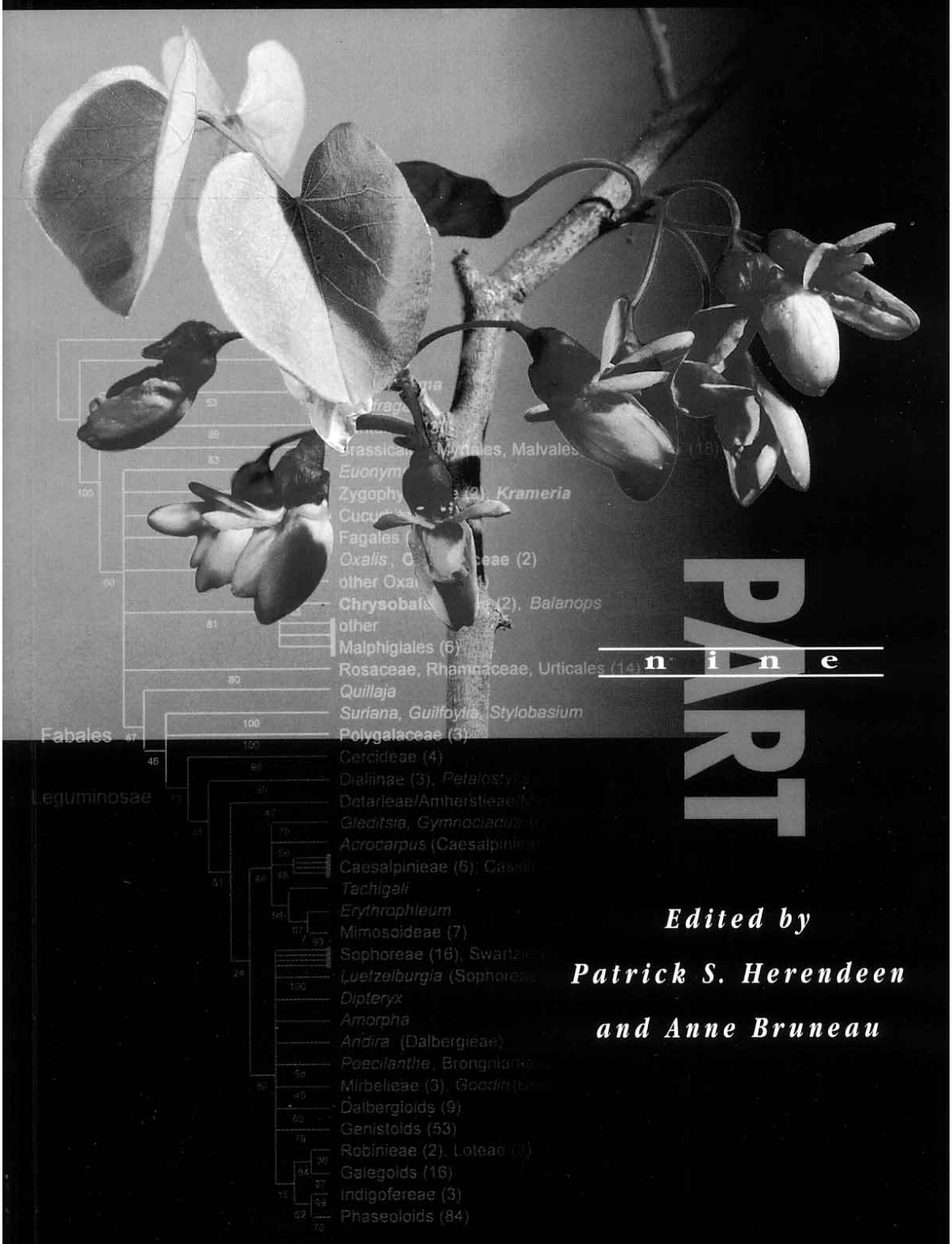
ADVANCES IN Legume Systematics



PART nine

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MOLECULAR PHYLOGENETICS OF *ACACIA* (FABACEAE: MIMOSOIDEAE) BASED ON THE CHLOROPLAST *TRNK/MATK* AND NUCLEAR HISTONE H3-D DNA SEQUENCES

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Abstract

The tribe Acacieae Benth. (Fabaceae: Mimosoideae) contains two genera, the monotypic African *Faidherbia* A. Chev. and the pantropical *Acacia* Mill., which comprise about 1200 species with over 950 confined to Australia. *Acacia* is subdivided into three subgenera: subg. *Acacia*, subg. *Aculeiferum* Vassal, and the predominantly Australian subg. *Phyllodineae* (DC.) Seringe. Previous morphological studies have suggested the tribe Acacieae and genus *Acacia* are artificial and that some taxa may be more closely related to taxa of the tribe Ingeae Benth. Sequence analysis of 33 Acacieae, eight Ingeae, and two Mimoseae species from the chloroplast *trn*K/*mat*K and nuclear Histone H3-D regions, presented here, indicate that the tribe Acacieae and genus *Acacia* are not monophyletic. At least three distinct lineages within *Acacia* are evident corresponding to the three recognised subgenera, with the monophyly of subg. *Aculeiferum* equivocal as the subgenus is paraphyletic in this analysis. Subgenus *Acacia* is basal and shows high affinity to taxa in tribe Mimoseae Bronn. The data suggest that the Ingeae is paraphyletic and that it is closely related to *Acacia* subg. *Phyllodineae*. The Ingeae genera and *Faidherbia* form a grade with subg. *Phyllodineae*. Within subg. *Phyllodineae* sectional classification is not supported by the data. A close relationship of the taxa of sect. *Botrycephalae* (Benth.) Taub., Australian taxa with bipinnately compound leaves, is seen with several taxa with uninerved phyllodes and racemose inflorescences.

Introduction

The genus *Acacia* Mill. is a cosmopolitan genus with over 1200 species (Maslin and Stirton, 1997). The majority of the species (950) are endemic to Australia with other centers of diversity in Africa and the New World. Along with the monotypic genus *Faidherbia* A. Chev., *Acacia* comprises the tribe Acacieae Benth. However, the tribe was originally described by Bentham (1842) as containing many taxa that are today referable to tribe Ingeae Benth., but he later (1875) restricted its definition to the two genera. The distinguishing characteristic of Acacieae, that of having free filaments of the stamens, is not maintained in all taxa with some having filaments shortly united at base (Vassal, 1981). The tribes have been considered closely related and taxonomic relationships remain unresolved within both tribes.

Three subgenera are commonly recognised within *Acacia* (Table 1). Subgenus *Acacia* and subgenus *Aculeiferum* Vassal, with over 120 and 180 species respectively, are

TABLE 1. A synoptic scheme of the classification of the Acacieae based on Vassal (1972) and Pedley (1978, 1986) as adopted in present paper. Under subgenus the parenthetical generic names are those adopted by Pedley (1986). [1]Number of species for the sections of subg. *Phyllodineae* is indicated in the parentheses. [2]For the purpose of this paper sections *Juliflorae* and *Plurinerve* have been divided into oligoneurous versus microneurous groups and [3]section *Phyllodineae* has been divided into racemose versus non-racemose groups following Maslin and Stirton (1997).

