

Phylogeny of *Hibiscus* and the Tribe Hibisceae (Malvaceae) Using Chloroplast DNA Sequences of *ndhF* and the *rpl16* Intron

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ABSTRACT. Circumscriptions of the genus *Hibiscus* and the tribe Hibisceae (Malvaceae) are based on morphological features that are not unique in the family. An examination of the literature regarding putatively ancestral morphological features revealed that *Hibiscus* and Hibisceae may be defined by shared ancestral features, and thus are unlikely to be monophyletic groups. These phylogenetic hypotheses were tested using two chloroplast DNA sequences (a coding region—*ndhF* and a non-coding region—the *rpl16* intron). Several genera usually placed in Hibisceae were found to occupy positions sister to the rest of the family, as was predicted from our reevaluation of their morphological features. Although the earliest divergences in the family were not resolved by chloroplast DNA topologies alone, several morphological features, when analysed in combination with *ndhF* suggested a possible resolution of the basal polytomy. These early divergences are represented by extant genera with relatively restricted distributions, which all possess Australasian species that are sister to more widespread and diverse lineages. This suggests the novel hypothesis that eastern Gondwana may be the centre of origin of the family. The pollen fossil record is consistent with this possibility, but does not support it unambiguously. Unexpectedly the tribes Decaschistieae and Malvavisceae as well as other genera of Hibisceae nest within *Hibiscus*. Nomenclatural upheavals concerning *Hibiscus*, one of the world's most popular horticultural plant genera, will be difficult to avoid.

The charismatic genus *Hibiscus* L. (Malvaceae) is familiar to many people, particularly in tropical and sub-tropical regions of the world. While the genus is distributed widely, its diversity is concentrated in the tropics (Table 1). Several members of the genus are important as fiber crops, livestock feed, human food sources, folk medicines, and ornamentals (Sivarajan and Pradeep 1996; Wilson 1999). The widely-cultivated ornamental shrub *Hibiscus rosa-sinensis* L., with its bewildering array of cultivars, is perhaps the best known species of the genus. Species of *Hibiscus* range from annual herbs (e.g., *H. lobatus* Kuntze) to trees (e.g., *H. macrophyllus* Roxb.), although most species are perennial sub-shrubs or shrubs. Many species have hairy or prickly stems (e.g., *H. mastersianus* Hiern), although some may be glabrous (e.g., *H. schizopetalus* Hook.f.). The genus as it is currently understood is united by the presence of the following features, which when taken together distinguish *Hibiscus* from the rest of Malvaceae: staminal column with five apical teeth, five-branched style, usually capitate stigmas, ovary with more than one ovule per cell, calyx and epicalyx (if present) persistent after flowering, a five- to ten-celled loculicidally dehiscent wingless fruit, petals basally fused to the staminal column, epicalyx usually eight or more lobed, petals not forming a tube around the staminal column.

Hibiscus as a generic name was first used by Linnaeus (1753, 1754), who included 20 species in his circumscription. Following this, De Candolle (1824) proposed a sectional classification that subdivided the 117 species known to him. However, it was not until Hochreutiner's (1900) monograph that the genus received a

comprehensive systematic treatment. Hochreutiner added five new sections to the six published by De Candolle, and a seventh recognized by Garke (1849) (Table 2). While Hochreutiner (1900) treated 197 species, over 300 species may be currently assignable to *Hibiscus*.

Hibiscus, as originally circumscribed, contained a diverse assemblage of morphologically distinct species groups, some of which have subsequently been segregated. Some of these species have been placed in other tribes (e.g., *Malvaviscus arboreus* Cav., in Malvavisceae), while others have been segregated to genera considered closely related to *Hibiscus* (e.g., *Abelmoschus* Medik. and *Fioria* Mattei, both now in Hibisceae). This trend has continued with the publication of a number of species in *Hibiscus* that have since been transferred to other genera but kept within Hibisceae (e.g., *Lagunaria patersonia* (Andr.) G. Don., *Alyogyne hakeifolia* (Giord.) Alef., *Radyera farragei* (F.Muell.) Fryxell & S.H.Hashmi [Fryxell 1968]). Other species not first published in *Hibiscus* have also come to be placed in Hibisceae (e.g., *Howittia trilocularis* F.Muell., which had been placed in the Malveae [Fryxell 1968]).

The circumscription of *Hibiscus* is closely linked with that of other genera of Hibisceae. This tribe was first recognized by Reichenbach (1828) and was delimited from the other tribes in Malvaceae by the possession of capsular rather than schizocarpic fruit. At that time Hibisceae contained nine genera, and was split into two sub-tribes on the basis of the presence or absence of an epicalyx (Reichenbach 1828). The current understanding of two groups of capsular-fruited Malvaceae, one related to *Hibiscus* and the other to *Gos-*

TABLE 1. Number of species and percentage endemism of *Hibiscus* in tropical and nontropical areas. No attempt to account for different taxonomic concepts has been made.

Area	Approx. # of species	Approx. % endemics	Reference
Mostly or completely within the tropics:			
India	23	22	Paul 1993
Madagascar	47	62	Hochreutiner 1955
Malesia	45	36	Borssum Waalkes 1966
Mexico	37	38	Fryxell 1988
Mostly or completely outside the tropics:			
Argentina	4	0	Instituto de Botánica Darwinion 1999
Iran, Afghanistan, etc	7	0	Riedl 1976
Texas	11	9	Correll and Johnston 1970

sypium L., originated with Alefeld (1861), who placed five genera in a new tribe Gossypieae (as "Gossypidae"). This tribe shares a number of features unique in the family (e.g., presence of gossypol glands, concuplicate embryo folding, involucre nectaries, etc; Fryxell 1968). Subsequent analysis has shown strong support for the monophyly of Gossypieae (Seelanan et al. 1997).

The features that hold Hibisceae together are: loculicidally dehiscent fruit (a 'capsule'); gossypol glands absent, five-toothed staminal column apex, styles usually branching at the apex, stigmas usually terminal, style branches equal in number to the carpels (Bentham 1863; Ulbrich 1921; Hochreutiner 1955; Borssum Waalkes 1966; Riedl 1976; Fryxell 1988; Paul 1993; Sivarajan and Pradeep 1996).

In reaffirming Alefeld's segregation of Gossypieae from Hibisceae, Fryxell (1968) noted that most of the Malvaceae have seed with an "advanced" structure, having complex embryos and little or no endosperm at maturity. However, he also observed that the seeds of two Hibisceae genera (*Alyogyne* Alef. and *Lagunaria* G.Don) have copious endosperm, a feature that he interpreted as ancestral (Fryxell 1968). This suggests that Hibisceae, as defined by Fryxell (1968), may be a heterogeneous assemblage of genera, some diverging early from the rest of the family, while others may be more derived. *Radyera* Bullock (Hibisceae) also has copious endosperm (Fryxell and Hashmi 1971), and may occupy a similar position. If *Alyogyne*, *Lagunaria*, and *Radyera* have been correctly interpreted as early divergences, then the fact that they share many of the tribal features with other Hibisceae (although *Alyogyne* and *Radyera* lack branching styles; Fryxell and Hashmi 1971) suggests that these features are also ancestral. If this is so, the tribe has been characterized by shared ancestral features (symplesiomorphies sensu Hennig 1966), and is therefore unreliable.

A similar examination of the features that distinguish *Hibiscus* from the other Hibisceae highlights the uncertainty in what actually constitutes *Hibiscus*. The smaller segregate genera in Hibisceae for which there

is reasonable information can each be defined by a few derived features, but this leaves a suite of ancestral features to circumscribe *Hibiscus*. For example, *Fioria* and *Kosteletzkya* C.Presl have winged capsules, *Abelmoschus* Medik. has an asymmetrical deciduous calyx, and *Kydia* Roxb. has five antheriferous arms atop the staminal column (Fryxell 1988; Paul 1993). This leaves *Hibiscus* without unique synapomorphies, and suggests it may be a paraphyletic assemblage with other members of the tribe nested within it.

The tribe Decaschistieae, erected to accommodate *Decaschistia* Wight & Arn. (Fryxell 1975), shares many features with Hibisceae but also possesses a number of potentially synapomorphic features, including an increase in carpel and style branch numbers to 10 (or sometimes only six or eight) and a fruit that disintegrates at maturity (Sivarajan and Pradeep 1996). As the genus was previously placed in Hibisceae (Hutchinson 1967), its situation is comparable to other Hibisceae genera discussed in the previous paragraph, that is, potentially arising from within *Hibiscus*.

