Oxylobium/Gastrolobium (Fabaceae : Mirbelieae) conundrum: further studies using molecular data and a reappraisal of morphological characters

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Abstract

A molecular phylogeny of *Oxylobium*, *Gastrolobium* and related genera, including *Brachysema*, *Jansonia*, *Nemcia* and *Podolobium,* is presented. The study was conducted using five molecular regions and 48 taxa, utilizing chloroplast DNA (the *trn*K 5′ intron, *psb*A/*trn*H intergenic spacer, *trn*L intron and adjacent *trn*L/*trn*F intergenic spacer) and nuclear ribosomal DNA (ETS and ITS) markers. *Oxylobium* is shown to be polyphyletic, while *Gastrolobium* is paraphyletic, containing within it the genera *Brachysema*, *Jansonia* and *Nemcia*, as well as *Oxylobium lineare*. This concurs with a previous molecular analysis and the results of the two analyses are compared. Morphological traits, such as subtending bracts, ovule number and fluoroacetate content, are shown to be homoplastic and suites of characters for defining the different genera using different morphological characters are presented. Our results support a recent monograph that expands *Gastrolobium* to include *Brachysema*, *Jansonia*, *Nemcia* and *Oxylobium lineare*. This revision leaves *Oxylobium* and *Podolobium* occurring exclusively in eastern Australia, while *Gastrolobium* occurs almost exclusively in south-western Australia, with only two species, *G. brevipes* and *G. grandiflorum*, occurring outside this area.

Keywords: Fabaceae, *Gastrolobium*, Mirbelieae, molecular phylogeny, *Oxylobium*.

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Introduction

The tribe Mirbelieae (Fabaceae) is endemic to Australia and comprises a major component of the flora in many of the continent's temperate ecosystems. Most genera in the Mirbelieae exhibit a typical, bee-pollinated papilionoid flower with yellow and red markings and all but one of the *c*. 35 genera (*Gompholobium* Sm.) have simple leaves or phyllodes.

Two problematic genera in this tribe are *Oxylobium* Jacks. and *Gastrolobium* R.Br. Described as a monotypic genus by Jackson (1807), *Oxylobium* is traditionally defined by opposite or whorled phyllotaxis and the possession of more than two ovules. This became the basis of classification used by many authors (e.g. de Candolle

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1825; Bentham 1837, 1839, 1864; Turczaninow 1853; Gardner & Bennetts 1956) until Crisp and Weston (1987, 1995) reviewed the tribe using morphological cladistic analyzes, redefining many genera. *Oxylobium* sensu lato (s.l) appears to be a heterogeneous genus in many morphological features and has perhaps been a dumping ground for taxa with plesiomorphic character states (see taxonomic history, Fig. 1). *Gastrolobium* is traditionally defined as possessing strictly two ovules and a stipitate ovary, the latter of which is shared by numerous genera in the tribe (e.g. Brown 1811; Bentham 1864; Crisp & Weston 1987, 1995; Chandler *et al*. 2001, 2002).

Prior to recent revisions, both *Oxylobium* and *Gastrolobium* contained species that produced toxic sodium monofluoroacetic acid, known commercially as Compound 1080, which is widely used in Australia to bait vertebrate pests such as foxes and cats (Twigg & King 1991). *Oxylobium* was redefined by Crisp & Weston (1987) to contain only non-toxic species, with the toxic species transferred to *Gastrolobium*. The non-toxic species of *Oxy-*

	Ventenat 1805	Jackson 1807	Brown 1811	De Candolle 1825	Bentham 1837, 1839	Turczaninow 1853	Bentham 1864	Kuntze 1891	Domin 1923	Crisp & Weston 1987	Crisp & Weston 1995	Chandler et al. 2002
	Callistachys	*Callistachys*	*Callistachys*	*Callistachys*	*Callistachys*	*Callistachys*		*Callistachys*		*Callistachys*	*Callistachys*	*Callistachys*
		Oxylobium	*Oxylobium*	*Oxylobium*			*Oxylobium*		*Oxylobium*	*Oxylobium*	*Oxylobium*	*Oxylobium*
			Brachysema	*Brachysema*	*Oxylobium*	*Oxylobium*					*Brachysema*	*Gastrolobium*
			Gastrolobium	*Gastrolobium*	*Brachysema*	*Brachysema*				*Brachysema*	*Gastrolobium*	
					Gastrolobium	*Gastrolobium*			*Brachysema*	*Gastrolobium*		
							Brachysema					
									Gastrolobium			
							Gastrolobium	*Brachysema*				
						Jansonia	*Jansonia*	*Jansonia*	*Jansonia*	*Jansonia*	*Jansonia*	
									Nemcia	*Nemcia*	*Nemcia*	
			Podolobium	*Podolobium*	*Podolobium*	*Podolobium*					*Podolobium*	*Podolobium*

Fig. 1 Taxonomic history and classification systems of *Gastrolobium*, *Oxylobium*, and related genera. The latest is the classification system of Chandler *et al*. (2001). The relative sizes of genera are shown; shaded areas show groups that were not included and also show how many taxa have been added over time.

lobium from Western Australia, except *O. lineare*, were transferred to the genus *Nemcia* Domin, which left *Oxylobium* occurring along the east coast and adjacent tablelands of eastern Australia and Tasmania, and *O. lineare* in Western Australia.

