	MDC8935	AF518134	AF287664[b]

[a] ANBG = Australian National Botanic Gardens (cultivated), RB = Rhyonen Butcher, GTC = Greg Chandler, JC = Jenny Chappill, MDC = Mike Crisp, PF = Paul Fryxell, GAUBA = Gauba Herbarium, Australian National University, KH = Katarzyna Hempel, TL = Terena Lally, ML = Mike Lazarides, AM = Anna Monro, JMT = Joan Taylor

[b] Sequence produced previously in this lab

[c] Sequence obtained from GenBank

The NJ analysis was run on the full data set using the LogDet transformation to correct for non-stationarity (Lockhart *et al.*, 1994). Initially, the proportion of invariant sites was determined using ModelTest (I = 0.2126), then re-estimated from the NJ tree through several iterations until stable (I = 0.3100).

Inference of Ancestral Embryo Sac Types

All lineages within the *Daviesia* group and Bossiaeeae were inferred to have GA embryology as the ancestral state (Figs 1–2). This is the only type found in 33 species sampled across most genera in these groups (Cameron and Prakash, 1994). NA embryo sacs were inferred as ancestral in all lineages within the *Isotropis* + *Mirbelia* group clade (Figs 1–2) because this was the only type in 67 species examined across 18 genera (Cameron and Prakash, 1994). Ancestral states could not be inferred unambiguously for FNES and MES types, so only the terminal branches are mapped for species with known type (Figs 1–2). Both species of *Indigofera* are mapped as having GA embryo sacs, although only *I. australis* has been examined.

Results

Sequence Variation and Phylogenetic Analysis

The ITS alignment comprised 1119 sites (including offset unaligned regions), of which 429 were parsimony-informative. Base frequencies were relatively uniform (Table 2). There was significant (p = 0.0000) base composition bias (non-stationarity) among taxa (Table 3) which was slightly greater when uninformative sites were excluded. Within the ingroup, bias occurred between the embryo-sac groups (p = 0.0000), but not within them (p = 0.9999 in both). Base composition was the same in the *Daviesia* group (Mirbelieae) and Bossiaeeae (Table 3), both of which have giant antipodals. The MP search of the ITS data found > 20,000 trees (with uninformative positions excluded, CI = 0.33, RI = 0.59). The strict consensus tree (not shown) contained no well supported groups that conflicted with supported groups in the *trn*L data partition. The NJ analysis using the LogDet transformation to correct for non stationarity did not result in a different tree (not shown).

The *trn*L intron alignment comprised 1029 sites (including offset unaligned regions) of which 199 were parsimony informative. There was a strong A+T bias (Table 2) with A in the highest proportion (37%) but there was no base composition bias among taxa (p = 1.00). The MP search of the *trn*L intron data found > 20,000 trees (with uninformative positions excluded, CI = 0.50, RI = 0.73).

TABLE 2. Base composition (all sites) and distribution of parsimony informative sites among partitions.

Region	A	C	G	T	Base pairs	Parsimony informative		
						All taxa	Mirbelieae	Bossiaeeae
***trn*L intron**	0.37	0.17	0.17	0.29	1025	199	134	58
ITS	0.21	0.27	0.29	0.23	1119	429	303	115

TABLE 3. ITS base composition and tests for non-stationarity

Region	A	C	G	T	Probability (P)
All	0.21	0.27	0.29	0.23	0.0000
Outgroups	0.21	0.27	0.29	0.24	0.0000
Ingroup	0.21	0.27	0.29	0.23	0.0000
Bossiaeeae	0.23	0.23	0.26	0.28	0.9999
Daviesia group	0.23	0.23	0.26	0.28	0.9999
Giant antipodals group	0.23	0.23	0.26	0.28	0.9999
Mirbelia group	0.20	0.28	0.30	0.21	0.9999