Genus	Subgenus	Section[1]
Faidherbia		
Acacia	subg. *Acacia* (*Acacia*)	
	subg. *Aculeiferum* (*Senegalia*)	sect. *Aculeiferum*
		sect. *Monacanthea*
		sect. *Filicinae*
	subg. *Phyllodineae* (*Racosperma*)	sect. *Alatae* (21)
		sect. *Botrycephalae* (42)
		sect. *Juliflorae*[2] (235)
		sect. *Lycopodifoliae* (17)
		sect. *Phyllodineae*[3](387)
		sect. *Plurinerve*[2] (212)
		sect. *Pulchellae* (27)

pantropical while subgenus *Phyllodineae* (DC.) Ser., with over 950 species, is mainly Australian (Ross, 1981; Maslin and Stirton, 1997). Subgenus *Acacia* has bipinnate leaves, stipular spines, colporate pollen with a smooth exine with columellae, whereas subgenus *Aculeiferum* has bipinnate leaves, no stipular spines, but may have 2–3 prickles near the stipules, and porate pollen with a smooth exine but without columellae (Vassal, 1981).

Subgenus *Phyllodineae* is the more diverse and variable of the subgenera. Most species have leaves reduced to vertically flattened phyllodes in a diverse range of sizes and shapes, but others have bipinnately compound leaves. They do not have prickles but can be spinescent, and have extraporate or porate pollen with the exine reticulate without columellae (Vassal, 1981). Among the phyllodinous taxa sections have been derived based on phyllode nervature and inflorescence structure (Pedley, 1978). While the sections may not be considered as natural groups (Pedley, 1986; Brain and Maslin, 1996; Chappill and Maslin, 1997) they form a useful framework for investigation (Table 1). Section *Phyllodineae* contains species with single-nerved phyllodes while sects. *Juliflorae* (flowers in spikes) and *Plurinerves* (Benth.) Maiden & Betche (flowers in heads) taxa have multinerved phyllodes. Within these plurinerved taxa differences can be noted between microneurous phyllodes (numerous, fine, longitudinal nerves) and oligoneurous phyllodes (few, distant, longitudinal nerves).

While there is growing agreement among researchers that the genus needs revision (Maslin and Stirton, 1997) there is little agreement about how this should be accomplished, especially with regard to the interrelationships of the three subgenera. However, the close relationship to Ingeae is evident. Pedley (1986) hypothesised that *Acacia* is polyphyletic, and suggested that subg. *Phyllodineae* is derived from subg. *Aculeiferum*. Pedley then suggests that the subgenera *Phyllodineae*/*Aculeiferum* lineage and the subg. *Acacia* had separate origins from the Ingeae.

A cladistic analysis of morphological characteristics presents a sister relationship between subgenera *Aculeiferum* and *Phyllodineae*, which is nested in the Ingeae separate from the subgenus *Acacia* (Chappill and Maslin, 1995). Alternatively, a cladistic analysis of floral development morphology suggests a close relationship of subgenera *Acacia* and *Aculeiferum* nested in the Ingeae separate from subg. *Phyllodineae* (Grimes, 1999). A cpRFLP study by Bukhari et al. (1999) indicates a sister relationship between subg. *Acacia* and subg. *Phyllodineae*, however this study included only Acacieae species so no inferences on the monophyly of the genus can be concluded.

The purpose of this study is to test the monophyly of the genus *Acacia* and the tribe Acacieae by the use of DNA sequence data from the chloroplast *mat*K/*trn*K region and the nuclear Histone H3-D sequence. Additionally these data will be used to study the phylogenetic interrelationships and intrarelationships of the *Acacia* subgenera.

Materials and Methods

Taxon sampling

The Acacieae ingroup taxa were selected based on a generic and infrageneric classification that outlined "a list of critical species on which to build a comparative data set" (Maslin and Stirton, 1997). This list describes morphological groups within each subgenus that could be used to systematically sample the large number of species in the tribe. Species were sampled from all three subgenera of *Acacia* (Table 2) and the monotypic *Faidherbia albida* was also included. Within the large subgenus *Phyllodineae* (over 950 species), five of the seven sections are sampled. Multiple species of each of the three large phyllodinous sections (*Phyllodineae*, *Juliflorae*, and *Plurinerves*) are included.

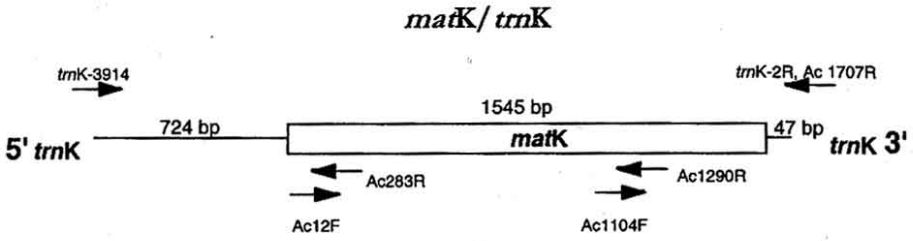
The outgroup selection was based on morphological evidence (Chappill and Maslin, 1995; Grimes, 1999). Seven genera from the Ingeae and two genera of the Mimoseae were included as outgroup taxa. A preliminary analysis indicated that the Ingeae taxa and *Neptunia monosperma* were ingroup taxa as they nested within the Acacieae in all analyses. *Mimosa tenuiflora* was used as the outgroup in subsequent analyses.

Seeds were acquired from various seed banks (Table 2), scarified, placed into a Petrie dish, and germinated at 25°C with 12 hours of light per day. The first true leaf was detached and pulverised in liquid nitrogen. DNA was extracted using a Plant DNAzol Reagent kit (GIBCOBRL Inc. Grand Island, New York).

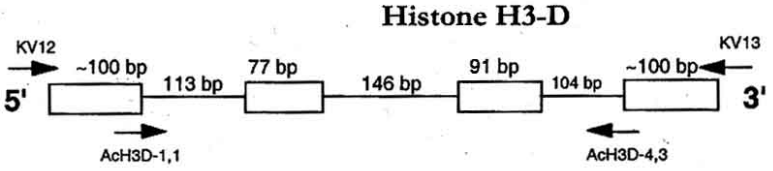
*trn*K/*mat*K

The cpDNA intron of the transfer RNA gene for lysine (*trn*K) contains the coding region for the maturase encoding gene (*mat*K) and flanking noncoding regions. The 1500 bp coding region for *mat*K has been found to evolve two-to-three times faster than *rbc*L (Johnson and Soltis, 1994; Plunkett et al., 1997). This *mat*K sequence has been mainly used in infrageneric studies while the flanking noncoding regions provide a potentially faster evolving sequence for lower level phylogenetic resolution.

The initial DNA amplification used the *trn*K-3914 and *trn*K–2R (Johnson and Soltis, 1994) and an *Acacia* specific primer (Ac1707R, Fig. 1) was created internal to *trn*K–2R and used in all subsequent PCR reactions. The *trn*K intron region was amplified via the polymerase chain reaction (PCR) using Taq DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, Connecticut.) The PCR reaction mixture consisted of 5 µL of 20X reaction buffer, 6 µL of 25 mmol/L magnesium chloride solution, 16 µL of a 1.25 mmol/L dNTP solution in equimolar ratio, 25 pmol of each primer, 10–50 ng of template DNA, and 1.0 unit of polymerase in a total volume of 100 µL. The PCR samples were heated to 94°C for 3 min prior to the addition of DNA polymerase to denature unwanted proteases and nucleases. The double-stranded PCR products were produced via 30 cycles of denaturation (94°C for 1 min), primer annealing (48°C for



trnK 3914[1]	GGG GTT GCT AAC TCA ACG G
trnK -2R[1]	AAC TAG TCG GAT GGA GTA G
Ac283R	CAC TGA CGG CAA GCC CCT CTG
Ac12F	GGT GCA (A/C)AA TCT AGG TTA TGA C
Ac1290R	AAT ACA AGA AAG CCG AAG
Ac1104F	CCT CTA ATT AGA TCA TTG GC
Ac1707R	TGC ACA CGG CTT TCC CTA TG



KV12[2]	ATG GCC CGC AC(C/G) AAG CAG AC
KV13[2]	AGC TGG ATG TCC TTG GGC AT
AcHisH3-1,1	ACT CGC CAC TAA GGT TTG TTT
AcHisH3-4,3	TGG AAA CGA AGG TCG GTC TG

1 min), and extension (72°C for 2 min). A 7 min final extension cycle at 72°C followed the 30th cycle to ensure the completion of all novel strands.