Finally, the characters that group the tribe and serve to group *Hibiscus* by default are shared by other tribes in the family. The branching style is shared with Malveae, Malvavisceae, and Decaschistieae; loculicidally dehiscent fruit with Gossypieae and Decaschistieae; carpel number equaling style branch number with Gossypieae, Malveae and Decaschistieae; absence of gossypol synthesis with Malveae, Malvavisceae, and Decaschistieae; staminal column surmounted by five teeth (or other sterile tissue) with Gossypieae, Malvavisceae, and Decaschistieae; possession of five carpels with part of Gossypieae (Bentham 1863; Ulbrich 1921; Hochreutiner 1955; Borssum Waalkes 1966; Riedl 1976; Fryxell 1988; Paul 1993; Sivarajan and Pradeep 1996).

Thus *Hibiscus* and Hibisceae present identical problems to the revisionary taxonomist. They are large groups that cannot be defined by unique characters and largely comprise the problematic species left behind after more clearly defined taxa have been segregated. As they lack convenient morphological synapomorphies, it is difficult to establish the evolutionary

TABLE 2. Synopsis of recent sectional classifications by various authors. Repeat marks (") indicate that the concept directly to the left of the mark was also used by the subsequent author, although they may have further subdivided the sectional concept. N/A indicates that the section was not present in the area treated by that author.

Hochreutiner (1900)	Ulbrich (1921)	Borsum Waalkes (1966)	Fryxell (1988)	Type or representative species	Species sampled in this study
Abelmoschus DC.	Abelmoschus Mekik. (as a genus)	"	"	A. moschatus (type)	A. ficulneus
Azanza DC.	"	"	Azanzae	H. azanzae (type)	H. tiliaceus
Bombycella DC.	Africanæ lobophyllæ Ulbr.	under Hibiscus	Bombicella	H. phoenicis (type)	H. brachysiphonius
	Eubombycella Ulbr.	N/A		H. pedunculatus	H. pedunculatus
	Syriaca Ulbr.	N/A		H. micranthus	
Columnaris Hochr.	"	under Hibiscus	Hibiscus	H. syriacus (type)	H. syriacus
		under Ketmia or Trionum	N/A	H. columnaris (type)	
Furcaria DC.	Furcaria furcellata Ulbr.	Bombycidendron Bors. Waalk.	N/A	H. grevifolius (type)	H. surattensis
	Furcaria cannabina Ulbr.	"	"	H. surattensis (type)	
	Furcaria sabdariffa Ulbr.	"	"	H. surattensis (type)	H. radiatus
	Furcaria diversifolia Ulbr.	"	"	H. cannabimus	H. sabdariffa
	Friesia Ulbr.	"	"	H. sabdariffa	
	Saxicolae Ulbr.	"	"	H. diversifolius	
	Gigantocalyx Ulbr.	"	"	H. friesii	
Ketmia DC.	"	"	"	H. saxicola	
Lilibiscus Hochr.	"	"	"	H. brichettii	
	Parapavonia Ulbr.	"	under Hibiscus	H. pruriens (type)	H. physaloides
	"	"	"	H. pruriens (type)	H. rosa-sinensis
Pterocarpus Garcke	under Furcaria	"	N/A	H. rosa-sinensis (type)	
Sabdariffa DC.	"	"	N/A	H. adenosiphon	
Solandra Hochr.	"	"	N/A	H. vitifolius = Fioria vitifolia	Fioria vitifolia
Spatula Hochr.	"	"	N/A	H. solandra (type)	
	Pentacalycinus Ulbr.	"	N/A	H. normani	H. schinzii
	"	"	N/A	H. rainerianus	H. normani
Trichospermum Hochr.	"	under Ketmia	Trionastrum Grisebach	H. sorortius (type)	
		"	N/A		
	Aristivalvus Ulbr.	"	N/A	H. intermedium	
	Calyphylli Ulbr.	"	N/A	H. calyphyllus	H. calyphyllus
	Panduriformes Ulbr.	"	N/A	H. panduriformis	H. apodus ms
	"	"	"	H. trionum (type)	H. trionum
	"	"	"	H. clypeatus (type)	
	Cucurbitina Ulbr.	"	Clypeati O.J. Blanch.	H. urens = Radyera urens (type)	Radyera farragei
		N/A	N/A (elsewhere under Radyera)	H. palustris (type)	
	Venusii Ulbr. (inc. H. mutabilis)	H. mutabilis under Trionum	Muenchusia O.J. Blanch.	H. striatus (type)	H. striatus
		"	Siriati O.J. Blanch.	H. striatus (type)	
		"	Venusii Ulbr.	H. venustus (type)	

TABLE 3. Taxa used in this study, including voucher information and Genbank numbers. Previously published ndhF Genbank accessions from Alverson et al. (1999) and Seelanan et al. (1997), and rp116 intron from Baum et al. (1998) are bracketed. Abbreviations as follows: ANBG = Australian National Botanic Gardens living collections, BEP = B.E. Pfeil, CLB = C.L. Brubaker, Gos = CSIRO *Gossypium* germplasm collection, LAC = L.A. Craven, LL = CSIRO long term storage collection, PI = US-D.A. Plant Introduction, PRP = Pilbara Ranges Project collection.

Species	ndhF	rp116	Voucher
Bombacaceae			
<i>Adansonia digitata</i> Linn.		(GI 2689440)	
<i>Adansonia grandidieri</i> Baill.		(GI 2589436)	
<i>Adansonia madagascariensis</i> Baill.		(GI 2589452)	
<i>Bombax buonopozense</i> Beauv.	(AF111726)		
<i>Campostemon schultzei</i> Mast.	(AF111727)		
<i>Mafisia cordata</i> Humb. & Bonpl.	(AF111724)		
<i>Pachira aquatica</i> Aubl.		(GI 2589432)	
<i>Pluragmotheca ecuadorensis</i> W.S. Alverson	(AF111725)		
<i>Pseudobombax marginatum</i> (A. St.-Hil., A. Juss. & Cambess.) A. Robyns	(AF111723)		
<i>Quararibea costaricensis</i> W.S. Alverson			
Malvaceae			
Decaschistieae			
<i>Decaschistia occidentalis</i> A.S. Mitch. ex Craven & Fryxell		AF384568	LAC, Stewart & CLB 9222
Gossypieae			
<i>Cienfuegosia tripartita</i> Günke			
<i>Gossypioides kirkii</i> (Mast.) Skovsted	(U 55324)		
<i>Gossypium hirsutum</i> L.	(U 55329)	AF384572	Seelanan 3
<i>Gossypium sturtianum</i> J.H. Willis	(U 55340)		
<i>Hampea appendiculata</i> Standley	(U 55327)	AF384573	Gos-5071
<i>Kokia drynarioides</i> Lewton	(U 55330)		
<i>Lebronnecia kokoioides</i> Fosberg & Sachet	(U 55325)		
<i>Thespesia populnea</i> (L.) Soland. ex Correa	(U 55328)		
<i>Thespesia thepesioides</i> (R. Br. ex Benth.) Fryxell		AF384625	n.v. (Seelanan, et al. 1997)
Hibisceae			
<i>Abelmoschus manihot</i> (L.) Medik.		AF384639	Mitchell 195
<i>Abelmoschus ficulneus</i> (L.) Wight & Arn.	AF384638	AF384560	Forster 14862
<i>Alyogyne huegelii</i> (Endl.) Fryxell	AF384657	AF384565	ANBG 9800039c
<i>Alyogyne hakeifolia</i> (Giord.) Alef.	AF384658	AF384564	LAC & BEP 10334
<i>Alyogyne pinoniana</i> (Gaudich.) Fryxell	AF384659	AF384566	LAC & BEP 10339
<i>Alyogyne cravenii</i> Fryxell	AF384648	AF384563	Fryxell, LAC & Stewart 4870
<i>Fioria vitifolia</i> (L.) Mattiei	AF384640	AF384570	Palmer & LAC 8609
<i>Hibiscus apodus</i> ms	AF384635	AF384574	Zich 153
<i>H. brachysiphonius</i> F. Muell.	AF384644	AF384575	Mitchell PRP 801
<i>H. burtonii</i> F.M. Bailey		AF384576	Mitchell Oct 1995
<i>H. calyphyllus</i> Cav.	AF384655	AF384577	Hornshy s.n.
<i>H. coatesii</i> F. Muell.	AF384645		Payne PRP 703
<i>H. costatus</i> A. Rich.	(U55323)		

TABLE 3. Continued.