A Mirbelieae + Bossiaeeae clade was recovered in both the ITS and *trn*L intron analyses. Bossiaeeae was monophyletic in both data sets but Mirbelieae was paraphyletic with respect to Bossiaeeae in the *trn*L intron analysis and polyphyletic in the ITS analysis (trees not shown). The data partitions were not incongruent (ILD test, p = 0.27) and were combined for further analysis. The MP search of the combined data found 1680 trees (with uninformative sites excluded, CI = 0.36, RI = 0.62). In the strict consensus tree, the Mirbelieae + Bossiaeeae comprised a weakly supported monophyletic group (Fig. 1) which was also recovered in the NJ LogDet analysis (Fig. 2). Bossiaeeae was strongly supported and well resolved in all analyses (Figs. 1–2). The *Daviesia* group and Bossiaeeae formed an unsupported clade in the NJ LogDet analysis (Fig. 2) but were part of a polytomy with the other Mirbelieae taxa in MP analyses (Fig. 1). Some genera in the *Daviesia* group (*Daviesia, Gompholobium* and *Sphaerolobium*) and the Bossiaeeae (*Goodia*) were well supported clades (Figs. 1–2). There was poor resolution among most of the *Daviesia* group genera except that *Erichsenia* and *Viminaria* formed a well-supported clade (Figs. 1–2).

Isotropis was sister to a strongly supported clade of the remaining non-*Daviesia* group Mirbelieae taxa (the *Mirbelia* group) (Figs. 1–2). Within the *Mirbelia* group, there was strong support for the monophyly of *Gastrolobium*, *Dillwynia*, *Jacksonia* and *Oxylobium* in MP and NJ analyses (Figs. 1–2). However, there was little or no support for some other currently recognised genera in the *Mirbelia* group (Figs. 1–2). *Chorizema* was paraphyletic with *C. carinatum* placed as sister to *Oxylobium*, and *Mirbelia* was rendered paraphyletic by the inclusion of *Callistachys* and two species of *Podolobium*. The other three species of *Podolobium* formed a separate clade. The clustering of some *Mirbelia* group taxa also differed among reconstruction methods. For example, *Euchilopsis linearis* was placed as sister to the rest of the *Mirbelia* group in MP analyses (Fig. 1) but within the group in the NJ LogDet analysis (Fig. 2).
The lack of supported resolution within the *Mirbelia* group is mainly the result of very short internal nodes at the base (e.g., Fig. 2). The genetic distance among *Mirbelia* group clusters is much less than that between other genera of the Mirbelieae and Bossiaeeae. However, LogDet distance variation within some *Mirbelia* group genera is similar to that within other genera (*Daviesia, Gompholobium, Goodia, Isotropis, Sphaerolobium*) (Table 4).

The ML analysis (not shown) did not provide any alternative, supported resolution. As in the MP and NJ analyses, Mirbelieae + Bossiaeeae formed a clade and Bossiaeeae was well supported but there was no supported resolution among the *Daviesia* group genera. Basal nodes in Mirbelieae + Bossiaeeae and in the *Mirbelia* group were very short.