The genera *Callistachys* Vent., *Gastrolobium*, *Podolobium* F. Muell., *Nemcia*, *Oxylobium lineare*, *Brachysema* R.Br. and *Jansonia* Kipp. Ex Lindl. comprise the 'Callistachys group' of Crisp and Weston (1995). This group was supported by the presence of filiform stipules, trifid bracts (*Gastrolobium* having entire and trifid bracts) and multiple embryo sacs (Cameron & Prakash 1990, 1994). The analysis of Chandler *et al*. (2001) did not support the existence of the 'Callistachys group', instead finding three other distinct groups: the *Gastrolobium* group, the *Oxylobium* group and the *Podolobium ilicifolium* group. Several of these genera, *Brachysema*, *Jansonia*, *Gastrolobium (*1 sp.) and *Nemcia,* contain putatively bird-pollinated species and up until Chandler *et al*. (2001), these were considered separate lineages in which bird pollination had arisen independently. Very little pollination biology has been reported for these groups (see Keighery 1980, 1982, 1984), so much of the evidence for entomophily is based on morphological features typical of this pollination syndrome, including a reduced standard petal, red or green flowers, an enlarged keel petal and the presence of nectaries. Chandler *et al*. (2001) found that these genera fall inside *Gastrolobium*, and the bird-pollinated lineages represented by *Brachysema*, *Jansonia* and the bird-pollinated species of *Nemcia* may form a single lineage within *Gastrolobium*.

Phylogenetic Analysis

Based on morphological cladistic analyzes, species of *Oxylobium* have been transferred to *Gastrolobium*, *Nemcia* and *Podolobium* (Crisp & Weston 1987, 1995). In a molecular phylogenetic analysis, Chandler *et al*. (2001) demonstrated that *Gastrolobium* is paraphyletic, containing within it the genera *Brachysema*, *Jansonia* and *Nemcia*, along with *Oxylobium lineare*. This study will test the relationships of *Oxylobium* sensu Crisp & Weston (1995), particularly in regard to *Podolobium*, which may be polyphyletic (Chandler *et al*. 2001). We use sequences from the *psb*A/*trn*H intergenic spacer and *trn*K 5′ intron from cpDNA and the external transcribed spacer (ETS) from nrDNA [as used by Chandler *et al*. (2001)] and sequences from the *trn*L intron and *trn*L/*trn*F intergenic spacer from cpDNA and the internal transcribed spacer (ITS) region from nrDNA [as used by Crisp *et al*. (1999, 2000)].

Additionally, we re-examine the morphological features used in past classifications, including ovule number, subtending bract type and pollination syndrome, for taxonomic and phylogenetic utility, as well as examining other characters for use in classifying genera, including trichome type, bracteoles, leaf tubercles and embryological characters.

Materials and Methods

A total of 48 taxa were used in this analysis and vouchers of all specimens were deposited at the Australian National Herbarium (Canberra) (Table 1). Authorities for all specific epithets are provided in Table 1. Three outgroups were selected based on the analysis of the genistoid legume tribes by Crisp *et al*. (2000) and the analysis of the Mirbelieae and Bossiaeeae by Crisp and Weston (1995). *Isotropis cuneifolia* was used to root the tree, as this genus occurs at the base of the '5-nucleate embryo-sac clade' of Crisp *et al*. (2000), while other outgroups included *Jacksonia horrida* and *Pultenaea dentata*. Five of six species were sampled from *Oxylobium* in the restricted

Table 1 List of taxa used in the analysis, with their collector voucher information and GenBank accession numbers.