Species	ndhF	rp116	Voucher
<i>H. divaricatus</i> Graham		AF384579	ANBG 9608949c
<i>H. dongolensis</i> Caill. ex Delile	AF384634	AF384580	PI 364900
<i>H. drummondii</i> Trucz.	AF384647	AF384581	LAC, Zich & Lyne 8940
<i>H. engleri</i> K. Schum.	AF384641	AF384582	PI 364901
<i>H. forsteri</i> F.D. Wilson		AF384583	Wrigley & Telford NQ1386
<i>H. fryxellii</i> D.J. Mabbertley	AF384632	AF384584	LL 1571
<i>H. furcellatus</i> Lam.	AF384629	AF384585	PI 585158
<i>H. heterophyllus</i> Griff.	AF384631	AF384586	ANBG 9608925
<i>H. insularis</i> Endl.		AF384587	ANBG 9201206
<i>H. ludwigii</i> Eckl. & Zeyh.	AF384656	AF384588	BEP 266
<i>H. macrophyllus</i> Roxb.	AF384636	AF384589	LAC 10202
<i>H. masterisianus</i> Hiern		AF384590	PI 585154
<i>H. mœusei</i> Exell		AF384591	PI 344235
<i>H. meraukensis</i> Hochr.	AF384627	AF384592	J.M. Bowles 98.010
<i>H. microchlaenus</i> F. Muell.	AF384633	AF384593	Zich 66
<i>H. nigricalis</i> E.G. Baker		AF384594	PI 500796
<i>H. normanii</i> F. Muell.	AF384628	AF384595	LAC & BEP 10338
<i>H. pedunculatus</i> L. f.	AF384649	AF384596	PI 364903
<i>H. pentaphyllus</i> F. Muell.	AF384651	AF384597	LAC, Stewart, Wendel & CLB 9146
<i>H. peralbus</i> Fryxell	AF384652	AF384598	Mitchell 2911
<i>H. physaloides</i> Guill. & Perr.	AF384643	AF384599	PI 533007
<i>H. radiatus</i> Cav.		AF384600	PI 585159
<i>H. rosa-sinensis</i> L.	AF384653		BEP 310
<i>H. rostellatus</i> Guill. & Perr.		AF384601	CLB 820
<i>H. sabdariffa</i> L.		AF384602	Mitchell June 1992
<i>H. saponarius</i> Craven		AF384603	Cowie & Neldner 8107
<i>H. schinzii</i> Hochr.		AF384604	PI 500714
<i>H. sp1 'bombicella'</i>	AF384642	AF384605	Purdie 4347
<i>H. splendens</i> Fras. ex Graham		AF384606	BEP 273
<i>H. striatus</i> Cav.		AF384607	PI 372212
<i>H. sturtii</i> Hook.		AF384608	Cowie & Baker 6423
<i>H. surattensis</i> L.	AF384626	AF384609	PI 585138
<i>H. syriacus</i> L.	AF384650	AF384610	BEP s.n.
<i>H. thibaeus</i> L.	AF384637	AF384611	LAC 10200
<i>H. trionum</i> L.		AF384612	PI 500697
<i>H. waimeae</i> A. Heller	AF384654	AF384613	cult. Punjob Ag. Uni., India, n.v.
<i>H. zonatus</i> F. Muell.	AF384630	AF384614	LL 1565
<i>Howittia trilobularis</i> F. Muell.	AF384662	AF384615	ANBG 08910071
<i>Lagunaria patersonia</i> (Andrews) G. Don	AF384664	AF384616	ANBG s.n.
<i>Macrostelia grandifolia</i> Fryxell-Leo Creek	AF384646	AF384619	LAC & BEP 10085
<i>Macrostelia grandifolia</i> Fryxell-Garraway Creek		AF384618	LAC 10344
<i>Macrostelia</i> aff. <i>grandifolia</i> -Bolt Head		AF384617	LAC 10343

TABLE 3. Continued.

Species	<i>ndhF</i>	<i>rpl16</i>	Voucher
<i>Radyera farragei</i> (F. Muell.) Fryxell & S.H. Hashmi	AF384663	AF384623	Fryxell, Craven & Stewart 4462
Malvaceae			
<i>Anotea flavida</i> Ulbr.	(U55322)		
<i>Malviscus arboreus</i> Cav.	(AF111718)	AF384621	BEP 265
<i>Pavonia hastata</i> Cav.	(AF111719)	AF384622	Purchased (n.v.)
<i>Pavonia multiflora</i> A. St.-Hil.			
Malveae			
<i>Abutilon fraseri</i> (Hook.) Walp.	(AF111716)	AF384562	BEP 262
<i>Abutilon hybridum</i> Hort.			
<i>Anoda cristata</i> (L.) Schtdl.	(AF111717)	AF384567	PI 372184
<i>Malope trifida</i> Cav.	AF384660	AF384620	Local weed, n.v.
<i>Malva neglecta</i> Wallr.		AF384624	Lepschi & Lally 2350
<i>Sida hookeriana</i> Miq.	AF384661		BEP 327
<i>Sida acuta</i> Burm. f.			
Sterculiaceae			
<i>Dombeya spectabilis</i> Boj.	(AF111752)		BEP 267
<i>Dombeya tiliacea</i> Planch.		AF384569	
<i>Fremontodendron californicum</i> (Torrey) Cov.	(AF111721)		BEP 339
<i>Fremontodendron californicum</i> (Torrey) Cov. × <i>mexicanum</i> Davidson		AF384571	

relationships among the remaining species, or between these species and the segregate taxa. Additionally, the long-standing uncertainties regarding the relationships between species with schizocarpic and capsular fruits has never been resolved although it is fundamental to our understanding of the Malvaceae (e.g., Edlin 1935; Judd and Manchester 1997). Without new and independent data, re-evaluation of the morphological variation will only perpetuate current ambiguities. While previous studies used molecular characters to elucidate relationships among some malvaceous taxa (e.g., La Duke and Doebley 1995; Seelanan et al. 1997; Alverson et al. 1999; Bayer et al. 1999), their sampling was not designed to test the monophyly of *Hibiscus* or *Hibisceae*.

To address these questions, two chloroplast DNA (cpDNA) sequences, one considered to be more slowly evolving (the gene *ndhF*), and the other evolving more quickly (the intron from *rpl16*), were obtained from representatives of each major section of *Hibiscus* (sensu Hochreutiner [1900] with modifications by Ulbrich [1921], Borssum Waalkes [1966] and Fryxell [1988]) and from key genera in the Hibisceae. These data allow an independent assessment of whether *Hibiscus* represents an independent evolutionary lineage without the well-defined segregate genera (e.g., *Fioria*, *Abelmoschus*), or whether it is a polyphyletic morass within which these genera are nested. By including representatives of other tribes, these data also will allow a re-assessment of the tribe Hibisceae.

MATERIALS AND METHODS

Sampling. Sequences from the cpDNA regions *ndhF* and the *rpl16* intron were generated for 39 and 63 taxa respectively, and to these 20 and nine sequences, respectively, were added from GenBank (Table 3). The final matrix comprises *ndhF* sequences from 59 taxa, and *rpl16* intron sequences from 72 taxa. Of these, 41 sequences of each marker were from the same species, and six more were representatives from the same genus using different species, but in this case only in non-Hibisceae genera (Table 3).

The advantage of using both a protein-coding region and an intron to infer the phylogeny of chloroplasts lies in the differences in the way selection operates on the products of these regions. Selection on protein-coding regions (such as *ndhF*) occurs on the protein molecule, which is quite different from the encoding DNA, and is mediated by the peculiarities of codon position—amino acid translation. Group two introns (e.g., the *rpl16* intron) have strong functional constraints that restrict the type and location of changes in order to preserve their structure, from which they derive their function (Michel et al. 1989). Furthermore, the functional molecule is an RNA molecule, where a one to one correspondence between DNA substitutions and RNA differences occurs. If congruent signal is found despite the selection differences and possible sequence bias differences (e.g., 3rd codon position bias in genes [Olmstead et al. 1998]; stem vs. loop substitution and insertion/deletion bias in introns [Kelchner 2000]), we can have increased confidence that the topologies found accurately reflect the history of the chloroplasts.

Within *Hibiscus*, several species were sampled from the largest sections (*H. Sect. Azanzae* DC., *H. Sect. Bombicella* DC., *H. Sect. Furcaria* DC., *H. Sect. Ketmia* DC., *H. Sect. Lilibiscus* Hochr.), as well as one or two species from several smaller sections (*H. Sect. Ca-*

TABLE 4. Primers used in this study. PCR amplification primers are in bold.