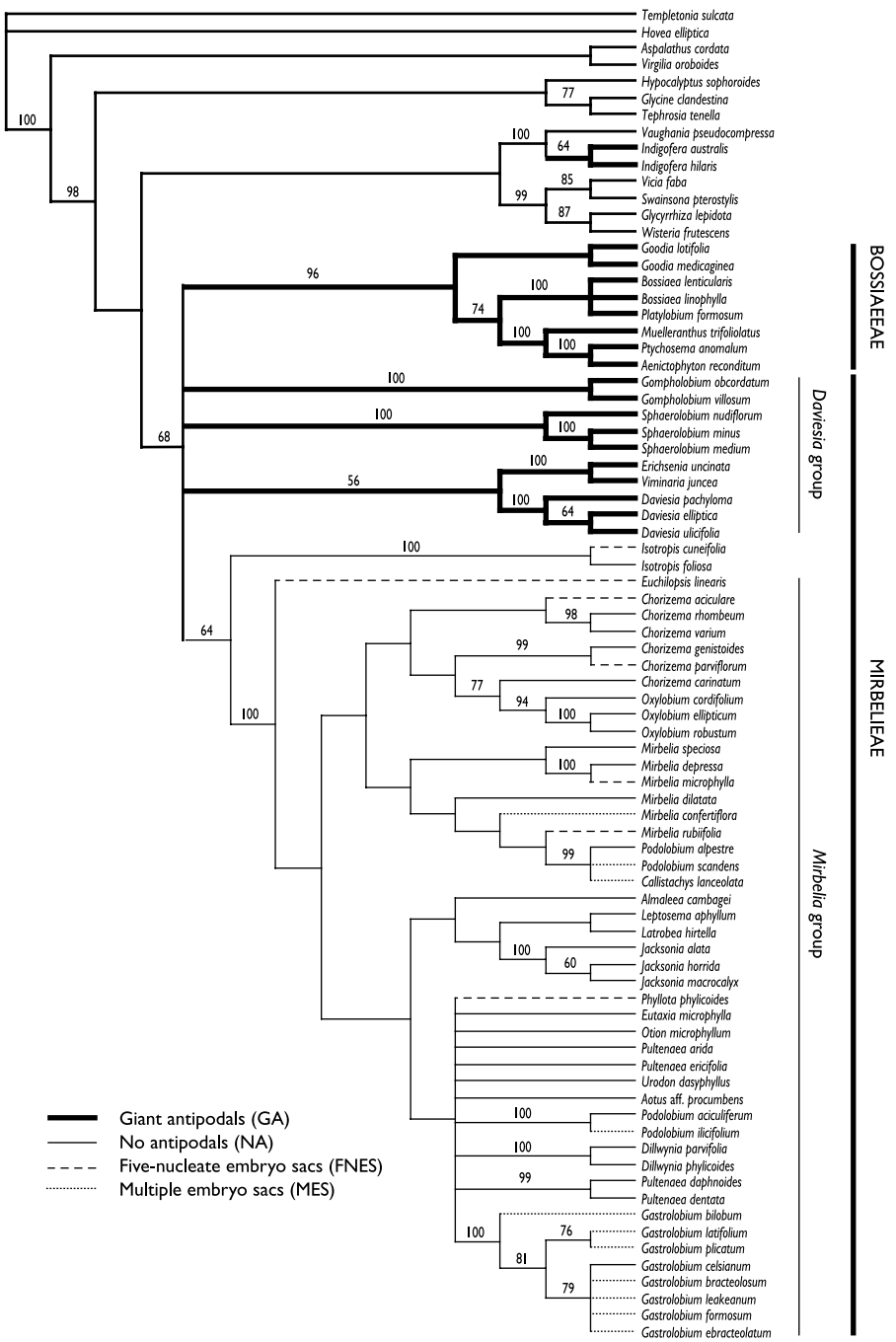


FIG. 1. Strict consensus of 1680 most parsimonious trees from combined ITS and *trnL* intron sequences (CI = 0.36, RI = 0.62). Numbers on branches are bootstrap scores from a 1000 replicate search.

Embryo sacs and phylogeny

Taxa with NA-type embryology were recovered as a monophyletic group in all analyses (Figs. 1–2). However, neither the FNES nor MES types formed a monophyletic group; instead, both types were scattered throughout the *Mirbelia* group + *Isotropis* clade.

Mirbelieae + Bossiaeeae taxa with GA embryology appeared as a monophyletic group in NJ LogDet analysis (Fig. 2) but as a polytomy in the MP analysis (Fig. 1) and paraphyletic in the ML analysis. All analyses placed *Indigofera* (giant antipodals) among the outgroup taxa (normal antipodals) rather than with the GA Mirbelieae + Bossiaeeae taxa (Figs. 1–2).

Discussion

Tribal Classification and Embryo Sac Evolution

It seems likely that giant antipodals have evolved at least twice amongst the taxa included in this study: once in the *Indigofera* clade and at least once in the Mirbelieae + Bossiaeeae clade. In this study, Indigoferae was placed not as sister to Mirbelieae + Bossiaeeae, but either in a clade with Hologalegina and the 'millettioid/phaseoloid' tribes (ML and NJ) or as sister to Hologalegina (MP). Although support for these relationships was weak, they have also been identified in previous studies (Hu, 2000; Wojciechowski *et al.*, 2000; Kajita *et al.*, 2001). Persistent, enlarged antipodals are uncommon but widespread among angiosperms and probably result from endopolyploidisation (d'Amato, 1984). It is therefore not surprising that GA may have evolved at least twice in this group of legumes. However, it remains uncertain whether the Mirbelieae + Bossiaeeae taxa with GA-type embryo sacs are monophyletic or paraphyletic. In contrast, there appears little doubt that taxa with NA type form a monophyletic group.