Double stranded PCR products were cleaned with the QIAquick PCR kit (QIAgen Inc. Hilden, Germany) and were sequenced using the dideoxy chain termination method with the use of the Big Dye Terminator RR Kit® and an ABI automated sequencer (Perkin-Elmer Applied Biosystems, Norwalk, Connecticut) at CSIRO, Plant Industry. An annealing temperature of 57°C was used for sequencing reactions. Initial sequences were generated with primer *trn*K-3914. Four *Acacia*-specific internal sequencing primers (Fig. 1) were designed based on initial sequence data. The cycle sequencing protocol followed manufacturer's instructions.

Histone H3-D

The Histone H3-D sequence is part of a multigene family. Most members of the family are intronless but this single-copy intron containing member has been shown to be phylogenetically useful. The intron sequences have been used successfully to produce gene trees in *Glycine* in order to investigate species complexes (Doyle et al., 1996, in press).

The Histone H3-D locus was amplified using the KV12 [5'-ATGGCCCGCAC (C/G)AAGCAGAC-3'] and KV13 (5'-AGCTGGATGTCCTTGGGCAT-3') primers of Kanazin et al. (1996). A single band of approximately 550 bp was amplified in several taxa, while multiple bands or no products were produced in other taxa. The single bands were sequenced and internal primers flanking the first intron/exon boundary (AcHisH3D-1,1) and the third intron/fourth exon boundary (AcHisH3D-4,3) were synthesised (Fig. 1). Amplification and sequencing of this Histone H3-D fragment followed the same procedure used for the *trn*K/*mat*K sequence except that an annealing temperature of 55°C was used for the PCR amplification. Some amplified products were cloned using the pGem®-T Easy Vector System II cloning kit (Promega Inc, Madison WI) and sequenced according to manufacturer's protocol using the vector primers.

Data analysis

Chromatographic traces and contiguous alignments were edited using Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, Michigan). The *mat*K coding region was determined by comparison to *Rosa persica* (Genbank number AB011974). Sequences were aligned manually with minimal gaps and base substitutions. The presence or absence of *trn*K/*mat*K indels were scored as separate characters. The *mat*K coding region and the flanking spacer region were analysed separately and the entire sequence analysed together. The Histone H3-D sequence was highly polymorphic and many small 1–2 bp insertions were used in the alignment. These indels were not scored as separate characters due to questionable homology. The data were analysed with no character weighting.

Maximum parsimony analyses were performed on the aligned sequences using the heuristic search option (excluding uninformative characters) in PAUP 4.02 (Swofford, 1999). A four-step search method for multiple islands was performed using 10000 random replicates (Olmstead and Palmer, 1994). Support for internal branches was evaluated by using the fast bootstrap method with 1000 replicates (Felsenstein, 1985). The incongruity indices I_M (Swofford, 1991) and I_{MF} (Mickevich and Farris, 1981) were used to test the congruity of the *trn*K/*mat*K and Histone H3-D datasets.

FIG. 1. Primers used in the study of phylogenetic relationships of *Acacia* and close relatives. (A.) Structure of the chloroplast *trn*K intron including the *mat*K coding sequence. Arrows above figure represent primers used for PCR and arrows below indicate sequencing primers. Primer *trn*K-3914 was used for both PCR and sequencing reactions. [1]Primers from Johnson and Soltis (1994). (B.) Structure of the nuclear Histone H3-D sequence. Arrows above figure represent primers used for PCR and arrows below indicate sequencing primers. [2]Primers from Kanazin et al. (1996).

TABLE 2. Sources of DNA used in this study. [1]ANBG = Australian National Botanic Gardens Canberra; ATSC = CSIRO Australian Tree Seed Centre, Canberra; DLEP = Boyce Thompson Desert Legume Program Tucson AZ; KP = King's Park Botanic Garden, Perth, OFI = Oxford Forestry Institute, QH = Queensland Herbarium. [2]All sequences have been deposited in Genbank. First number is the Histone H3-D sequence and the second is the *trn*K/*mat*K sequence.[3]Taxa are native to the New World. Seed taken of cultivated plants in U.S. [4]This taxon is native to the New World but is introduced in Australia.

Tribe	Subgenus	Section	Species	DNA Source[1]	Country of Origin	Voucher	Genbank Acc. #
Mimoseae			*Mimosa tenuiflora* (Willd.) Poir.	OFI 24/83	Honduras	CANB 615541	AF274167 AF274120
Mimoseae			*Neptunia monosperma* F. Muell. ex Benth.	ANBG 9102794	Australia	CANB 615542	AF274168 AF274209
Ingeae			*Albizia sinaloensis* (Vell.) Morong	DLEG 890413	Mexico	CANB 615543	AF274169 AF274121
Ingeae			*Cathormion umbellatum* (Vahl) Kosterm.	ANBG 9206371	Australia	CANB 615544	AF274170 AF274122
Ingeae			*Ebenopsis ebano* (Berland) R. C. Barneby & J. W. Grimes	DLEG 890179	New World cultivated[2]	CANB 615545	AF274171 AF274123
Ingeae			*Enterolobium contortisiliquum* (Vell.) Morong	DLEG 90113	Paraguay	CANB 615546	AF274172 AF274124
Ingeae			*Havardia pallens* Britton & Rose	DLEG 950001	Mexico	CANB 615547	AF274173 AF274125
Ingeae			*Albizia versicolor* Welw. ex Oliver	DLEG 950017	Zimbabwe	CANB 615548	AF274174 AF274210
Ingeae			*Pararchidendron pruinosum* (Benth.) I. C. Nielsen	ANBG 8200992	Australia	CANB 615549	AF274175 AF274127

TABLE 2 continued

Ingeae			*Paraserianthes lophantha* subsp. *lopantha* (Willd.) I. C. Nielsen.	ANBG 7901474	Australia	CANB 615550	AF274176 AF274128
Acacieae			*Faidherbia albida* (Delile) A. Chev.	OFI 138/94	Ethiopia	CANB 615551	AF274177 AF274129
Acacieae	*Acacia*		*A. caven* (Molina) Molina	DLEG910473	Argentina	CANB 615552	AF274178 AF274131
Acacieae	*Acacia*		*A. pennatula* (Cham. & Sch.) Benth.	DLEG 960002	New World cultivated[2]	CANB 615553	AF274179 AF274134
Acacieae	*Aculeiferum*	*Aculeiferum*	*A. senegal* (L.) Willd.	DLEG 910052	Zimbabwe	CANB 615554	AF274180 AF274143
Acacieae	*Aculeiferum*	*Filicinae*	*A. boliviana* Rusby	QH	New World cultivated[3]	CANB 615555	AF274181 AF274144
Acacieae	*Aculeiferum*	*Monacanthea*	*A. glomerosa* Benth.	DLEG 910150	Brazil	CANB 615556	AF274182 AF274147
Acacieae	*Aculeiferum*	*Monacanthea*	*A. schweinfurthii* Brenan & Exell	DLEG 950015	Zimbabwe	CANB 615557	AF274183 AF274211
Acacieae	*Phyllodineae*	*Botrycephalae*	*A. elata* A. Cunn. ex Benth.	ATSC 16673	Australia	CANB 615558	AF274184 AF274149
Acacieae	*Phyllodineae*	*Alatae*	*A. glaucoptera* Benth.	ATSC 15473	Australia	CANB 615559	AF274185 AF274217
Acacieae	*Phyllodineae*	*Botrycephalae*	A. leucoclada *Maslin*	ATSC 18067	Australia	CANB 615560	AF274186 AF274212
Acacieae	*Phyllodineae*	*Botrycephalae*	A. parramattensis *Tindale*	ATSC 17711	Australia	CANB 615561	AF274187 AF274150
Acacieae	*Phyllodineae*	*Botrycephalae*	*A. spectabilis* A. Cunn. ex Benth.	ATSC 12057	Australia	CANB 615562v	AF274188 AF274213