	5' to 3' sequence	Reference
<i>rp116</i> intron		
F71	GCT ATG CTT AGT GTG TGA CTC GTT G	Jordan et al. 1996
F627	CGG AAC AAA CCA GAG ACC AC	Seelanan et al. 1999
R699	TCG CGG GCG AAT ATT TAC	Seelanan et al. 1999
R1516	CCC TTC ATT CTT CCT CTA TGT TG	Kelchner and Clark 1997
<i>ndhF</i>		
F1	GAA TAT GCA TGG ATC ATA CC	Seelanan et al. 1997
F803	CTA TGG TAG CGG CGG GAA TTT TTC	Olmstead and Sweere 1994
R972	CAT CAT ATA ACC CAG TTG GGA C	Olmstead and Sweere 1994
R1318	CGA AAC ATA TAA AAT GC(AG) GTT AAT CC	Olmstead and Sweere 1994

lyphylli Ulbr., *H. Sect. Hibiscus*, *H. Sect. Panduriformes* Ulbr., *H. Sect. Solandra* Hochr., *H. Sect. Spatula* Hochr., *H. Sect. Striati* O.J. Blanch., *H. Sect. Trionum* DC.) to encompass as much morphological variation as possible (Table 2). Most species from Hibisceae genera suspected of representing early divergences also were sampled (four of five *Alyogyne*, one of two *Radyera*, one of one *Lagunaria*), as well as those genera which have been placed elsewhere at some time (one of three *Campostemon* Mast., one of one *Howittia*). Representatives of *Decaschistia* and several Hibisceae which may be derived from within *Hibiscus* were sampled (*Abelmoschus*, *Floria*, *Macrostelia* Hochr.), as well as several representatives of Malvaceae (*Anotea* Kunth, *Malvaviscus* Fabr., *Pavonia* Cav.). Gossypieae and Malveae also were sampled, although less thoroughly, as these tribes have been more comprehensively examined in previous studies and appeared to be monophyletic based on cpDNA evidence (La Duke and Doebley 1995; Seelanan et al. 1997). Given the long-standing ambiguities regarding the division between Bombacaceae and Malvaceae, several potential outgroups from within Bombacaceae were sampled based on work by Alverson et al. (1998, 1999) and Bayer et al. (1999).

Leaf tissue used for DNA extraction came from a variety of sources, either fresh from glasshouse grown plants, from previously frozen fresh collections, from silica gel or from saturated NaCl—CTAB (hexadecyltrimethylammonium bromide [Rogstad 1992]) collections. Seeds, cuttings, or leaf material came from our own or our colleagues' field collections, the United States Department of Agriculture (USDA) seed store, or the following botanic gardens: Darwin Botanic Gardens, Brisbane Botanic Gardens, Australian National Botanic Gardens (Australia) and Punjab Agricultural University (India).

DNA Extraction and Sequencing. Genomic DNA was extracted as follows: 80–100 mg of leaf tissue was ground in liquid N₂ in 1.5 ml eppendorf tubes, followed by 20 min. incubation at 65° in 1 ml of CTAB lysis buffer (Paterson et al. 1993) and two chloroform: isoamyl (24:1) extractions, followed by precipitation of the DNA with 100% ethanol. Fresh material of some species (such as *Abelmoschus* and some *Hibiscus*) and herbarium or silica-dried material proved more difficult as a source of usable DNAs, so the DNeasy kit (QIAGEN, Germany) was used on this material. Polymerase chain reactions (PCR) were performed using a Hybaid Express machine. For *ndhF* 30 cycles of 95°C (1 min), 48°C (1 min), 72°C (1 min 45 sec, with a time increase of 3 sec per cycle), was followed by a final 72°C (1 min) step. For *rp116*, a touchdown program began with a single cycle of 94°C (4 min), followed by 10 cycles of 94° (25 sec), 55–50° (25 sec) touchdown (-0.5°C per cycle), with a slow ramp increase of 0.25°C per sec, 65° (3 min). This was followed by a further 20 cycles of 94°C (25 sec), 50°C (25 sec), with a slow ramp increase of 0.25°C per sec, 65° (3 min), followed finally by a single cycle of 65°C (3 min). Sequence data were obtained by sequencing a cleaned (Qiaquick PCR cleanup kit—QIAGEN, Germany), PCR-amplified product using the primers shown in Table 4. Sequences were read on an ABI 377 Prism automated sequencer operated by CSIRO Plant Industry, Black Mountain. Possible sequence misreads were checked by comparison of the forward strand with the reverse strand sequence in all cases.

Alignment. Sequences were aligned manually using the pro-

gram SeqLab (Wisconsin Package Version 10.1, Genetic Computer Group, Madison, Wisc.). The *ndhF* alignment was trivial, as no insertions or deletions (indels) were inferred in the first 1260 bp of the gene (the portion of the gene sequence obtained in this study). The *rp116* intron alignment was modified slightly to accommodate the integrity of conserved regions involved in secondary and tertiary interactions, which is crucial for preservation of the group two chloroplast intron self-splicing mechanism (Michel et al. 1989). Four *rp116* intron regions that could not be aligned unambiguously were excluded from the analysis: the D3 bulge in domain I and three small parts of the domain IV loop. Inferred indels in the *rp116* intron data were encoded as binary characters at the end of the aligned sequence. These characters were each given the same weight as a single nucleotide position. The alignments are available on request.

Analysis. Equally-weighted parsimony was employed as the tree assessment criterion. The search for most parsimonious (MP) trees was conducted in two stages using PAUP* (4.0b6) (D. Swofford; Sinauer Associates, Inc. Publishers). 1) A heuristic search using tree bisection-reconnection (TBR) and MULPARS (which saves all MP trees, rather than only one from each replicate), 100 random addition sequence (RAS) replicates (two trees held at each step) holding a maximum of 100 trees at each replicate, branches were collapsed if the maximum branch length=0. The best trees from each RAS replicate were saved, and used as starting trees in the second search. 2) A heuristic search using TBR and MULPARS. While the second search was limited to 10,000 trees, branch swapping was completed on all of these trees. As the tree number was limited (meaning that there may be some unwarranted resolution in the strict consensus trees), only branches with significant bootstrap support values should be considered when inferring phylogenetic relationships.

The two data sets were initially analyzed separately, and then combined by adding the sequences together end-to-end in one matrix and then analyzed as above. Each nucleotide from both regions and the inferred indels from the *rp116* intron were treated equally in the combined analysis. A partition homogeneity test (implemented in PAUP*) was used to determine whether the partitions were providing different signal in the combined analysis.

To assess the relative measure of clade support in the topologies, a bootstrap (Felsenstein 1985; BS) search was conducted using 20 RAS replicates, with 100 bootstrap replicates.

Using the combined data set, a (T-PTP) test (Faith 1991; implemented in PAUP*) was used to test the hypothesis that those genera with copious endosperm (*Alyogyne*, *Lagunaria*, and *Radyera*) are excluded from the rest of the family. The monophyly of the rest of the family was therefore used as a constraint tree. *Howittia*, which is sister to *Lagunaria* in each analysis with strong support (see results), but upon examination was found to be lacking copious endosperm, was included in the copious-endosperm group of genera in this test.

Morphology. The unexpected placement of several genera unresolved at the base of the family in the cpDNA trees (see results, Figs. 1–3) prompted us to investigate a selection of 11 morphological characters for taxa which have not all been examined before (Table 5). The characters used were those which provided infor-