Taxon*trnL/trnF*	Voucher	*trnK*	5'	*psbA/trnH*	3'-ETS	ITS
Brachysema bracteolosum	F.Muell. G.T. Chandler 426	AF298424	AF298330	AF298236	AY015063	AY015180
Brachysema celsianum	Lem. M.D. Crisp 9009	AF298425	AF298331	AF298237	AY015064	AY015181
Brachysema latifolium	R.Br. G.T. Chandler 365	AF298426	AF298332	AF298238	AY015065	AY015182
Brachysema melanopetalum	F.Muell. M.D. Crisp 8470	AF298427	AF298333	AF298239	AY015066	AY015183
Brachysema minor	Crisp M.D. Crisp 8922	AF298428	AF298334	AF298240	AY015067	AY015184
Brachysema modestum	Crisp M.D. Crisp 8465	AF298429	AF298335	AF298241	AY015068	AY015185
Brachysema praemorsum	Meisn. G.T. Chandler 729	AF298430	AF298336	AF298242	AY015069	AY015186
Brachysema sericeum	Domin J.M. Taylor 1959	AF298431	AF298337	AF298243	AY015070	AY015187
Brachysema subcordatum	Benth. M.D. Crisp 8511	AF298432	AF298338	AF298244	AY015071	AY015188
Callistachys lanceolata	Vent. G.T. Chandler 474	AF298433	AF298339	AF298245	AY015072	AY015189
Gastrolobium bilobum	R.Br. G.T. Chandler 724	AF298437	AF298343	AF298249	AY015073	AY015190
Gastrolobium brownii	Meisn. G.T. Chandler 726	AF298438	AF298344	AF298250	AY015074	AY015191
Gastrolobium calycinum	Benth. G.T. Chandler 544	AF298440	AF298346	AF298252	AY015075	AY015192
Gastrolobium congestum	G.Chandler & Crisp G.T. Chandler 404	AF298441	AF298347	AF298253	AY015076	AY015193
Gastrolobium cuneatum	Henfr. M.D. Crisp 8937	AF298443	AF298349	AF298255	AY015077	AY015194
Gastrolobium grandiflorum	F.Muell. G.T. Chandler 598	AF298448	AF298354	AF298260	AY015078	AY015195
Gastrolobium parviflorum (Benth.)	Crisp G.T. Chandler 760	AF298458	AF298364	AF298270	AY015079	AY015196
Gastrolobium pusillum	Crisp & P.H.Weston M.D. Crisp 8921	AF298461	AF298367	AF298273	AY015080	AY015197
Gastrolobium spinosum	Benth. G.T. Chandler 548	AF298469	AF298375	AF298281	AY015081	AY015198
Gastrolobium truncatum	Benth. M.D. Crisp 8919	AF298478	AF298384	AF298290	AY015082	AY015199
Isotropis cuneifolia	Heynh. M.D. Crisp 8459	AF298481	AF298387	AF298293	AY015083	AY015200
Jacksonia horrida	DC. M.D. Crisp 8934	AF298482	AF298388	AF298294	AY015084	AY015201
Jansonia formosa	Kippist ex. Lindl. M.D. Crisp 8933	AF298483	AF298389	AF298295	AY015085	AY015202
Mirbelia depressa	E.Pritz. M.D. Crisp 9020	AF298485	AF298391	AF298297	AY015086	AY015203
Mirbelia dilatata	R.Br. M.D. Crisp 8491	AF298486	AF298392	AF298298	AY015087	AY015204
Nemcia alternifolia	ms. M.D. Crisp 8512	AF298487	AF298393	AF298299	AY015088	AY015205
Nemcia coriacea	Domin G.T. Chandler 723	AF298488	AF298394	AF298300	AY015089	AY015206
Nemcia hookeri (Meisn.)	Crisp M.D. Crisp 8907	AF298491	AF298397	AF298303	AY015090	AY015207
Nemcia leakeana (J.Drumm.)	Crisp M.D. Crisp 8481	AF298492	AF298398	AF298304	AY015091	AY015208
Nemcia luteifolia	Domin M.D. Crisp 9407	AF298493	AF298399	AF298305	AY015092	AY015209
Nemcia obovata (Benth.)	Crisp G.T. Chandler 657	AF298494	AF298400	AF298306	AY015093	AY015210
Nemcia plicata (Turcz.)	Crisp G.T. Chandler 623	AF298495	AF298401	AF298307	AY015094	AY015211
Nemcia pulchella (Turcz.)	Crisp M.D. Crisp 8480	AF298496	AF298402	AF298308	AY015095	AY015212
Nemcia pyramidalis (T.Moore)	Crisp G.T. Chandler 488	AF298497	AF298403	AF298309	AY015096	AY015213
Nemcia reticulata	Domin G.T. Chandler 540	AF298498	AF298404	AF298310	AY015097	AY015214
Nemcia spathulata (Benth.)	Crisp M.D. Crisp 8448	AF298501	AF298407	AF298313	AY015098	AY015215
Nemcia vestita	Domin M.D. Crisp 8489	AF298502	AF298408	AF298314	AY015099	AY015216
Oxylobium arborescens	R.Br. G.T. Chandler 616	AF298504	AF298410	AF298316	AY015100	AY015217
Oxylobium ellipticum	R.Br. G.T. Chandler 603	AF298505	AF298411	AF298317	AY015101	AY015218
Oxylobium lineare (Benth.)	Benth. M.D. Crisp 8471	AF298506	AF298412	AF298318	AY015102	AY015219
Oxylobium pulteneae	DC. M.D. Crisp 9046	AF298507	AF298413	AF298319	AY015103	AY015220
Oxylobium robustum	Joy Thomps. I.R. Telford 4294	AF298508	AF298414	AF298320	AY015104	AY015221
Podolobium aestivum	Crisp & P.H.Weston G.T. Chandler 612	AF298511	AF298417	AF298323	AY015105	AY015222
Podolobium alpestre (F.Muell.)	Crisp & P.H.Weston G.T. Chandler 1039	AF298512	AF298418	AF298324	AY015106	AY015223
Podolobium ilicifolium (Andr.)	Crisp & P.H.Weston G.T. Chandler 308	AF298513	AF298419	AF298325	AY015107	AY015224
Podolobium procumbens (F.Muell.)	Crisp & P.H.Weston B. Hadlow 461	AF298514	AF298420	AF298326	AY015108	AY015225
Podolobium scandens	DC. G.T. Chandler 309	AF298515	AF298421	AF298327	AY015109	AY015226
Pultenaea dentata	Labill. M.D. Crisp 9053	AF298516	AF298422	AF298328	AY015110	AY015227

All specimens are housed at Canberra.

sense of Crisp and Weston (1995), as well as 5/6 species of *Podolobium*, while two out of 40 species from the closely related *Mirbelia* Sm. were sampled. Within the 'Callistachys group' of Crisp and Weston (1995), 10/60 species of *Gastrolobium* were used, as were 12/40 species of *Nemcia*, 9/10 species of *Brachysema*, plus *Jansonia formosa* and *Callistachys lanceolata* (both monotypic), and *Oxylobium lineare*. It was considered that this sample should be sufficient to test the monophyly of each genus, as well as evaluate the results from the analysis of Chandler *et al.* (2001).

Total DNA from fresh or preserved tissue was isolated as outlined in Bayer *et al.* (1996) while the protocol of Gilmore *et al.* (1993) was used to isolate DNA from herbarium specimens. All regions were amplified via the polymerase chain reaction (PCR) using *Taq* DNA polymerase. See Chandler *et al.* 2001) for PCR and cycle-sequencing protocols for the *psb*A/*trn*H intergenic spacer, the *trn*K 5′ intron and ETS regions, and Crisp *et al.* (1999, 2000) for the *trn*L/*trn*F intergenic spacer and the *trn*L intron and ITS.

Sequences were assembled using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI), then manually aligned following the principles of non-coding sequence alignment (Golenburg *et al.* 1993). Indels were placed where they minimized the number of inferred length mutations, unless clear evidence was seen for non-homologous length mutations. Sequence data were analyzed using PAUP 4.0b10 (Sinauer, Sunderland, MA) (Swofford 2001). The data matrix contained 45 ingroup taxa and three outgroup taxa. Parsimony analysis was performed on unweighted characters by heuristic searches with simple addition of taxa, with TBR branch swapping and MULPARS in effect. An island search was employed to search for the most parsimonious trees, with a random addition sequence of 100 replicates using a heuristic search (Maddison 1991). Decay analysis (Bremer 1988) and bootstrapping (Felsenstein 1985) were used to estimate the robustness of clades. The decay analysis was facilitated by the program AutoDecay (Eriksson 1998), and visualized by using the tree-drawing package Tree-View (Page 1996). Bootstrapping was performed using 500 replicates using the same parsimony criteria as for the heuristic search. Constraint analyses were also performed on the data using PAUP, testing the monophyly of the groups that were found through the analysis. An incongruence-length difference (ILD) test was performed (implemented as the partition homogeneity test in PAUP 4.0b10) to test for data set incongruence (Farris *et al.* 1994). Comparative morphological data were taken from the data matrices of Crisp and Weston (1987, 1995) and supplemented from the monograph of Chandler *et al.* (2002).