TABLE 2 continued

Acacieae	*Phyllodineae*	*Juliflorae* (microneurous)	*A. aulcocarpa* A. Cunn. ex Benth.	ATSC 13865	Australia	CANB 615563	AF274189 AF274214
Acacieae	*Phyllodineae*	*Juliflorae* (oligoneurous)	*A. colei* A. Cunn ex G.Don	KPBG 19920691	Australia	CANB 615564	AF274190 AF274215
Acacieae	*Phyllodineae*	*Juliflorae* (oligoneurous)	*A. leiocalyx* (Domin) Pedley	ATSC 14743	Australia	CANB 615565	AF274191 AF274216
Acacieae	*Phyllodineae*	*Juliflorae* (oligoneurous)	*A. lysiphloia* F. Muell. ex Benth.	ATSC 13769	Australia	CANB 615566	AF274192 AF274151
Acacieae	*Phyllodineae*	*Juliflorae* (oligoneurous)	*A. monticola* J. M. Black	ATSC 14609	Australia	CANB 615567	AF274193 AF274152
Acacieae	*Phyllodineae*	*Juliflorae* (oligoneurous)	*A. pachycarpa* F. Muell. ex Benth.	ATSC 15749	Australia	CANB 615568	AF274194 AF274153
Acacieae	*Phyllodineae*	*Phyllodineae* (non-racemose)	*A. rossei* F. Muell.	ANBG 7902341	Australia	CANB 615569	AF274195 AF274162
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose)	*A. binervata* DC.	ATSC 16245	Australia	CANB 615570	AF274196 AF274218
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose)	*A. cultriformis* A. Cunn. ex G.Don	ANBG 9607201	Australia	CANB 615571	AF274197 AF274219
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose)	*A. victoriae* Benth.	ATSC 15043	Australia	CANB 615572	AF274198 AF274226
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose) "bivenosa group"	*A. ligulata* A. Cunn.. ex Benth	ANBG 8210071	Australia	CANB 615573	AF274199 AF274155
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose) "microbotyra group"	*A. bancroftii* Maiden	ATSC 14756	Australia	CANB 615574	AF274200 AF274156

TABLE 2 continued

Acacieae	*Phyllodineae*	*Phyllodineae* (racemose) "microbotyra group"	*A. microbotrya* Benth.	ATSC 17649	Australia	CANB 615575	AF274201 AF274157
Acacieae	*Phyllodineae*	*Phyllodineae* (racemose) "microbotyra group"	*A. notabilis* F. Muell.	ANBG 8210199	Australia	CANB 615576	AF274202 AF274158
Acacieae	*Phyllodineae*	*Plurinerves* (microneurous)	*A. calcicola* Forde & Ising	ATSC 14033	Australia	CANB 615577	AF274203 AF274220
Acacieae	*Phyllodineae*	*Plurinerves* (microneurous)	*A. nuperrima* Baker f.	ATSC 16722	Australia	CANB 615578	AF274204 AF274164
Acacieae	*Phyllodineae*	*Plurinerves* (microneurous)	*A. suaveolens* (Sm.) Willd.	ATSC 10031	Australia	CANB 615579	AF274205 AF274221
Acacieae	*Phyllodineae*	*Plurinerves* (oligoneurous)	*A. melanoxylon* R. Br.	ATSC 17230	Australia	CANB 615580	AF274206 AF274166
Acacieae	*Phyllodineae*	*Plurinerves* (oligoneurous)	*A. platycarpa* F. Muell.	ANBG 8115716	Australia	CANB 615581	AF274207 AF274223
Acacieae	*Phyllodineae*	*Plurinerves* (oligoneurous)	*A. retivenea* F. Muell.	ATSC 13841	Australia	CANB 615582	AF274208 AF274224

TABLE 3. Nucleotide character statistics for the *trn*K/*mat*K and Histone H3-D regions in species of *Acacia* and relatives.

	5' *trnK* intron region	*matK*	3' *trnK* intron region	All *matK/trnK*	Histone H3-D Intron 1	Histone H3-D Exon 2	Histone H3-D Intron 2	Histone H3-D Exon 3	Histone H3-D Intron 3	All Histone H3-D	total
Aligned length (bp)	825	1557	87	2415	113	77	169	91	104	482	2958
Length, range (bp)	658–724	1506–1545	43–47	2232–2319	70–109	77	113–146	91	80–94	434–455	–
G+C content mean %	34.1	31.9	29.2	32.5	32.2	28.0	36.8	42.6	37.4	41.0	34.0
Mean divergence %	2.8 (0.3–12.5)	1.7 (0.1–8.3)	3.6 (0–15.4)	2.2 (0.1–7.0)	14.4 (1.0–46.8)	3.8 (0–14.3)	20.6 (1.3–53.8)	4.1 (0–12.1)	16.9 (0–44.2)	12.1 (1.3–29.5)	3.7 (0.5–9.1)
Variable sites (%)	21.3	25.4	36.2	23.3	76.1	22.1	86.2	25.3	80.0	60.1	29.4
Informative sites (%)	5.1	9.6	17.0	7.5	50.4	16.9	67.8	15.4	57.7	43.6	13.2
Constant sites (%)	78.7	74.6	63.8	76.6	23.9	77.9	13.8	74.7	20.0	39.9	70.6
Autapomorphic sites (%)	16.2	15.8	19.2	15.8	25.7	5.2	18.4	9.9	22.3	16.5	16.2
Indels	13	5	1	19	–	–	–	–	–	–	–
Indel size range (bp)	2–19	6–30	4	2–30	–	–	–	–	–	–	–
Informative base subsitutions	71	82	8	161	57	13	59	14	60	203	364
Total informative characters	84	87	9	180	57	13	59	14	60	203	383

Results

*mat*K sequence characteristics

The aligned length of the sequenced portion of the *trn*K intron was 2415 bp with 1557 bp forming the *mat*K coding region and an additional 912 nucleotides sequenced in the flanking intron region (Table 3). Areas of questionable homology, including poly A regions (54 bp) were omitted from the analysis. The sequence contained 161 informative base substitutions and 19 indels. Most of the indels (13/19) were in the 5' noncoding region. The mean divergence among taxa was greatest in the small 47 bp section downstream from the *mat*K coding region. The *mat*K coding region codes from 502 to 515 amino acids. For the entire *trn*K/*mat*K region the highest divergence (7.0%) among ingroup taxa was between *Acacia senegal* and *Albizia versicolor*, while the lowest divergence (0.1%) was between *Acacia leucoclada* and *A. parramattensis*, both bipinnate Australian species referable to *Acacia* subgenus *Phyllodineae* sect. *Botrycephalae*.