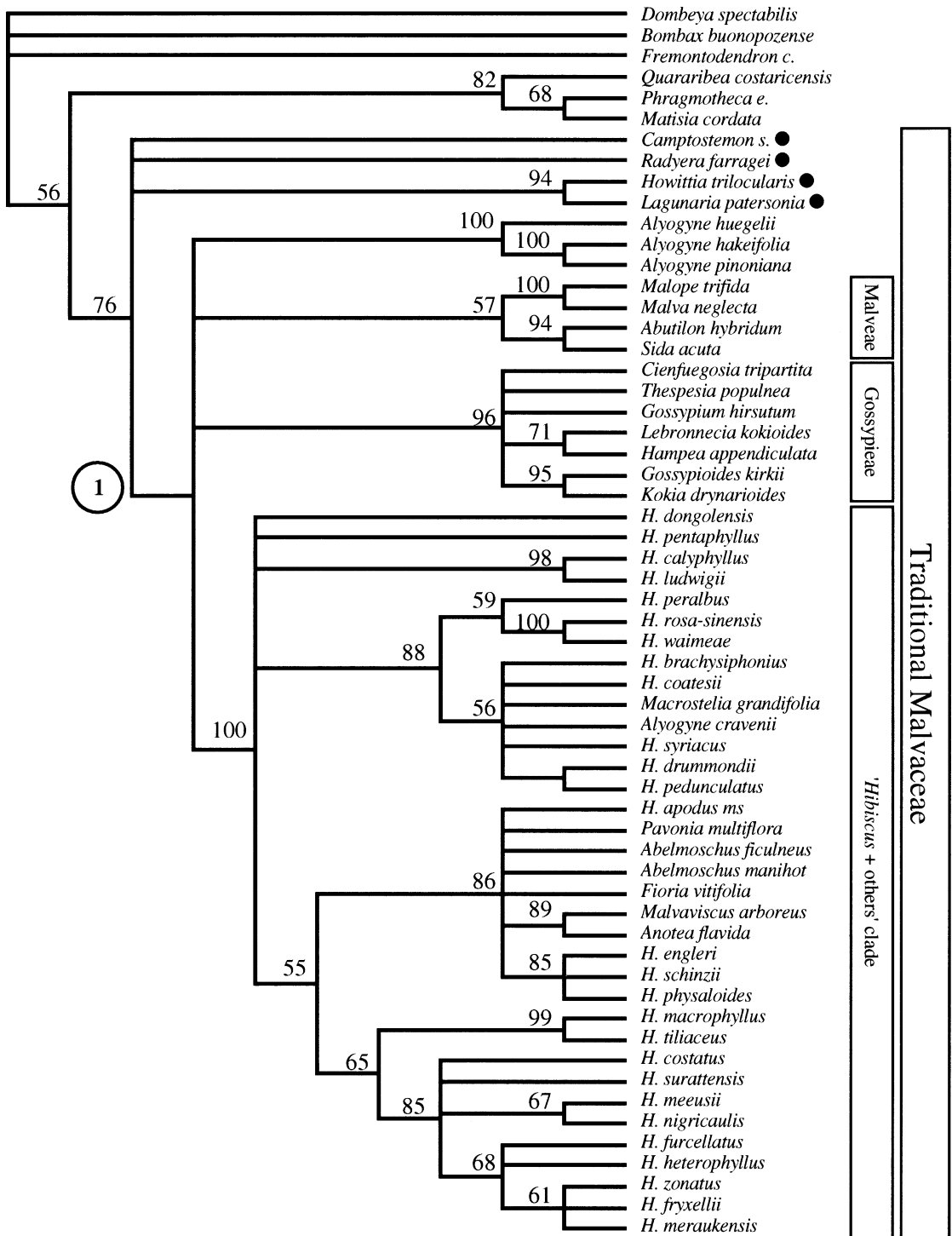


FIG. 1. Strict consensus cladogram of 10,000 MP trees found using *ndhF*. MP trees are each 338 steps, CI=0.76 and RI=0.86. Traditional Malvaceae sensu Hutchinson (1967). *Camptostemon*, which forms a monophyletic group with all of the malvaceous genera in this analysis, has since been transferred to Hibisceae (Malvaceae) by Fryxell (1968). Early-diverging genera marked by solid circles are discussed in text. Major clades corresponding with tribes Gossypieae and Malveae are boxed at the right, whereas the clade containing all *Hibiscus* and several Hibisceae genera is also boxed at the right. Bootstrap support for each branch above 50% is shown. The node marked by a circled number one is discussed in the results.

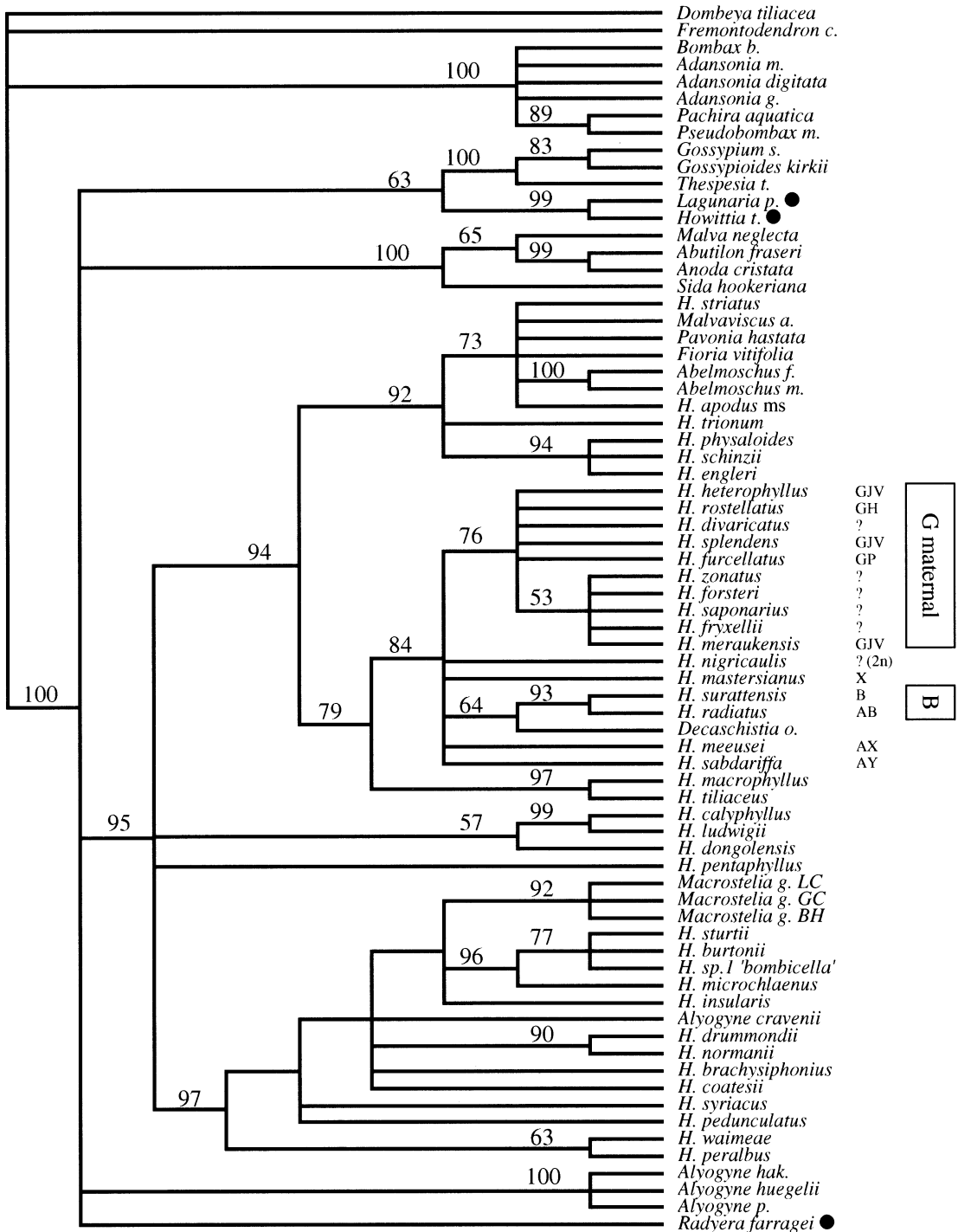


FIG. 2. Strict consensus cladogram of 6,279 MP trees found using the *rpl16* intron. MP trees are each 371 steps, CI=0.82 and RI=0.91. Chromosome and chloroplast groups of *Hibiscus* section *Furcaria* are indicated (for chromosome groups one letter, e.g., 'B' indicates a diploid, two letters a tetraploid, three a hexaploid; for chloroplast groups see discussion). Early-diverging genera marked by solid circles are discussed in text. Bootstrap support for each branch above 50% is shown.