TABLE 4. Sequence divergences within genera

Taxa	Distance (range)*
***Mirbelia* group**	
Within genera	
Jacksonia	4–5
Dillwynia	2
Gastrolobium	1–2
Chorizema	3–8
Oxylobium	1–3
Other Mirbelieae/Bossiaeeae	
Within genera	
Daviesia	6
Gompholobium	8
Sphaerolobium	4–16
Goodia	3
Isotropis	3

* LogDet: % mean substitutions over all sites of combined data; 31% of sites assumed to be invariant

Reconstructing the path of embryo sac evolution depends upon resolution of relationships in the Bossiaeeae and Mirbelieae. A paraphyletic GA group containing the NA group (some MP and ML trees; not shown) implies that embryo sacs with giant antipodals gave rise to those lacking antipodals. In this case, the GA embryology may have played a role in the evolution of the anomalous NA-type embryology. If the GA and NA groups are monophyletic sister taxa (e.g., Fig. 2), then the two embryo sac types may have evolved independently in each lineage, or there may have been a sequence from one to the other in their common ancestor. It seems unlikely that, in a large family with almost universal presence of normal *Polygonum*-type embryology (Prakash, 1987), originations of two different types in the same clade were independent events, unless their common ancestor had evolved a predisposition to new kinds of development.

It is likely that the FNES type is plesiomorphic for the NA-type group. This form occurs in many *Mirbelia* group taxa (Cameron and Prakash, 1994) and in the sister taxon to the *Mirbelia* group, *Isotropis* (Figs. 1–2). However, the evolutionary relationships of FNES and MES embryology remain unclear because the internal relationships of the *Mirbelia* group are poorly resolved. Taxa with MES embryology do not form a clade (Figs. 1–2) and some monophyletic genera such as *Dillwynia* and *Jacksonia* have both types (Cameron and Prakash, 1994). It therefore appears likely that MES may have evolved more than once in the *Mirbelia* group although reversible changes between FNES and MES cannot be discounted. Comparison of embryo sac development in the well-distinguished sister taxa *Isotropis* and the *Mirbelia* group may give some insight into the evolution of NA embryo sacs.

In this study, Mirbelieae was paraphyletic with respect to Bossiaeeae (see also Crisp *et al.*, 2000). Although Bossiaeeae is a strongly supported monophyletic group, it is either nested within Mirbelieae (ML and NJ) or unresolved at its base (MP). If taxa within Mirbelieae and Bossiaeeae fall into two monophyletic sister groups which can be defined by embryo sac type (GA or NA) (e.g., Fig. 2), then the tribes may need to be re-circumscribed accordingly. Bossiaeeae could be expanded to include the *Daviesia* group genera, and Mirbelieae could be restricted to genera lacking antipodals (*Isotropis* and the *Mirbelia* group). However, a taxonomic change should not be made unless monophyly of the GA group is confirmed.

Diversification and Generic Delimitation in the *Mirbelia* Group

There is very little structuring within the *Mirbelia* group, probably reflecting a rapid diversification into many lineages at the base of the crown group. In contrast to the strong support for monophyly of genera in the *Daviesia* group and the Bossiaeeae, few *Mirbelia* group genera are supported here. In addition, apparent synapomorphies for many genera are homoplastic with respect to other *Mirbelia* group taxa and thus circumscription is primarily based on combinations of characters, rather than unique features (Crisp and Weston, 1987). This introduces a taxonomic problem because many currently recognised genera cannot be supported or resolved with the currently available data.

One solution to the poor differentiation among genera within the *Mirbelia* group might be to maintain only the well supported monophyletic genera (e.g., *Dillwynia*, *Gastrolobium*, *Jacksonia*, and *Oxylobium*). However, recognition of only these taxa would result in a paraphyletic residue within the *Mirbelia* group. To maintain monophyletic genera, the remainder would have to be assigned to an increased number of smaller genera. Already there are five genera with five or fewer species (*Almaleea, Euchilopsis, Oxylobium, Stonesiella* and *Urodon*), and only recently the monotypic *Jansonia* was sunk into *Gastrolobium* (Chandler *et al.*, 2002). This appears to be an unnecessary over-splitting of the group and, if genera within the Bossiaeeae and Mirbelieae are to be equivalent, should also be applied to species-groups within *Daviesia*, *Bossiaea* and others.