Topology of *mat*K/*trn*K tree

Topologies of the cladograms derived from the intron and *mat*K coding regions were congruent with slightly better resolution from the *mat*K coding region. Maximum parsimony analysis using the 180 informative characters of the entire dataset found at least 1000 trees of 842 steps with a CI of 0.78, a RI of 0.65 and G-fit of -134.2 (Farris, 1989). The topology of the maximum parsimony trees have four basic components (Fig. 2): 1) a clade (A) of *Acacia* subg. *Acacia* with *Neptunia monosperma*, 2) a paraphyletic grouping (B) of *Acacia* subg. *Aculeiferum*, with sects. *Aculeiferum* and *Monacanthea* forming a monophyletic clade, 3) a grade (C) including *Faidherbia albida*, and all Ingeae genera included in the study, and 4) a clade (D) comprising *Acacia* subg. *Phyllodineae*.

Constraint analyses tested the monophyly of Acacieae and *Acacia*. When the *Acacia* and Ingeae were constrained as monophyletic and the analysis repeated an additional 44 steps were added to the most parsimonious tree.

Acacia subg. *Acacia* and the Australian *Neptunia monosperma* of tribe Mimoseae, comprise the basal clade in the strict consensus tree (Fig 2). This grouping is supported by five synapomorphies (SYN = 5); and 63% bootstrap support (BV = 63%). The two New World species of subgenus *Acacia*, *A. caven* and *A. pennatula*, are strongly supported as sister species by two synapomorphic indels and two homoplasious indels (Fig. 2).

Acacia subg. *Aculeiferum* forms a monophyletic group when *A. boliviana* of sect. *Filicinae* Benth. is excluded (B, Fig. 2). *Acacia boliviana* appears as part of a basal grade (C) to subg. *Phyllodineae*. The rest of the subgenus is supported as monophyletic (SYN = 10; BV = 82%) and is sister to the Ingeae*Filicinae*\ *Faidherbia**Phyllodineae* clade. The two taxa referable to sect. *Monacanthea*, *A. glomerosa* and *A. schweinfurthii*, are well supported as sister taxa (SYN = 14; BV = 97%). They are joined by *A. senegal* of sect. *Aculeiferum* (SYN = 10; BV = 82%), which contains an autapomorphic indel in the 5' intron.

A grade consisting of *Faidherbia albida*, and the seven Ingeae genera (C, Fig. 2) also contains the monophyletic subg. *Phyllodineae*. *Faidherbia albida* is supported as sister to the Ingeae and subg. *Phyllodineae* clade. The Ingeae taxa are paraphyletic as they form a polytomy with *Acacia* subg. *Phyllodineae*. Resolution within the Ingeae is limited with only clear support for the sister relationship of *Havardia* and *Ebenopsis* (BV = 98). The two species *Albizia*, one from Africa and the other from Mexico, do not form a monophyletic group.

Acacia subg. *Phyllodineae* form a monophyletic group (B, Fig. 2). The support for this clade is lower than for clades representing the other subgenera (SYN = 3; BV = 57%). Because this subgenus contains over 950 species (Maslin and Stirton, 1997), the 25 taxa sampled here cannot represent the complexity of the subgenus. Five of

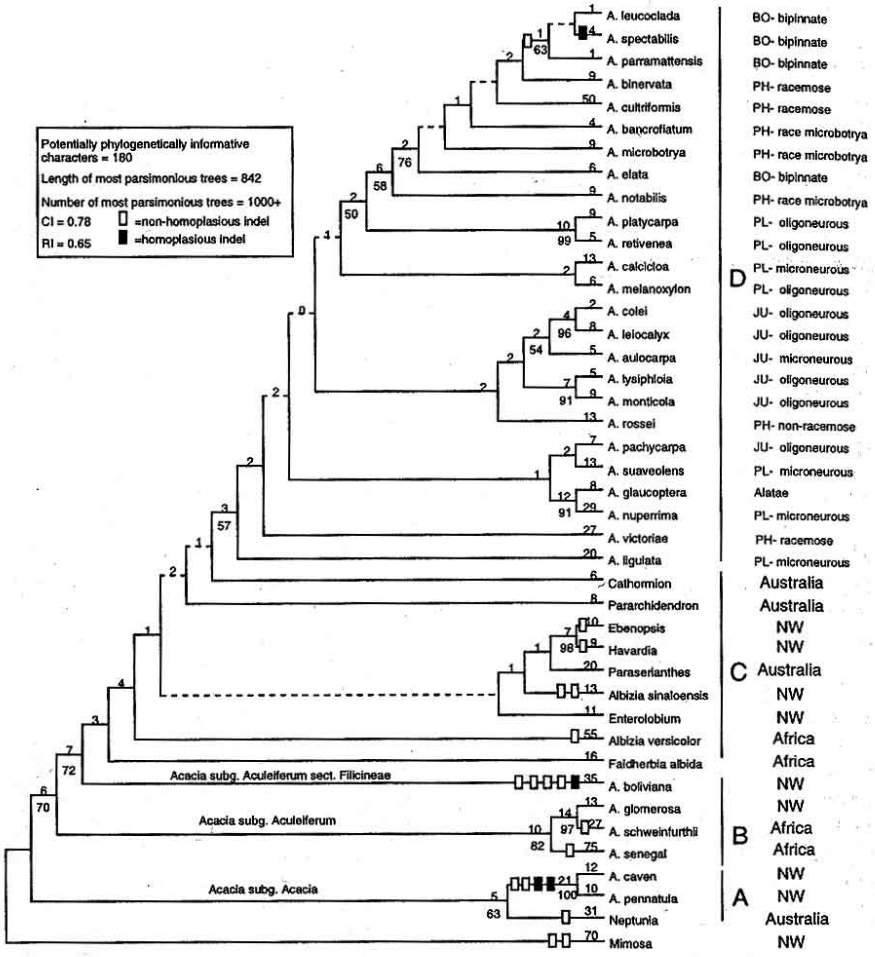


FIG. 2. One of more than 1000 most parsimonious trees from the *trnK/matK* sequence data. A dashed line indicates branches that collapse in the strict consensus tree. Numbers above the lines indicate branch length. Numbers below some lines indicate bootstrap support value. Indels are indicated by boxes. Letters A–D indicate clades or grades discussed in the text. A = *Acacia* subg. *Acacia*, B = *Acacia* subg. *Aculeiferum*, C = Ingeae/*Faidherbia*, D = *Acacia* subg. *Phyllodineae*. BO = sect. *Botrycephalae*, JU = sect. *Juliflorae*, PH = sect. *Phyllodineae*, PL = sect. *Plurinerves*, PU = sect. *Pulchellae*, see Tables 1, 2. See text for discussion of character groupings.

the seven sections (Tables 1, 2) recognised by Pedley (1978) are represented, including uninerved and plurinerved phyllodinous species as well as species with bipinnately compound leaves.

The basal portion of clade D is represented by two uninerved species referable to section *Phyllodineae*: *A. ligulata* of the *A. bivenosa* group (Chapman and Maslin, 1992) and *A. victoriae* of the *A. victoriae* group (Maslin, 1992). The strict consensus tree contains a five-part polytomy above these taxa. Most internal branches are short with support in most cases for taxa already recognised as closely related.