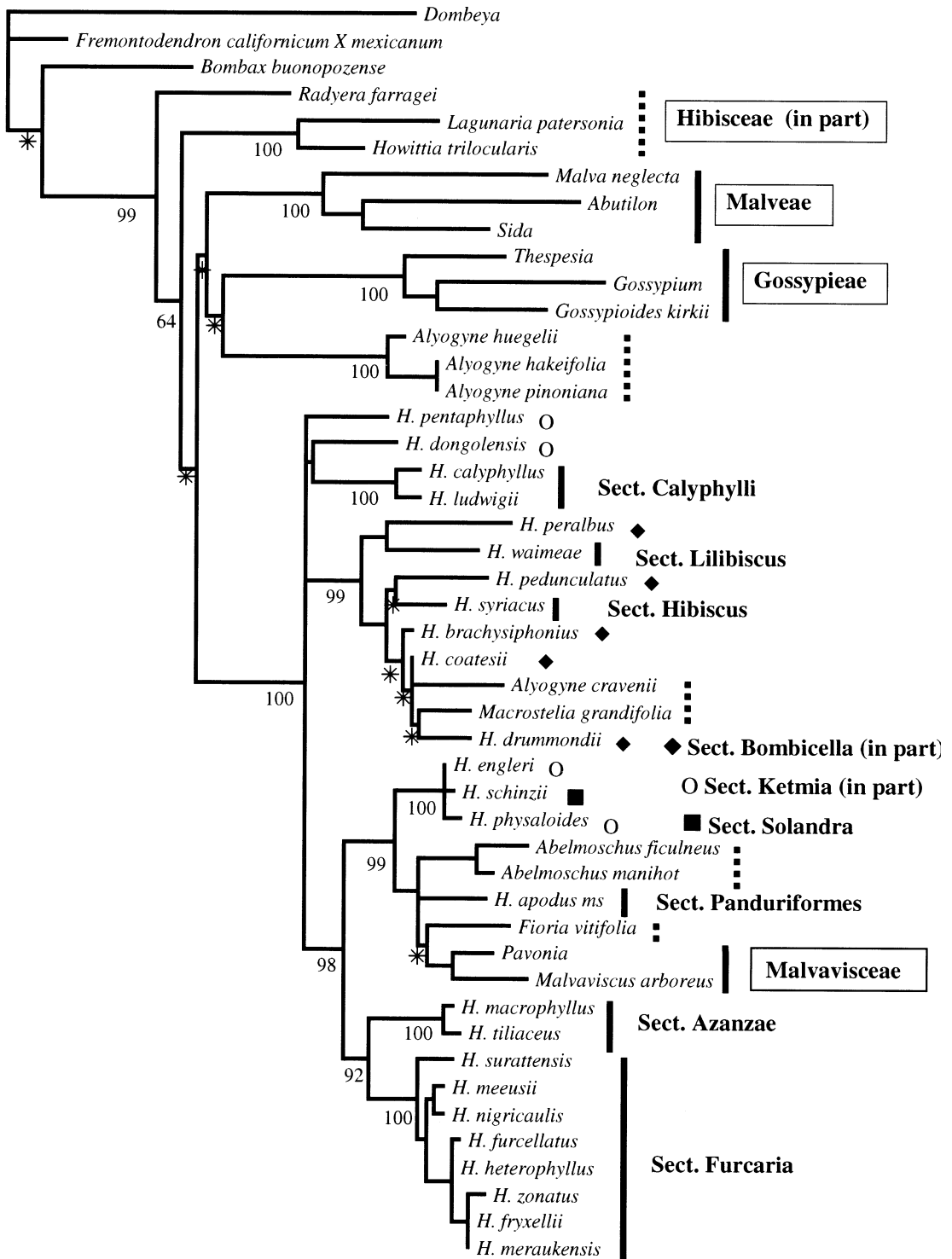


FIG. 3. Phylogram of a randomly selected MP tree found using the combined data sets of *ndhF* and the *rpl16* intron. Branches marked by an asterisk collapse in the strict consensus of 2,982 MP trees. MP trees are each 587 steps, CI=0.81 and RI=0.87. Branch lengths are estimated using ACCTRAN optimisation. Bootstrap support for each branch above 50% is shown. Tribal placements to the right of clades are boxed, sections of *Hibiscus* are not boxed. Hibisceae genera are indicated by dashed vertical lines. Those genera without epithets are a composite of sequences of two different species from that genus (see Table 3).

TABLE 5. Morphological data matrix. Species marked by an asterisk have data derived from different species for morphology and *ndhF*. The species listed here are the source of the morphological information. States for the characters are as follows: 1) Staminal Column Teeth: 0 = absent, 1 = present; 2) Pollen Spines: 0 = absent, 1 = present; 3) Pollen Aperture Distribution: 0 = zono, 1 = panto; 4) Pollen Aperture: Type 0 = porate, 1 = colpate, 2 = colpate; 5) Styles: 0 = confluent, 1 = divided; 6) Stigmata Shape: 0 = filiform, 1 = lobed or flat, 2 = capitate, 3 = club-shaped; 7) Stigmata Position: 0 = interior, 1 = top, 2 = top and exterior; 8) Hypocotyl: 0 = straight, 1 = bent; 9) Endosperm: 0 = copious, 1 = reduced or absent; 10) Gossypol Glands: 0 = absent, 1 = present; 11) Pollen Shape: 0 = triangular, 1 = oblate or spherical. Multiple states indicate a polymorphic taxon. Pollen terminology sensu Erdtman (1969). Approximately 26% of the matrix is missing (unknown).

Taxon	1	2	3	4	5	6	7	8	9	10	11
<i>Dombeya tiliacea</i> *	0	1	?	0	1	0	0	0	0	0	?
<i>Bombax ceiba</i> *	0	0	0	2	1	0	0	0	0	0	0
<i>Fremontodendron californicum</i> × <i>mexicanum</i>	0	0	?	?	0	0	0	0	0	0	?
<i>Quararibea costaricensis</i>	1	0	0	0	0	1	1	?	?	0	1
<i>Phragmotheca ecuadorensis</i>	?	?	?	?	0	1	1	?	?	0	?
<i>Matisia cordata</i>	0	0	0	1	0	2	1	?	?	0	1
<i>Campostemon schultzei</i>	0	1	0	1	0	1	1	0	0	0	1
<i>Hibiscus costatus</i>	?	1	?	?	1	2	1	1	1	0	1
<i>H. surattensis</i>	1	1	1	?	1	2	1	?	?	0	1
<i>H. meeusei</i>	?	1	?	?	1	2	1	1	1	0	1
<i>H. nigricaulis</i>	?	1	?	?	1	2	1	?	?	0	1
<i>H. furcellatus</i>	1	1	?	?	1	2	1	1	1	0	1
<i>H. zonatus</i>	?	?	?	?	1	2	1	?	?	0	?
<i>H. heterophyllus</i>	1	1	1	?	1	2	1	?	?	0	1
<i>H. fryxellii</i>	?	1	?	?	1	2	1	?	?	0	1
<i>H. meraukensis</i>	1	1	?	?	1	2	1	1	?	0	1
<i>H. dongolensis</i>	?	?	?	?	1	2	1	?	?	0	?
<i>H. apodus</i> ms	1	1	?	?	1	2	1	1	1	0	1
<i>H. macrophyllus</i>	1	?	?	?	1	2	1	?	?	0	?
<i>H. tiliaceus</i>	1	1	?	?	1	2	1	1	?	0	1
<i>Pavonia hastata</i> *	1	1	1	0	1	2	1	1	1	0	1
<i>Malvaviscus arboreus</i>	1	1	1	0	1	2	1	1	?	0	1
<i>Anotea flavida</i>	1	1	?	?	1	2	1	?	?	0	1
<i>Abelmoschus ficulneus</i>	1	1	1	0	1	2	1	1	?	0	1
<i>A. manihot</i>	1	?	?	?	?	2	1	?	?	0	?
<i>Fioria vitifolia</i>	1	1	1	0	1	2	1	1	1	0	1
<i>H. engleri</i>	1	1	?	?	1	2	1	?	?	0	1
<i>H. schinzii</i>	1	1	?	?	1	2	1	1	1	0	1
<i>H. physaloides</i>	1	1	?	?	1	2	1	?	?	0	1
<i>H. brachysiphonius</i>	?	1	?	?	1	2	1	1	1	0	1
<i>H. coatesii</i>	1	1	?	?	1	2	1	?	?	0	1
<i>Macrostelia grandifolia</i> Leo Creek	1	1	?	?	1	2	1	?	?	0	1
<i>H. drummondii</i>	1	1	?	?	1	2	1	1	?	0	1
<i>Alyogyne cravenii</i>	1	1	?	?	1	2	1	1	1	0	1
<i>H. pedunculatus</i>	1	1	?	?	1	2	1	1	1	0	1
<i>H. syriacus</i>	1	1	1	0	1	2	1	?	?	0	1
<i>H. pentaphyllus</i>	1	1	1	0	1	2	1	1	?	0	1
<i>H. peralbus</i>	?	?	?	?	1	2	1	1	1	0	?
<i>H. rosa-sinensis</i>	1	1	1	0	1	2	1	?	?	0	1
<i>H. waimeae</i>	1	1	?	?	1	2	1	?	?	0	1
<i>H. calyphyllus</i>	1	1	?	?	1	2	1	1	1	0	1
<i>H. ludwigii</i>	1	1	?	?	1	2	1	1	1	0	1
<i>Alyogyne huegelii</i>	0	1	1	1	0	1	1	1	0	0	1
<i>Alyogyne hakeifolia</i>	0	1	1	1	0	3	2	1	0	0	1
<i>Alyogyne pinoniana</i>	0	1	1	1	0	1	1	1	0	0	1
<i>Cienfuegosia tripartita</i>	1	1	1	0	0 & 1	?	1 & 2	0	1	1	1
<i>Lebronnecia kokoioides</i>	?	?	?	?	?	?	?	0	1	1	?
<i>Hampea appendiculata</i>	?	?	?	?	?	?	?	0	1	1	?
<i>Thespesia populnea</i>	1	1	1	1	0	3	2	0	1	1	1
<i>Gossypoides kirkii</i>	?	?	?	?	?	?	?	?	?	1	?
<i>Kokia drynarioides</i>	1	1	?	?	0	?	?	?	?	1	1
<i>Gossypium sturtianum</i> *	1	1	1	1	0	3	2	0	1	1	1
<i>Malope trifida</i>	?	1	1	0	?	?	?	1	1	0	1
<i>Malva neglecta</i>	0	1	1	0	1	0	0	1	1	0	1
<i>Abutilon fraseri</i>	0	1	0	1	1	2	1	?	?	0	1
<i>Sida acuta</i>	0	1	0 & 1	0 & 1	1	2	1	1	0	0	1
<i>Howittia trilocularis</i>	0	1	?	?	0	1	1	1	1	0	1
<i>Radyera farragei</i>	0	1	0	1	0	1	1	0	0	0	1
<i>Lagunaria patersonia</i>	0	1	1	1	0	1	1	1	0	0	1