Given that the level of genetic differentiation within the *Mirbelia* group is approximately equivalent to that present in Bossiaeeae and *Daviesia* group genera

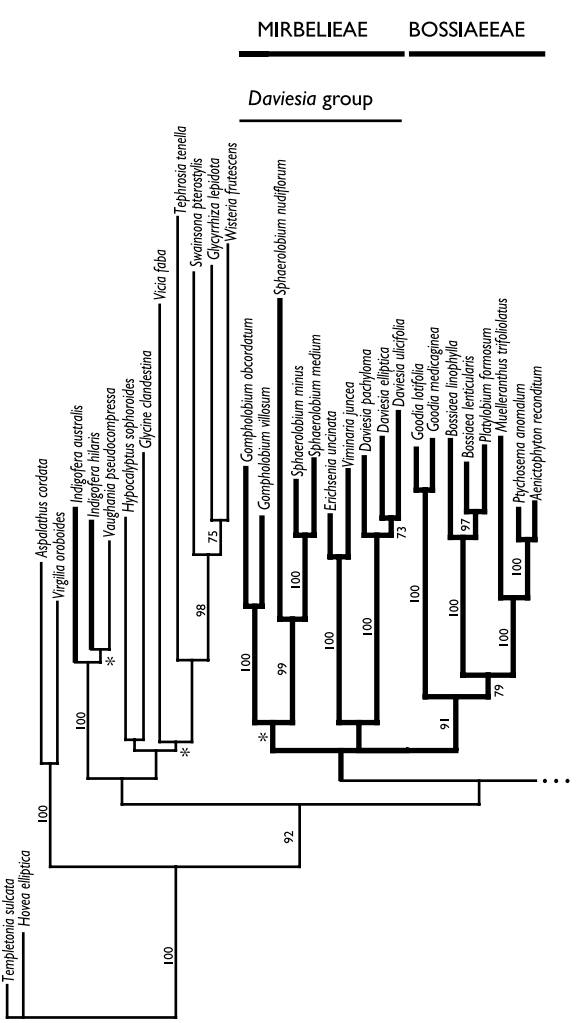
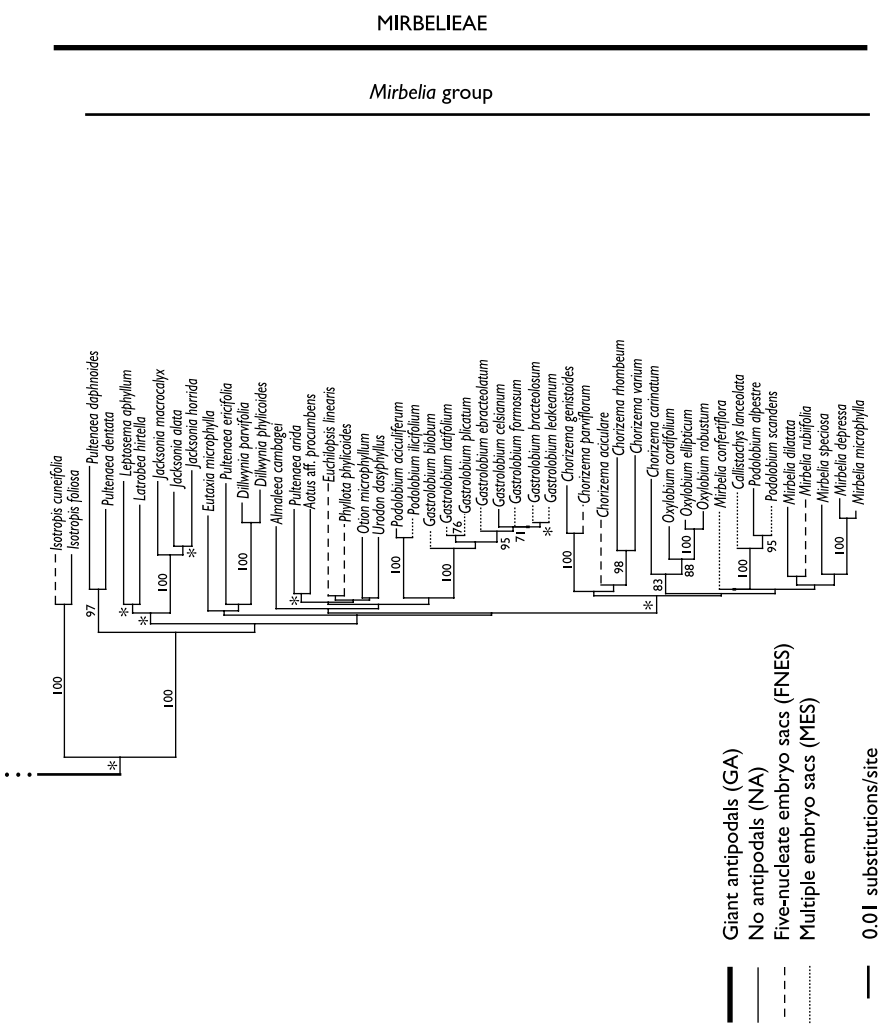