Acacia nuperrima (sect. *Plurinerves*) and *A. glaucoptera* of section *Alatae* are supported as sisters (SYN = 6; BV = 91%). The strict consensus tree joins five of the six species of section *Juliflorae* together with moderate support. Two of these, *A monticola* and *A. lysiphloia* are supported as sister species (SYN = 7; BV = 91%). This group was suggested by Maslin and Stirton (1997). The remaining species of section *Juliflorae*, *A. pachycarpa*, is part of a clade containing species of section *Plurinerves*.

The final clade is composed of five species with uninerved phyllodes and racemose inflorescences of sect. *Phyllodineae* and four bipinnate leaf species of sect. *Botrycephalae*. This clade is weakly joined (SYN = 2; BV = 50%) by two closely related species referable to section *Plurinerves*, *A. platycarpa* and *A. retivenea* (SYN = 10, BV = 99%). Three of the four *Botrycephalae* taxa form a clade (SYN = 1; BV = 63%) while the other, *A. elata*, is embedded within the phyllodinous taxa.

Histone H3-D sequence characteristics

The primers designed to flank the intron/exon boundaries of Histone H3-D amplified fragments from 434-455 bp (Table 3). The aligned length of the sequenced portion of the Histone H3-D gene was 482 bp. BLAST search of the sequences indicated close homology of the exon to both the Histone H3-D and Histone H3-B sequences in *Glycine*. PCR amplification resulted in a single band and phylogenetic analyses produced gene trees concordant with the *mat*K gene tree.

The sequence was highly polymorphic: 203 potentially informative base substitutions, with over 75% of the intron nucleotides polymorphic. The mean divergence among taxa was 12.1%. Numerous small, one to two bp indels, and the high degree of polymorphism made the alignment difficult. Areas of questionable homology (28 bp) were omitted from the analysis. The sequences from taxa in the major clades of the *mat*K/*trn*K cladogram were easily aligned, but aligning these groupings and the outgroup taxa was difficult, which was apparent in the strict consensus cladogram (Fig. 3). It is for this reason that indels were not scored as separate characters.

Topology of Histone H3-D tree

The strict consensus tree from the Histone H3-D sequence data (Fig. 3) was constructed from 256 trees of 924 steps each with a CI of 0.59, a RI of 0.57 and G-fit of -148.2. When *Acacia* and Ingeae were constrained as monophyletic and the analysis repeated an additional 62 steps were added to the most parsimonious tree. The topology of the cladogram derived from Histone H3-D was similar to the *mat*K/*trn*K tree except for the placement of several long branches which may reflect the difficulty of alignment. The positions of subgenera *Acacia*/*Neptunia* and *Aculeiferum* are transposed. *Acacia boliviana* of subg. *Aculeiferum* sect. *Filicinae* is placed as the basal ingroup taxon making subg. *Aculeiferum* paraphyletic. Support for branches within these two subgenera remain strong (SYN = 17–47 ; BV = 86–100%).

Compared to the *mat*K/*trn*K tree, the Histone H3-D has relatively high support for the basal components of the *Faidherbia*/Ingeae/subg. *Phyllodineae* grade. *Faidherbia* is supported as basal (SYN = 16; BV = 90%) while *Pararchidendron* is the basal taxa of the grade (SYN = 15; BV = 84%). *Cathormion*, *Enterolobium*, and *Albizia versicolor* are supported as sisters in the Histone H3-D tree, while this relationship was not indicated by the *mat*K/*trn*K data.

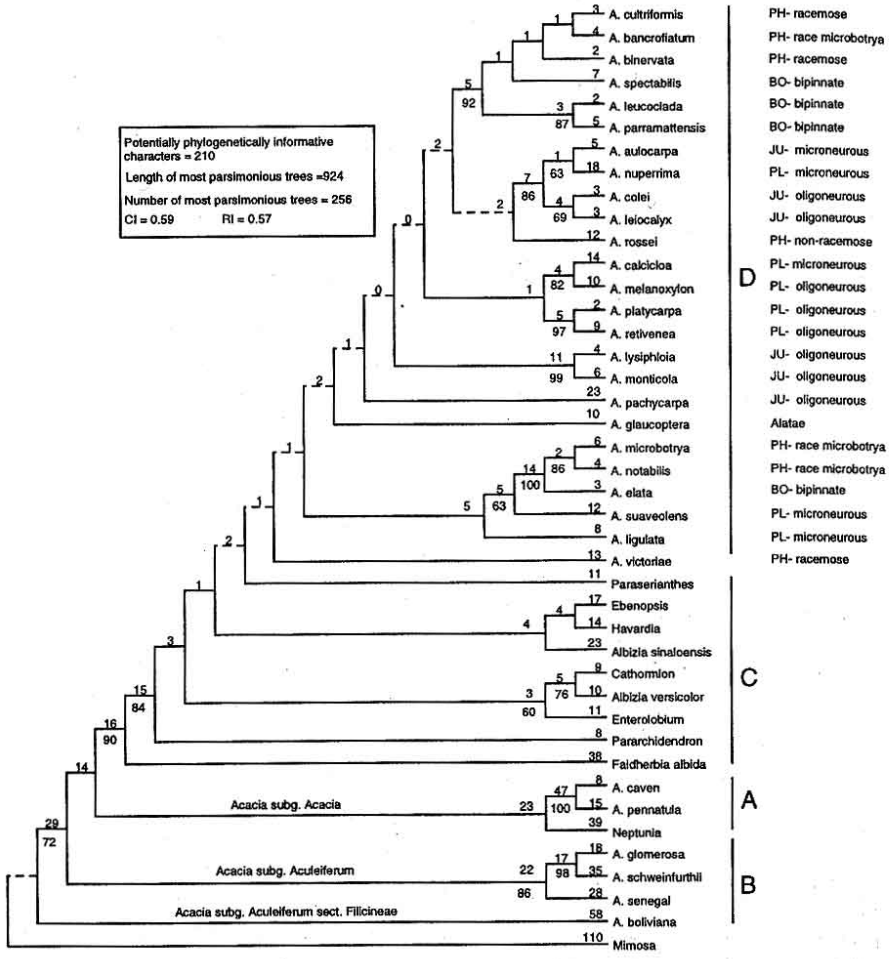


FIG. 3. One of 256 most parsimonious trees from the histone H3-D dataset. See Fig. 2 for an explanation of symbols and section affiliation.

Subgenus *Phyllodineae* is resolved as monophyletic but with only one synapomorphy and less than 50% bootstrap support. Within the subgenus most internal branches collapse (Fig 3. dashed lines) with a few species groups indicated. The sect. *Botrycephalae* and certain racemose taxa of the uninerved phyllode sect. *Phyllodineae*, including the "*A. microbotrya* group" are separated into two clades. One clade contains the bipinnate *A. elata* and two species of the "*A. microbotrya* group" (SYN = 14; BV = 100%). They group with two species of sect. *Plurinerves* with microneurous venation. The second clade of *Botrycephalae*/uninerved racemose taxa is comprised of the remaining taxa found in the corresponding clade in the *mat*K/*trn*K strict consensus tree (Fig. 2).

Within subgenus *Phyllodineae* the close relationship of *A. glaucoptera* to *A. nuperrima* in the *mat*K/*trn*K data is not maintained by the Histone H3-D data. While the *mat*K/*trn*K clade of the *Juliflorae* taxa is not maintained by the Histone H3-D data, a clade of sect. *Plurinerves* taxa (*A. calcicola, A. melanoxylon, A. platycarpa* and *A. retivenea*), not present in the *mat*K/*trn*K tree, is supported.