mation regarding relationships at the base of the Malvaceae or its immediate sister groups. Characters have been drawn from the androecium, gynoecium, seed, and pollen. Character information was drawn from the following sources: Nilsson and Robyns (1974), van Heel (1966), Fryxell (1968, 1988), Fryxell and Hashmi (1971), Robyns (1964), Muller (1981), Christensen (1986), Alverson (1989, 1991), Judd and Manchester (1997), and our own scanning electron and light microscopic observations. Some of the characters investigated have previously been used in tribal classifications, but may not have been reported for all of those genera found here to be occupying unusual positions. Other characters have been discussed in the literature previously, but their taxonomic implications have not been fully appreciated. As the morphological data set is limited, and chosen for a particular purpose, it was analysed in combination with the *ndhF* data (for which more taxa at the base or sister to the family were available than in the *rpl16* data). Doyle's (1992) method of combining the morphology with DNA sequence data coded as a single ordered multistate character was investigated, as well as a standard combination of data sets (the matrices simply combined end to end). We coded only those nodes with BS support of >75% as single character state changes in implementing Doyle's method.

RESULTS

The *ndhF* data set included 1260 bp of sequence at the 5' end of the exon (beginning from position 40 in the *Gossypium hirsutum* L. sequence, U55340; Seelanan et al. 1997) and contained 112 parsimony-informative characters. Approximately 0.7% of the data matrix was scored as missing (due to uncertain sequence reads). The *rpl16* intron included 1847 bp of aligned sequence. After several highly variable regions were excluded (see methods), this resulted in 1226 bp of unambiguously aligned sequence and 47 coded indels which were used together in all analyses of this data set. This sequence starts from the 15th nucleotide of the domain I 5' bulge and includes 39 nucleotides of the following exon at its end. The 5' bulge interrupts the domain I 5' stem between positions five and six (the 5th position corresponds to the second last nucleotide of the F71 primer [Table 4]). Including the indels, the *rpl16* intron data set contained 150 parsimony-informative characters. Approximately 0.2% of this data matrix was scored as missing (as above).

Strict consensus cladograms of the 10,000 trees found for the *ndhF* data set and the 6,279 trees found for the *rpl16* intron data set are presented (Figs. 1, 2). The consistency index (CI) and retention index (RI) values for these MP trees are 0.76 and 0.86 for *ndhF* and 0.82 and 0.91 for *rpl16* respectively. A randomly selected MP phylogram for the combined analysis is also shown (Fig. 3). The CI and RI values for this MP tree are 0.81 and 0.87. Branches which collapse in the strict consensus cladogram are indicated. A strict consensus cladogram of 10,000 trees found for morphology combined with *ndhF* is presented in Figure 4. The CI and RI values for these MP trees are 0.74 and 0.86. BS values above 50% are indicated on the strict consensus trees (Figs. 1–4) above the relevant clades.

ndhF. The strict consensus tree (Fig. 1) shows several novel results. All species of *Hibiscus* are grouped

in one clade; however, nested within this clade are all three representatives of Malvaceae as well as several genera of Hibisceae (*Abelmoschus*, *Fioria*, *Macrostelia*). One species of *Alyogyne* (*A. cravenii* Fryxell) is also nested within the *Hibiscus* clade, while its congeners form a separate monophyletic group. The remaining Hibisceae (*Camptostemon*, *Howittia*, *Lagunaria*, *Radyera*) form a polytomy at the base of the family, which is monophyletic in this tree. As expected from previous studies, the Gossypieae and Malveae are monophyletic (Seelanan et al. 1997; La Duke and Doebley 1995).

Of those sections of *Hibiscus* with multiple species sampled, some form monophyletic groups (*H. Sect. Furcaria*, *H. Sect. Azanzae*, *H. Sect. Lilibiscus* and *H. Sect. Calyphylli*), while others do not (*H. Sect. Bombicella* and *H. Sect. Ketmia*). All four species of *H. Sect. Bombicella* are in the same clade, but this clade also contains *Macrostelia grandifolia* Fryxell, *Alyogyne cravenii* (Hibisceae) and *Hibiscus syriacus* L. (*H. Sect. Hibiscus*).

rpl16. The results from the *rpl16* intron are largely consistent with those of *ndhF* (Fig. 2). As in *ndhF*, a clade containing all *Hibiscus* also contained taxa from other genera (Hibisceae: *Abelmoschus*, *Alyogyne cravenii*, *Fioria*, *Macrostelia*; Malvaceae: *Malvaviscus*, *Pavonia*). *Decaschistia occidentalis* Craven & Fryxell, the only representative of the tribe Decaschistieae available, was nested within a sub-clade containing all sampled members of *H. Sect. Furcaria*. One slight difference is the position of lineages at the base of the family. *Radyera* and *Alyogyne* are part of the basal polytomy that includes each major lineage in the family. *Lagunaria* and *Howittia* form a monophyletic group with Gossypieae, although there is only one character supporting this group. A *Camptostemon* sequence was not available for this data set.

Combined cpDNA Data. The partition homogeneity test found no significant difference between the *ndhF* and *rpl16* intron partitions of the combined data set ($p > 0.05$). Furthermore, the strict consensus tree produced by combining the data sets agrees with the trees produced by separate analysis (Fig. 3). The data presented here provide a robust estimation of the phylogenetic relationships of taxa in traditional Malvaceae. In particular, the congruence between the genic and intron cpDNA regions provides compelling evidence of relationships despite the sequence biases known to occur in these regions (see methods).

A minor difference is in the placement of the early divergent lineages. *Radyera* is sister to the rest of the family, whilst Gossypieae, Malveae, the *Hibiscus* clade, the *Lagunaria* + *Howittia* clade, and the *Alyogyne* clade form a polytomy (Fig. 3). *Camptostemon* was not included in this analysis, as only an *ndhF* sequence was available. Whilst the placement of *Radyera* sister to the rest of the family is very weakly supported (the monophyly of the rest of the family has 64% BS), the mono-