Results

Sequence characteristics

The combined sequences yielded a total 2750–3109 bp of unaligned sequence data (see Table 2). The ETS has the fewest indels (five, single-base pair indels) and also the shortest aligned length; the ITS also has relatively few, single-base indels. The *psb*A/*trn*H spacer has a low mean length (356 bp), but contains many indels, which led to an aligned length that is almost double that of the mean. Gaps ranged in size from 2 to 241 bp, with many occurring in areas of alignment ambiguity, which were excluded from the analysis. The *trn*K 5′ intron, *trn*L/*trn*F spacer and *trn*L intron contained a moderate number of indels, though only the *trn*K 5′ intron contained a significant number that were coded unambiguously, ranging in length from 5 to 20 bp; gaps not coded were excluded. Unambiguous indels were scored as binary characters.

Sequence divergence in the combined matrix, based on pairwise distances, varies from 3.3% between *Nemcia hookeri* and *N. obovata* to 19.2% between *Gastrolobium brownii* and *Isotropis cuneifolia*. Other sequence divergence rates of note include a c. 5.5% difference between the two *Podolobium* groups; *Callistachys lanceolata* is c. 3.5% different to the *Podolobium alpestre* group, but is c. 7.0% divergent from the *P. ilicifolium* group; species of *Gastrolobium sensu* Crisp and Weston (1995) are 2.0–4.5% divergent, while the *Nem-*

Table 2 Sequence characteristics of the DNA regions sequenced in this study. NA = not applicable

Sequence characteristic	*psb*A/*trn*H	*trn*K 5′	ETS	ITS	*trn*L/F	Combined
Length range (bp)	180–414	816–1016	315–345	560–573	622–906	2750–3109
Length mean (bp)	356	897	339	568	811	2960
Aligned length (bp)	604	1355	356	597	1042	3954
G + C content mean	29.5%	33%	58%	59%	33%	NA
Sequence divergence (%)	3.27–19.17	0.00–21.36	0.00–20.20	0.00–28.44	0.35–19.10	0.10–19.10
Number of variable sites	328	466	233	229	369	1469
Number of potentially informative sites	192	237	142	125	137	674
Number of unambiguously coded indels	5	11	0	0	0	16
Coded indel size range (bp)	2–241	5–20	NA	NA	NA	2–241
Ratio of coded indels to potentially informative sites	1 : 38	1 : 22	NA	NA	NA	1 : 42

cia species differ by 0.7–5.0% and the infraspecific rate between species of Brachysema ranges from 1.0 to 3.0%.

Phylogenetic reconstruction

A heuristic search of all nucleotide characters and scored indels produced four equally parsimonious trees of 2107 steps [excluding uninformative characters; consistency index (CI) = 0.48; retention index (RI) = 0.64; Fig. 2, including bootstrap and decay results]. The partition homogeneity test indicates that the chloroplast and nuclear data sets are combinable ($P = 0.07$) and the results of constraint analyses are summarized in Table 3. Forcing *Oxylobium* sensu Bentham (1864) to be monophyletic [including *Oxylobium* sensu Crisp & Weston (1995), *O. lineare*, *G. parviflorum* and *N. reticulata*] adds 173 steps to the tree; a monophyletic *Podolobium* would increase the length of the tree by 45 steps, while *Callistachys + Podolobium* adds a further 14 steps; constraining *Gastrolobium* sensu Crisp and Weston (1995) to be monophyletic required an additional 18 steps. Excluding either *Brachysema*, *Jansonia*, *Nemcia* or *Oxylobium lineare* from *Gastrolobium* sensu lato adds from 41 to 56 steps to the tree. Forcing *Brachysema* to be monophyletic adds 23 steps and constraining the bird pollinated group (Clade J + *B. latifolium*) needs another 15 steps, though if the large, orange-petaled species of *Nemcia* are included in this group (*N. coriacea* and *N. pyramidalis*; Clade H), just an additional 11 steps are required.

Selected morphological characters from the analyses of Crisp and Weston (1987, 1995) are traced onto the tree (Fig. 3). The two most important characters used in the past (bract shape and ovule number) are shown diagrammatically on the tree, while other characters are shows as

Table 3 Constraint analyzes performed on the 48 taxon data set, and the number of additional steps needed to obtain the desired topology (original tree length = 2107 steps)

Constraint	Tree length	Extra steps
Oxylobium sensu Bentham (1864)	2280	173
Gastrolobium s.s. monophyletic	2125	18
Gastrolobium s.l. excluding *O. lineare*	2131	56
Gastrolobium s.l. excluding *Brachysema*	2148	41
Gastrolobium s.l. excluding *Nemcia*	2157	50
Gastrolobium s.l. excluding *Jansonia*	2163	56
Brachysema monophyletic	2130	23
B-P† group (including *B. latifolium*) monophyletic	2122	15
B-P species (Clade H +*B. latifolium*) monophyletic	2119	11
Podolobium monophyletic	2152	45
Podolobium + Callistachys monophyletic	2121	14

†Bird-pollinated

numbered bars. MacClade (Sinauer, Sunderland MA) (Maddison & Maddison 2000) was used to map the characters parsimoniously.