Fig. 2. Phylogram found by Neighbor Joining analysis of same terminals as in Fig. 1 using a Log Det model to correct for non-stationarity. Numbers on branches are bootstrap scores from a 1000 replicate search. * 50 ≤ BS ≤ 69.


MIRBELIEAE
Mirbelia group
Isotropis cuneifolia
Isotropis foliosa
Pultenaea daphnoides
Pultenaea dentata
Leptosema aphyllum
Latrobea hirtella
Jacksonia macrocalyx
Jacksonia alata
Jacksonia horrida
Eutaxia microphylla
Pultenaea ericifolia
Dillwynia parvifolia
Dillwynia phylicoides
Almaleea cambagei
Pultenaea aridа
Aotus aff. procumbens
Euchilopsis linearis
Phyllota phylicoides
Orion microphyllum
Urodon dasyphyllus
Podolobium acuciferum
Podolobium ilicifolium
Gastrolobium bilobum
Gastrolobium latifolium
Gastrolobium plicatum
Gastrolobium ebracteolatum
Gastrolobium calcicum
Gastrolobium formosum
Gastrolobium bracteolosum
Gastrolobium leakeanum
Chorizema genistoides
Chorizema parviflorum
Chorizema aciculare
Chorizema rhombeum
Chorizema varium
Chorizema carinatum
Oxylobium cordifolium
Oxylobium ellipticum
Oxylobium robustum
Mirbelia coriifolia
Calistachys lanceolata
Podolobium alpestre
Podolobium scandens
Mirbelia dilatata
Mirbelia rubifolia
Mirbelia speciosa
Mirbelia depressa
Mirbelia microphylla
100
97
100
100
100
100
100
100
100
95
71
100
98
83
100
88
100
95
100
Giant antipodals (GA)
No antipodals (NA)
Five-nucleate embryo sacs (FNES)
Multiple embryo sacs (MES)
0.01 substitutions/site

(Table 4), it may be appropriate to treat the *Mirbelia* group as a single genus. In particular, it would then be equivalent to its sister taxon, the genus *Isotropis*. Although the number of species would be large (c. 450), this is much smaller than other Australian plant genera such as *Eucalyptus* and *Acacia*. The oldest generic name in the *Mirbelia* group, which should be adopted if it were treated as a single genus, is *Pultenaea*. In its current circumscription, this genus has more than 100 species.

Conclusion

Monophyly of the Australian tribal group Mirbelieae + Bossiaeeae is supported, albeit weakly, by this study. However, recognition of Mirbelieae as distinct from Bossiaeeae is unsustainable. Alternatively, it may be possible to achieve monophyletic tribes by treating the giant antipodals group (Bossiaeeae + the *Daviesia* group) as an expanded Bossiaeeae, and the no antipodals group (*Isotropis* + the *Mirbelia* group) as a reduced Mirbelieae. However, stronger evidence for monophyly of the giant antipodals group should be sought before adopting this option. The sequence of embryo sac evolution is unclear, however it seems likely that originations of the unusual types in Bossiaeeae and Mirbelieae are linked. The multiple embryo sac (MES) type was probably derived more than once from the five-nucleate (FNES) type. Longstanding problems in differentiating genera within the *Mirbelia* group have not been resolved by the large new set of data presented in this study. It may be preferable to reduce the entire group to a single genus, for which the correct name would be *Pultenaea*.

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