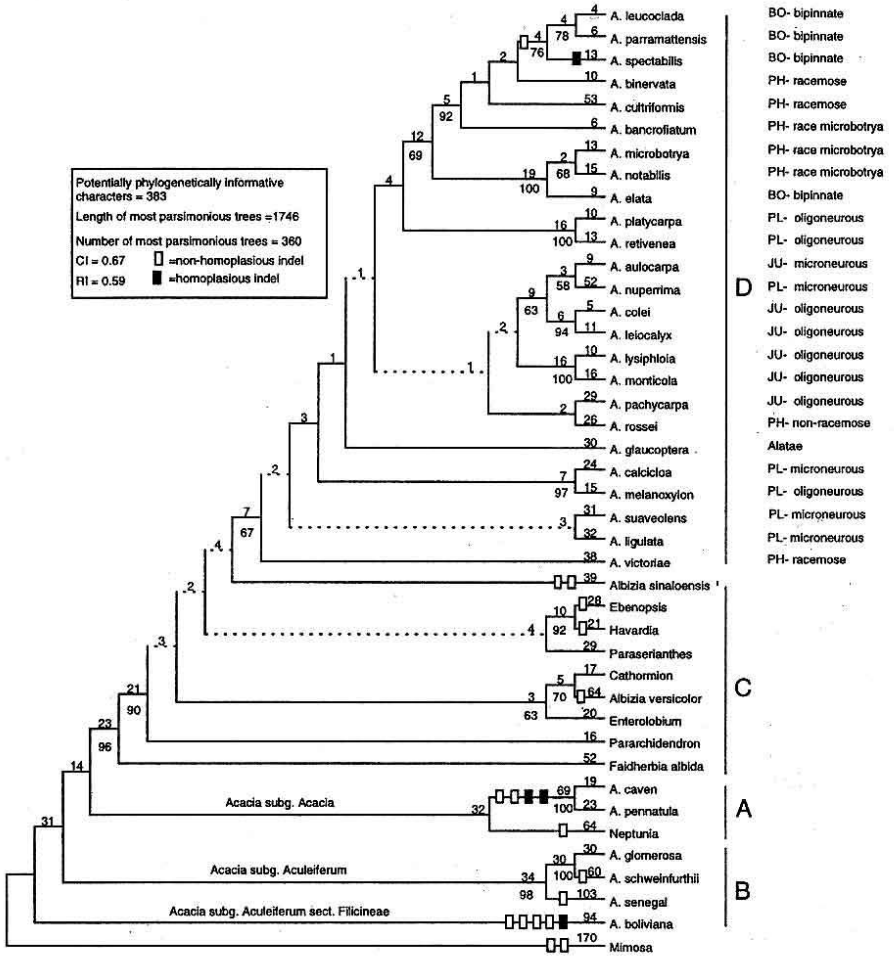


FIG. 4. One of 360 most parsimonious trees from combined analysis of *trn*K/*mat*K and histone H3-D dataset. See Fig. 2 for an explanation of symbols and section affiliation.

Congruence and total evidence tree

Incongruity analyses estimated only a small degree of incongruence between the two datasets (I_M = 1.0% and I_{MF} = 3.1%). The datasets were combined and a total evidence dataset was analysed. The resulting analysis produced 360 trees of 1746 steps with a CI of 0.67, a RI of 0.59 and G-fit of -272.2. When *Acacia* and Ingeae were constrained as monophyletic and the analysis repeated, an additional 44 steps were added to the most parsimonious tree. The strict consensus tree maintains features of both the *mat*K/*trn*K and Histone H3-D trees. Like the Histone H3-D data, subg. *Phyllodineae* is paraphyletic. *Acacia boliviana* of subg. *Aculeiferum* sect. *Filicinae* is sister to the remainder of the taxa analysed, followed by a clade of subg. *Aculeiferum* (*s. str*) (Fig. 4). The relationship of *Neptunia* to subg *Acacia* is also maintained. The Ingeae taxa form a paraphyletic grade with respect to a monophyletic *Acacia* subg. *Phyllodineae* (Fig. 4).

Within the subgenus *Phyllodineae* clade, the relationship of the bipinnate sect. *Botrycephalae* with certain racemose species referable to sect. *Phyllodineae* is maintained (Fig. 4). Also six *Juliflorae* taxa form a clade with a single representative each of sects. *Plurinerves* and *Phyllodineae*, but this not maintained in the strict consensus tree (Fig. 4). The single species of sect. *Alatae*, *A. glaucoptera*, does not group with *A. nuperrima* as in the *mat*K/*trn*K data alone. Other than the *Botrycephalae* clade the internal branches are not well supported within subg. *Phyllodineae* (Fig. 4). The discrepancies between the two datasets may indicate hybridisation or lineage sorting that need to be investigated with further taxon sampling and more DNA sequencing.

Discussion

Summary of major results

Cladistic analysis of sequence data from the chloroplast *trn*K/*mat*K and the nuclear Histone H3-D locus concur in finding the tribe Acacieae and the genus *Acacia* as polyphyletic (Fig. 4). At least three distinct lineages within *Acacia* are evident corresponding to the three recognised subgenera, except that subgenus *Aculeiferum* is paraphyletic with sect. *Filicinae* sister to the rest of subg. *Aculeiferum*. The data suggest that the Ingeae is paraphyletic and that it is closely related to *Acacia* subg. *Phyllodineae*.

Neptunia monosperma, an outgroup taxon representative of tribe Mimoseae, is supported as sister to *Acacia* subgenus *Acacia* and this in turn casts suspicion on the monophyly of the Mimoseae. *Neptunia* has been recognised as part of an informal grouping of Mimoseae taxa, the *Dichrostachys* group (Lewis and Elias, 1981), but cpDNA analysis features do not preclude the possibility of its alliance outside the *Dichrostachys* group to other mimosoids (Luckow, 1997).

The results clearly show a closer relationship of subgenus *Phyllodineae* to tribe Ingeae than to other *Acacia* subgenera. While these results clearly refute the monophyly of the Acacieae and *Acacia*, the results do not clearly demarcate relationships among the major clades. More taxa, especially Ingeae and Mimoseae, and more sequence data will be needed to answer these questions. Many long branches are evident in the phylogenetic trees especially in the Ingeae grade and *Phyllodineae*. Most taxa have many autapomorphies and few synapomorphies unite taxa on the tree, yet few branches collapse in the strict consensus tree. This may be due to the limited sampling of taxa and lack of sufficient informative characters (Bininda-Emonds et al., 1998; Hillis 1998). This may indicate the likelihood of rapid morphological radiation without substantial molecular divergence within these groups. The conflicting position of *A. boliviana* in the chloroplast and nuclear phylogenies may be a result of long branch attraction. Increased data collection and taxon sampling are needed to differentiate between hypotheses of an artificial placement and an alternative explanation of hybridisation.

The close relationship of subgenus *Phyllodineae* to the Ingeae was also indicated in the study of inflorescence development by Grimes (1999) which showed the *Phyllodineae* sister to the Ingeae. In the present study the Ingeae is paraphyletic with *Acacia* subg. *Phyllodineae* nested within it. However there is little variation among the Ingeae taxa and low support for the internal branches within this clade. Further sequence data are needed to determine whether the Ingeae is indeed non-monophyletic. However our results differ from Pedley (1986) and a cladistic analysis of morphological data (Chappill and Maslin, 1995), which suggested the derivation of subgenus *Phyllodineae* from subg. *Aculeiferum*. It should be noted that Asian representatives of subgenus *Aculeiferum* were not included in the present study. A cpRFLP study by Bukhari et al. (1999) indicates a sister relationship between subg. *Acacia* and subg. *Phyllodineae*, however this study included only Acacieae species so no inferences on the monophyly of the genus can be concluded.