The major clades are designated by letters shown on the strict consensus tree (Fig. 2). Clade A [Decay (D) = 4, synapomorphies (SYN) = 42, Bootstrap (BS) = 64] contains *Callistachys*, *Mirbelia*, *Oxylobium* and the *Podolobium alpestre* group. Clade B (D = 44, SYN = 70, BS = 100) comprises the *Podolobium ilicifolium* group. Note that *Podolobium* is separated into two strongly supported clades.

The *Gastrolobium* group makes up Clade C (D = 27, SYN = 47, BS = 100) and contains *Gastrolobium sensu* Crisp & Weston (1987), *Brachysema*, *Jansonia*, *Nemcia* and *O. lineare*. Ovule numbers of these taxa range from two to 21. The most basal position within Clade C is occupied by a single species, *G. pusillum*, which has four to 10 ovules. Clade D (D = 3, SYN = 20, BS = 60) is made up of a group of 'core' *Gastrolobium* species (*G. spinosum*, *G. cuneatum*, *G. bilobum*, *G. congestum*, *G. grandiflorum* and *G. parviflorum*), and the ovule number in this group ranges from two to five (mostly two), while the subtending floral bracts are all entire, except *G. parviflorum*, which has both entire and trifid bracts. Four species of *Nemcia* with morphological traits intermediate between *Gastrolobium* and *Nemcia* (Crisp & Weston 1987), form Clade E (D = 21, SYN = 25, BS = 65) and includes *N. hookeri*, *N. obovata*, *N. plicata* and *N. spathulata*. Ovule number is strictly two and the subtending floral bracts are all trifid.

Gastrolobium brownii and *G. truncatum* comprise Clade F (D = 5, SYN = 16, BS = 67), which have strictly two ovules, but these species have both entire and trifid bracts present on a given plant or inflorescence (often being entire at the base of the inflorescence and becoming trifid apically). Clade G (D = 2, SYN = 9, BS = 29) is made up of *N. alternifolia*, *N. reticulata* and *Oxylobium lineare*, which vary in ovule number from two to 18, but all have trifid bracts. Clade H (D = 5, SYN = 10, BS = 27) has two subgroups within it and contains species of *Nemcia* with large, orange petals (*N. coriacea* and *N. pyramidalis*), plus the red-petaled species of *Nemcia* (*N. leakeana*, *N. luteifolia* and *N. vestita*), *Jansonia* and all but one species (*B. latifolium*) of *Brachysema*. Within this clade, Clade I (D = 1, SYN = 3, BS = 50) contains the large, orange-petaled species of *Nemcia*, with two to four ovules and trifid bracts; and Clade J (D = 1, SYN = 3, BS = 34), contains the red-petaled, putatively bird-pollinated group of species from *Brachysema*, *Jansonia* and *Nemcia*, which vary from two to 21 in ovule number, and all of which have trifid bracts.

Discussion

This analysis supports the narrow circumscription of *Oxylobium* and the transfer of *O. lineare* into Gastrolobium. The 'core' species of *Oxylobium* included in this analysis
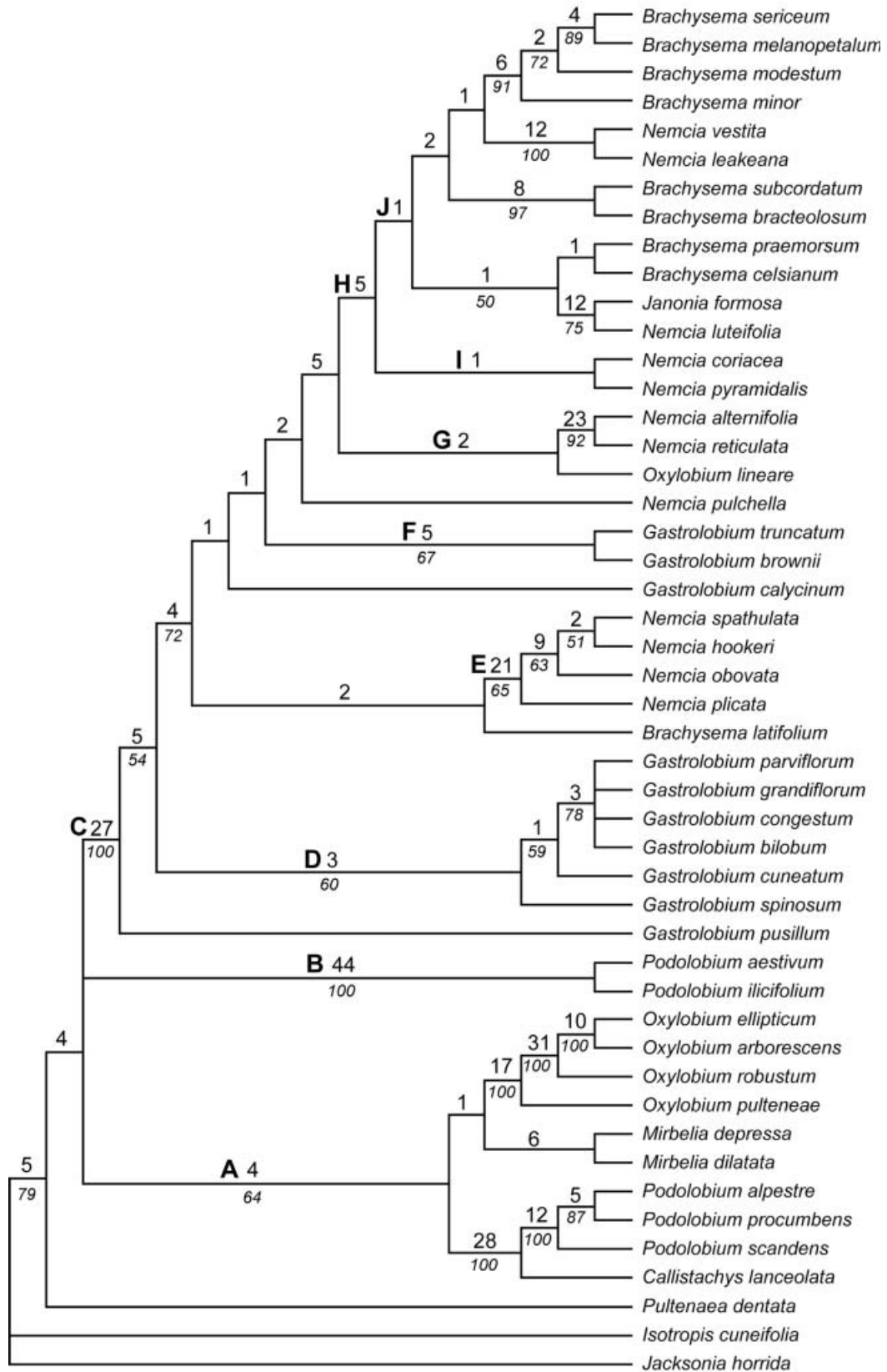
Fig. 2 Strict consensus tree of four trees, length 2107 steps. The major clades are marked A to J and are discussed in the text. Decay values are given above the line and bootstrap values are below the line and in italics. CI = 0.48, RI = 0.64.
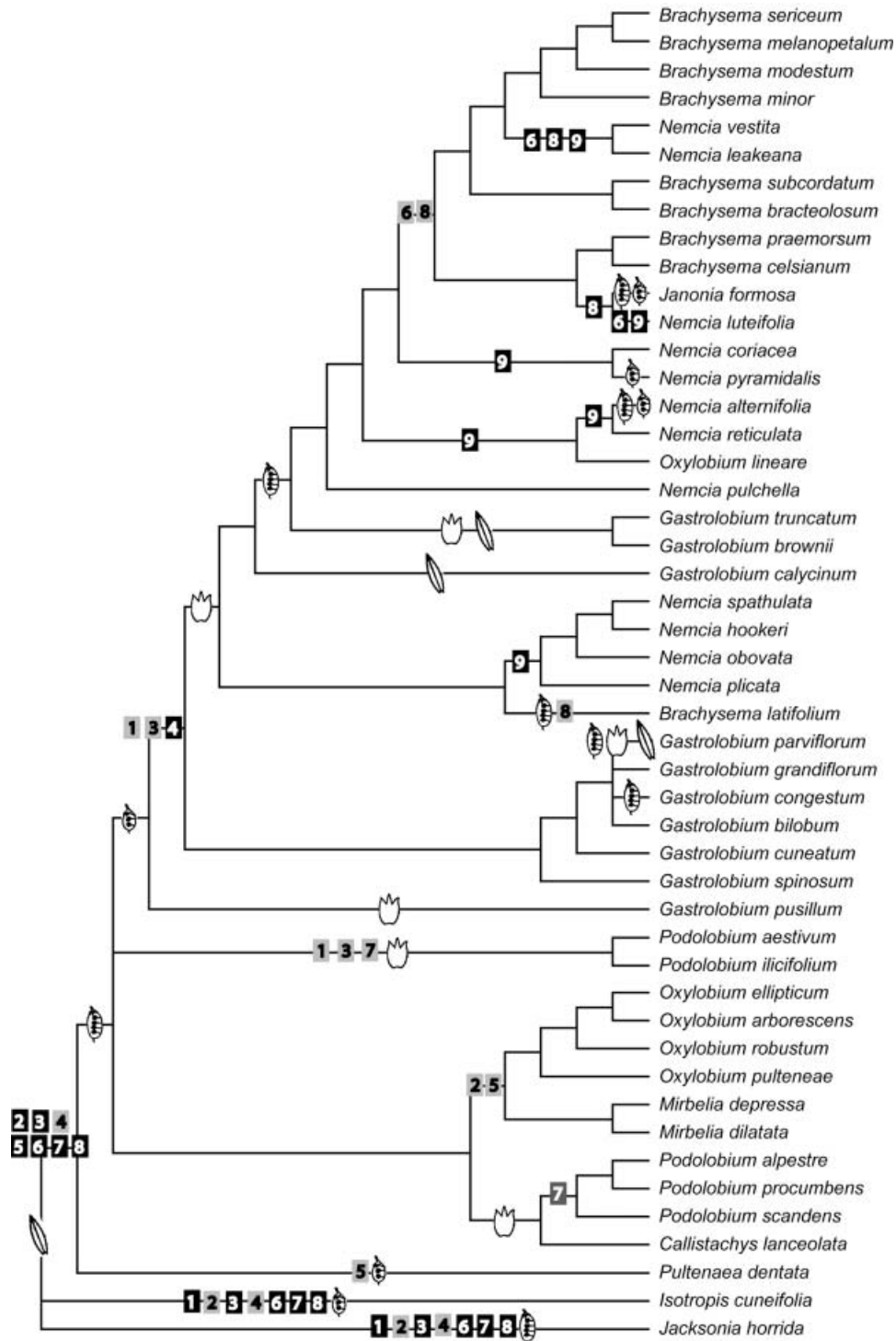
Fig. 3 Morphological characters of Crisp and Weston (1987, 1995) mapped onto the strict consensus tree. Bract shape (entire leaf = entire bracts, trifid leaf = trifid bracts) and ovule number (legume with two ovules = strictly two ovules, legume with four ovules = strictly more than two ovules) are shown diagramatically to emphasize these characters considered important in past analyses, while nine other characters are shown as bars with internal numbers (unless otherwise indicated, black bars indicate a character state is present, and light gray bars indicate a character state is absent): 1 = bracteoles (present or absent); 2 = stipules (present or reduced/absent); 3 = leaf tubercles (present or absent); 4 = aril (lobed or not lobed); 5 = multiple embryo sacs (black bar) or *Aotus*-type embryo sacs (light gray bar); 6 = standard petal prominent (black bar) or reduced (light gray bar); 7 = peltate hairs absent (black bar), present with two prominent arms (light gray bar) or present with one arm reduced to a lateral bulge (deep gray bar); 8 = cup-shaped nectaries (present or absent); 9 = *Nemcia*-type inflorescence (indicated where present only).