Until recently *Faidherbia* was treated as a species of *Acacia* and Ross (1979) suggested that the species is not closely related to other African *Acacia* species. Despite having shortly united filaments, instead of free stamens as in most *Acacia* species, Vassal (1981) placed the genus in the Acacieae instead of the Ingeae. While the cladistic morphological analysis (Chappill and Maslin, 1995) suggests a closer affinity to the Ingeae the present DNA sequence data are equivocal.

Acacia subgenus *Phyllodineae* consists of three of Bentham's series (*Pulchellae*, *Botrycephalae*, and *Phyllodineae*; Vassal, 1972). The separation of the *Botrycephalae* (a group of Australian bipinnate species) from the *Phyllodineae* is not supported by the present DNA sequence data as it would leave the *Phyllodineae* paraphyletic.

Subgenus *Phyllodineae* contains taxa that have bipinnately compound leaves and taxa with phyllodes. Numerous phyllode and inflorescence characters have been used to create convenient morphological groups, although it is not known if the groups are natural (Maslin and Stirton, 1997). Among the phyllode-bearing taxa there appears to be a natural division between the single-nerved phyllode species (sect. *Phyllodineae*) and the plurinerved species (sects. *Juliflorae* and *Plurinerves*; Pedley, 1986; Maslin and Stirton, 1997). The DNA sequence data presented here are not robust enough to test this division, but some grouping of the plurinerved taxa is seen (Fig. 4). Traditionally sect. *Phyllodineae* has been subdivided based on inflorescence structure, racemose or non-racemose. The single non-racemose species included in the present study, *A. rossei*, is weakly allied with plurinerved taxa. Serological work (Brain and Maslin, 1996) also showed some non-racemose species as being closely related to plurinerved taxa.

The plurinerved taxa have been divided based on inflorescence type, with flowers in spikes in sect. *Juliflorae* and flowers in heads in sect. *Plurinerves*. Recent studies have shown them to be closely related (Tindale and Roux, 1969, 1974; Vassal, 1972; Pettigrew and Watson, 1975; Tindale, 1980). In contrast, the immunological data of Brain and Maslin (1996) suggested that sect. *Plurinerves* may be a natural group but the *Juliflorae* is not supported as monophyletic. The DNA sequence data is equivocal as to the nature of these groups. The *Juliflorae* taxa are joined in a weakly supported clade with a taxon each from sects. *Plurinerves* and *Phyllodineae*, while other *Plurinerves* taxa form a paraphyletic basal grade within subgenus *Phyllodineae* (Fig. 4). It seems unlikely that the *Juliflorae* and *Plurinerves* are natural entities based on this small sampling. The plurinerved taxa have been further grouped by the pattern of nervature, either microneurous, with numerous, fine, almost longitudinal nerves, and the oligoneurous taxa, with only a few, distant, longitudinal nerves (Maslin and Stirton, 1997). These divisions do not form monophyletic entities (Fig. 4) however there are tendencies for species pairs to have similar phyllode form.

The chloroplast and nuclear DNA data presented here indicate that the *Botrycephalae* is not monophyletic and is derived from certain uninerved racemose species. This suggests multiple reversals from the phyllode to bipinnately compound leaves, which is the ancestral character state of the Mimosoideae (Pedley, 1986). This study allies certain uninerved racemose species referred to as the *A. microbotrya* group (*A. bancrofiatum*, *A. notabilis* and *A. microbotrya*) and other uninverved racemose species, *A. cultriformis and A. binervata*, to the *Botrycephalae*. The origin of the *Botrycephalae* from the *A. microbotrya* group is supported by other studies that considered morphological (Chappill and Maslin, 1995), phytochemical (Tindale and Roux, 1969, 1974) and serological data (Brain and Maslin, 1996). The serological analysis of Brain and Maslin (1996) shows a close relationship of *A. microbotrya* to the bipinnate *A. mearnsii*, but also to another racemose member of the sect. *Phyllodineae*, *A. binervata*, that has not been considered part of the microbotrya group. The DNA sequence analysis also groups these three taxa. Another racemose member of the sect. *Phyllodineae*, *A. cultriformis*, is included in this clade but was not sampled in previous studies. The long branch (53 steps; Fig. 4) of *A. cultriformis* may hint that its placement is artificial.

Subgroups have been recognised within series *Botrycephalae* based on flavanoids and tannins of heartwood (Tindale and Roux, 1969) and gum exudates (Anderson, 1978). However the lack of overlapping taxa among these and the present study limits interpretation, as *A. elata*, which occurs in a distinct clade from other species of series *Botrycephalae* in our analysis, was not included in the previous studies.

Congruence among datasets and applicability of Histone H3-D

Several discrepancies are noted between the chloroplast and nuclear phylogenies presented here. *Acacia boliviana* changes position in the resulting cladograms. Caution is suggested when interpreting this and other differences in phylogenies based on the two datasets. The position of *A. boliviana* is not well-supported in either cladogram and further sampling and DNA sequencing is critical for a better understanding of its phylogenetic placement.

The nuclear Histone H3-D sequence has been used in phylogenetic analysis in *Glycine* both within and among genome groups (Doyle et al., in press). The present study applies the sequence for phylogenetic studies at a generic level in *Acacia*. It is evident from difficulty in alignment that the use of Histone H3-D will be limited to species level studies. The problematic sequence alignment among taxa in different clades resulted in little resolution and allowed limited inference about the relationships among clades. While the *Acacia* specific primers successfully amplified single bands in *Acacia* and non-*Acacia* taxa not all species were successfully amplified. Another difficulty that arose with the use of this sequence is putative secondary structural problems confronted in the sequencing of the taxa. Several sequencing reactions, directly from PCR products, were successful through the first intron and second exon but were unreadable when the sequence entered the third intron. This pattern was seen with both the forward and reverse primers. The cloning and subsequent cloning of these PCR fragments attained good sequences but at a considerable cost of time and resources.

The congruence of the Histone H3-D and the *mat*K/*trn*K data suggests that the sequencing of paralogous rather than orthologous sequences was not a factor. Since the sequence is a single-copy, heterozygosity among alleles at the locus or polyploidy may provide polymorphisms within the PCR product of a single individual. This may lead to multiple peaks at a single nucleotide site due to the sequencing of more than one template. Such putative heterozygous sites have been identified in *A. parramattensis* (Miller, unpublished data). For this species two cloned Histone H3-D alleles differ by four base substitutions. This ability to sequence alleles within the predominately outcrossing subgenus *Phyllodineae* (Pettigrew and Watson, 1975) may provide detailed information in the form of gene phylogenies that may help resolve origins and evolution in hybrid species complexes.

Conclusions

DNA sequence data from the nuclear Histone H3-D and the chloroplast *mat*K/*trn*K region are congruent in refuting the monophyly of the tribe Acacieae and the genus *Acacia*. The *mat*K/*trn*K better resolved higher level relationships than the Histone H3-D data. The genus *Acacia* is composed of at least three evolutionary lineages intertwined with the Ingeae. More taxonomic sampling and more DNA sequence will be necessary to delimit relationships among the Acacieae, Ingeae and Mimoseae. The phylogeny based on these DNA sequences suggests plasticity in leaf (phyllode) and inflorescence characters as traditional taxonomic divisions within subg. *Phyllodineae* are not maintained. This also indicates the likelihood of rapid morphological radiation without substantial molecular divergence within these groups.

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