(*O. arborescens*, *O. ellipticum*, *O. pulteneae* and *O. robustum*) form a strongly supported clade (D = 17, SYN = 29, BS = 100), which is weakly sister to *Mirbelia*. *Oxylobium lineare* is placed well within *Gastrolobium* s.l., seemingly related to several species formerly in *Nemcia* (Clade G), though this is weakly supported, and constraint analyses show that excluding *O. lineare* from *Gastrolobium* adds 56 steps to the analysis, strongly supporting its inclusion in *Gastrolobium*. *Podolobium* is split into two clades, as suggested by Chandler *et al.* (2001). The *P. alpestre* group is sister to *Callistachys lanceolata* and together they form a strongly supported clade (D = 28, SYN = 51, BS = 100), while the *P. ilicifolium* group (Clade B) is part of a trichotomy, which includes the *Oxylobium* group (Clade A) and the *Gastrolobium* group (Clade C). This suggests that *Podolobium* should be split, the *P. alpestre* group being submerged into *Callistachys* (the presence of bracteoles uniting it with *Oxylobium s.s*) and the *P. ilicifolium* group (which includes the type of the genus) remaining as *Podolobium* s.s., a group that lacks bracteoles (shared with *Gastrolobium* s.l). *Nemcia* is polyphyletic within *Gastrolobium* s.l. and several taxa that were considered to be intermediate in morphology between *Gastrolobium* and *Nemcia* (*N. hookeri*, *N. obovata*, *N. plicata* and *N. spathulata*; Crisp & Weston 1987, 1995) occupy an intermediate position on the tree (Clade H, Fig. 2).

Reassessment of morphological characters

Traditionally, *Oxylobium* has been defined by its opposite or whorled phyllotaxis, but this is not an unequivocal synapomorphy for the genus. Morphological characters used by Crisp and Weston (1987, 1995) to delineate *Oxylobium* (with the exception of *O. lineare*, which differs in all of these features except for possessing more than four ovules) include a spirally arranged phyllotaxis (shared with *Podolobium*); the presence of leaf tubercles (shared with *Mirbelia*); stipules that are reduced or absent (except for *O. robustum* Joy Thomps.); entire subtending bracts (shared with most species of *Gastrolobium*); the presence of bracteoles (shared with *Callistachys* and *Podolobium*); an *Aotus*-type embryo sac (Cameron & Prakash 1990, 1994); and an unlobed aril [shared with *Callistachys* and the *Podolobium alpestre* group]. *Oxylobium lineare* shares most of its features with the 'Callistachys group' of Crisp and Weston (1995), including filiform stipules, branched inflorescences, bracteoles lacking, trifid bracts, a multiple embryo sac and a lobed aril. This illustrates the complexities involved in defining *Oxylobium*, as most of its features are shared with other genera.

Crisp and Weston (1987) expanded upon the circumscription of *Nemcia* by Domin (1923) (four to six ovules, trifid subtending bracts and condensed, racemose inflorescences), by transferring the non-toxic species of *Gastrolobium* and *Oxylobium* into *Nemcia*, so that *Nemcia* included species with reduced racemes (one or two flowers in the leaf axils or on short, axillary shoots), and nonstipitate ovaries, in addition to those characters of Domin (1923).

*Podolobium s.*l. is distinguished from *Oxylobium* by possessing peltate hairs and rigid, recurved or patent stipules (Crisp & Weston 1995). *Podolobium* shares with *Gastrolobium* sensu Crisp and Weston (1987) campanulate calyces with recurved lobes, but, unlike *Gastrolobium*, only occurs in eastern Australia (sympatric with *Oxylobium*). The *Podolobium alpestre* group (*P. alpestre*, *P. procumbens*, and *P. scandens*) shares characters with *Callistachys*, many of which are jointly shared with *Oxylobium*. The presence of bracteoles, however, is not an unequivocal synapomorphy for *Podolobium*, as the *P. ilicifolium* group lacks bracteoles (*P. aciculiferum*, *P. aestivum* and *P. ilicifolium*), while they are present in the *P. alpestre* group. Using the topology-dependent permutations tests of Faith (1991), Crisp and Weston (1995) found that *Podolobium* is more closely related to *Gastrolobium* than to *Oxylobium*, while the analysis of Chandler *et al.* (2001) showed that *Podolobium* falls into two separate groups, with the *Podolobium ilicifolium* group more closely related to *Gastrolobium* than to *Oxylobium*.

Brachysema and *Jansonia*, together with *Nemcia* and *Gastrolobium grandiflorum*, represent four genera that include bird-pollinated species. In a morphological cladistic analysis, Crisp (1994) concluded that these represent independent lineages that contain bird-pollinated species. Various orthophilous characters were shown to ally *Brachysema* and *Jansonia*, including a reduced standard petal, and red or green petals (Crisp & Weston 1987, 1995), but recent molecular work has caste doubt on this conclusion, including Chandler *et al.* (2001), who concluded that these lineages are all included within *Gastrolobium*. A major question that needs to be addressed is whether the bird-pollinated species all belong to the same lineage, or whether there is convergence among several independent lineages.

Ovule number, particularly the distinction between two and more than two ovules, has long been considered as fundamental in classifying the Mirbelieae, starting with Bentham (1837) and continuing until Crisp and Weston (1987), primarily because many genera in the tribe have strictly two ovules, though *Mirbelia*, *Jacksonia* R. Br. ex Sm. and *Leptosema* have long been accepted with two to several ovules (Crisp 1982; Crisp & Weston 1987). Within *Gastrolobium*, two species (*G. congestum* G. Chandler & Crisp and *G. appressum* C.A. Gardner) have two to three ovules, as does *Nemcia alternifolia* ms., while *Jansonia formosa* has between two and five. When ovule numbers are

mapped onto the tree, it becomes clear how homoplastic this character is (Fig. 3). All the species of *Oxylobium* s.s and *Podolobium* have more than two ovules, but *Gastrolobium* s.l. (Clade C) contains taxa with ovule number from two to more than 20. This character, while useful for the identification of species or species groups, does not appear to be phylogenetically informative at higher levels, though the overall trend within *Gastrolobium* appears to be towards more numerous ovules (Fig. 3).

The type of subtending floral bract has also been used as a subsidiary character at the generic level, with bracts being classified as entire or trifid (Fig. 3). *Gastrolobium parviflorum*, transferred by Crisp and Weston (1987) from *Oxylobium*, illustrates the variability of this character as it possesses inflorescences in which the lower bracts are trifid and the upper bracts are entire, a feature also shared by *G. brownii* and *G. truncatum*. A number of species of *Gastrolobium* and *Oxylobium* with trifid bracts were transferred into the reinstated and expanded *Nemcia*, leaving most species in the reduced *Gastrolobium* and all those in *Oxylobium s.*s., with entire bracts. Therefore, it appears that the type of subtending floral bract is homoplastic and of limited phylogenetic utility, though useful for diagnosing species groups.

While these more traditional defining characters do not appear to be overly useful at the generic level, other characters are. We can use a suite of characters for defining genera, rather than trying to find unequivocal synapomorphies, which may be very difficult to define. The *Gastrolobium* group (Clade C) can be defined by the absence of bracteoles, a lack of leaf tubercles and a lobed aril on the seed. The *Podolobium ilicifolium* group (*Podolobium* s.s) may be defined by the absence of bracteoles and leaf tubercles and the presence of two-armed, peltate hairs on the leaf, which differs from the *P. alpestre* group (*Callistachys* s.l)(which has bracteoles and leaf tubercles) and has modified peltate hairs that have one arm reduced to a lateral bulge. *Oxylobium* has bracteoles and leaf tubercles, leaf hairs that are not peltate, reduced or absent stipules (except for *O. robustum*), entire bracts and an *Aotus*-type embryo sac.

A reduced standard petal and the presence of nectaries (possible indicators of bird pollination; see Crisp (1994); Keighery (1980, 1982, 1984) may have arisen only once, though the topology of the tree shows two subsequent reversals, but the weak support in this part of the tree rends any hypothesis tentative. *Nemcia*-type inflorescence (Crisp & Weston 1987, 1995) was a character used to attempt to define the inflorescence structure of some species of the resurrected *Nemcia* that have trifid bracts and reduced racemes with only a few flowers. However, not all species of *Nemcia* actually possess this character and it otherwise seems to be very homoplastic.

Comparative analysis of Gastrolobium

Our results largely concur with those found by Chandler *et al.* (2001), though the sister group affinity of Gastrolobium to the *Podolobium ilicifolium* group is not supported, with the *P. ilicifolium* group (Clade B, Fig. 2), the *Oxylobium* group (Clade A) and *Gastrolobium* s.l. (Clade C) forming a polytomy (Fig. 2). Current work in the tribe shows that this is not an unusual pattern in the tribes Mirbelieae and Bossiaeeae (Crisp *et al.* 2000; M.D. Crisp, unpubl. data). Strongly supported genera or infrageneric groups are often found, yet the relationships between these clades are often tenuous, perhaps indicating a starburst radiation in this group, followed by intense speciation. Both the present study and that by Chandler *et al.* (2001) have found that *Oxylobium* s.s., *Callistachys* s.l (including. the *Podolobium alpestre* group), the *P. ilicifolium* group and *Gastrolobium* s.l. are well supported, but the interrelationships of these groups remain unclear. Within *Gastrolobium* s.l., *Nemcia* is polyphyletic (spread between Clades E, G, I and J), while Brachysema is paraphyletic (Clade J, with *B. latifolium* sister to Clade E).

More deeply nested within *Gastrolobium* s.l. is a clade of putatively bird pollinated species (Clade J), although this group has very low support (D = 1, BS = 34). This includes *Brachysema* (except *B. latifolium*), *Jansonia* and the red petaled species of *Nemcia* (*N. leakeana*, *N. luteifolia* and *N. vestita*). It had been thought that these genera represented multiple origins of bird pollination (see Crisp 1994) and even though support for this group in both the current analysis and that of Chandler *et al.* (2001) is low, it provides an alternate hypothesis for the future examination of the evolution of bird pollination in the Mirbelieae. Unfortunately, little of the pollination biology is known for these species, with few published reports documenting visitations by insects, birds or otherwise (e.g. Keighery 1980, 1982, 1984), making it difficult to draw evolutionary conclusions from our study, since we do not have any clear idea of the true pollination biology, e.g. whether these species are bird-pollinated, insect-pollinated, or some combination.

Brachysema itself is paraphyletic, with *B. latifolium* placed near a small group of *Nemcia* species (Fig. 2). This was an unexpected result, much as it was in the analysis of Chandler *et al.* (2001), as it shares with the rest of *Brachysema* various vegetative and floral characteristics, such as a reduced standard petal and red petals, and is similar vegetatively and in habit to *B. minor* Crisp (see Chandler *et al.* 2002). Multiple accessions of *B. latifolium* were sequenced from across its range and morphologies (not presented); all of these sequences are practically identical and cluster together and away from the rest of *Brachysema*. Constraining *Brachysema* to include *B. latifolium* adds 23

more steps to the tree and even constraining the so-called bird-pollinated clade to be monophyletic (so as to include *B. latifolium*) adds 15 steps. Further work is needed to examine the underlying cause of this placement.

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