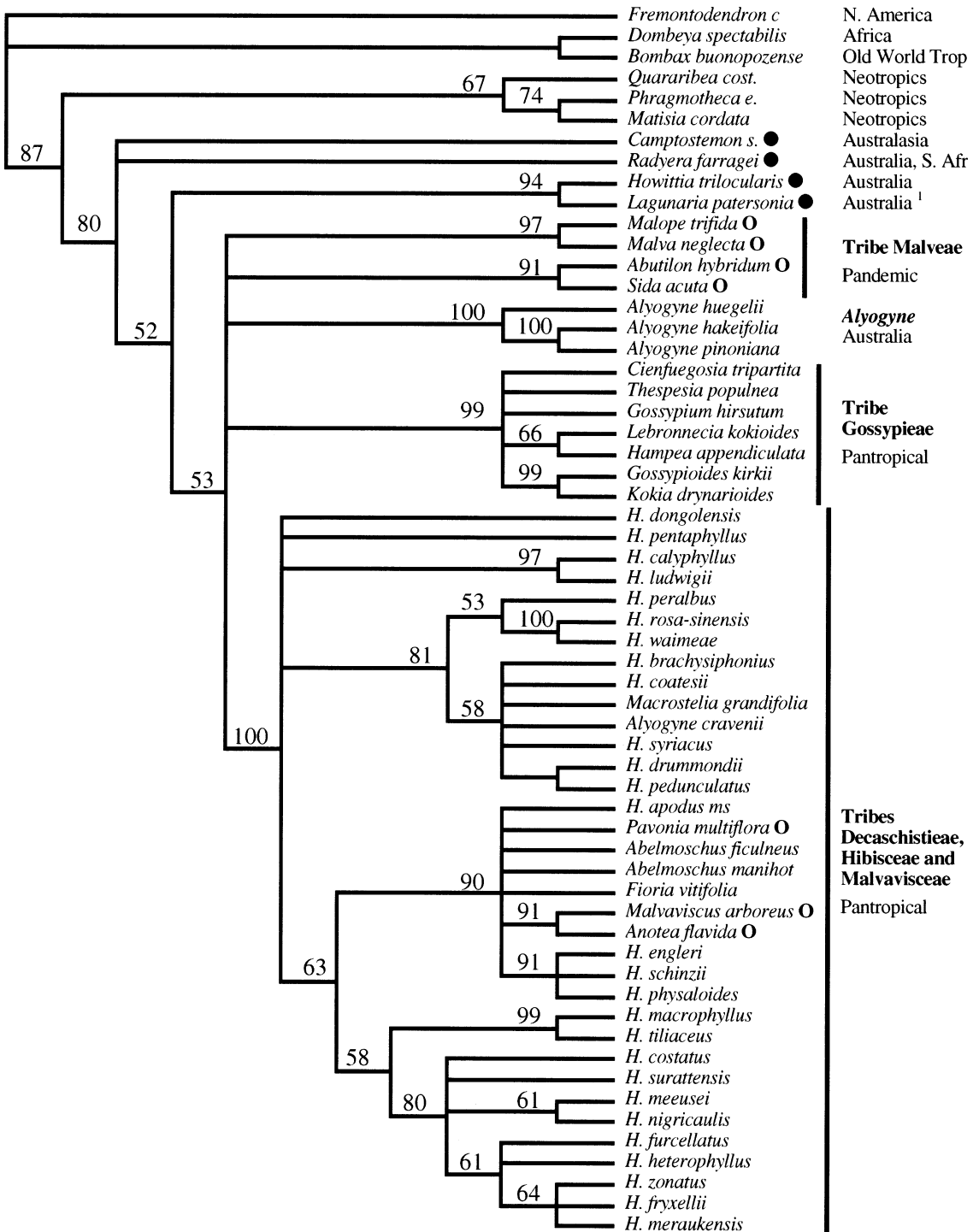


FIG. 4. Strict consensus cladogram of 10,000 MP trees found using 1260 *ndhF* sites and 11 morphological characters. MP trees are each 374 steps, CI=0.74, RI=0.86. Bootstrap support for each branch above 50% is shown. Genera from those tribes with members which have predominantly or completely schizocarpic fruit are marked with open circles (see discussion). Early-divergent genera marked by solid circles are discussed in text. ¹*Lagunaria* is also present on the Tasman Sea islands of Lord Howe and Norfolk.

phyly of the other clades mentioned is very well supported, as shown by 100% BS. The monophyly of the family is also strongly supported, with 99% BS.

T-PTP Test for the Monophyly of the Family Excluding Copious-endosperm Genera. The T-PTP test showed that the family may be a monophyletic group either excluding the copious-endosperm genera or including some or all of the copious-endosperm genera, i.e., neither hypothesis can be excluded from consideration by the combined data set ($p < 0.05$).

Possible Long Branch Attraction. Several clades included taxa separated by long internal branches (Gossypieae, Malveae, and *Lagunaria* + *Howittia*; Fig. 3), and may be examples of long branch attraction, which can mislead the parsimony criterion (Swofford et al. 1996). This possibility was discounted for Gossypieae and Malveae, as they have been more comprehensively sampled in other cpDNA-based studies and found to be monophyletic (La Duke and Doebley 1995; Seelanan et al. 1997). Although increased taxon sampling is our preferred method for stabilizing long branches, and appears to do so in other groups (e.g., Mirbelieae [Fabaceae], M.D. Crisp unpubl. data), this is not an option for *Lagunaria* and *Howittia* as they are monotypic genera. Instead, an examination of the characters supporting the clade in the combined data set revealed 16 synapomorphies, of which 11 showed no homoplasy. These 11 apparently unique synapomorphies included one complex inserted sequence of eight base pairs in IC region of the *rpl16* intron: TCAAGAAA (gap 14). This insertion is not a repeat of the immediately adjacent sequence, nor does the immediately adjacent sequence appear to create a stem that might cause a mutational trigger at this site (Kelchner and Clark 1997). Furthermore, the 16 synapomorphies for the clade are fairly evenly distributed between the two regions (nine from *rpl16* and seven from *ndhF*), suggesting that one region alone is not supplying a spurious signal.

Morphology Combined with *ndhF*. A combined analysis of *ndhF* and morphology (Fig. 4) added some resolution of the basal nodes in Malvaceae, compared with *ndhF* alone (Fig. 1), or morphology combined with *ndhF* coded as a single ordered multistate character (the method of Doyle 1992; not shown). This suggests that the *ndhF* data contain some information regarding the basal nodes in Malvaceae that is consistent with the morphology, but that this information is lost when only well supported nodes (BS > 75%) on the *ndhF* strict consensus tree are encoded as single character state changes for incorporation into a combined analysis with morphology.

In this analysis, three lineages, *Camptostemon* and *Radyera* and the remainder of the family are unresolved at the earliest node. Within the remainder of the family, *Lagunaria* and *Howittia* form a well-supported clade sister to the rest. The clade that includes *Lagunaria*,

Howittia, and the rest of the family, as well as the clade that includes the rest of the family alone are both only weakly supported (52% and 53% BS respectively), however, they have more support than the corresponding clade in the *ndhF* tree (Fig. 1, clade one [circled]; <50% BS). Additionally, the support for the clade that includes the traditional Malvaceae and its sister group (*Matisia*, *Phragmothea*, together with *Quararibea*) is markedly increased in this analysis (from 56% to 87% BS).

DISCUSSION

The Tribe Hibisceae is not Monophyletic. The data presented here illustrate clearly the paraphyly of Hibisceae. This finding confirms the predictions made by our reevaluation of morphological features of the genera currently placed in this tribe. As predicted, the copious-endosperm genera (*Alyogyne*, *Lagunaria*, and *Radyera*) diverge early from the remainder of the family, while other genera in Hibisceae occupy a more derived position, nested within *Hibiscus*. *Camptostemon* and *Howittia*, for which endosperm information was not previously available, also diverge early. *Camptostemon* had, however, been placed close to other Malvaceae in a previous analysis (Alverson et al. 1999). Seed of this genus has since been found to contain copious endosperm, supporting an early divergence, while the endosperm of *Howittia* is much reduced.

Morphology can Resolve the Basal Polytoamy in the cpDNA Trees. Additional resolution regarding the early divergences, although limited, was obtained by the combination of morphology and *ndhF* data sets. These results provide independent confirmation that the basal relationships found in the cpDNA data sets are reflective of the phylogenetic history of these taxa. This therefore provides additional support for the finding that the tribe Hibisceae is not monophyletic.

The taxa of Malvaceae, as traditionally circumscribed (Hutchinson 1967), almost all have spiny pollen. Spiny pollen also is shared with the early-diverging genera in this analysis (*Camptostemon*, *Radyera*, *Howittia*, and *Lagunaria*) but not by the sister group of the family found here (*Matisia*, *Phragmothea*, and *Quararibea*). As very few genera from the other core Malvacean families possess spiny pollen, this character appears to be useful in delimiting the family in the traditional classification.

Malvaceae may have an Australasian Origin. The morphology of pollen grains also was useful in estimating a possible minimum age of the family, given that Malvaceae almost certainly arose from a shared ancestor with some members of the Bombacaceae. Pollen of the *Bombax* L. type, which includes pollen from *Fremontodendron* Cov., *Bernoullia* Oliv., *Ochroma* Sw., and *Pseudobombax* Dugand (which are among the genera near to the base of the Malvaceae; Alverson et al. 1999),

is the oldest Bombacaceae type to be found (Muller 1981). This pollen type has been found in south-eastern U.S.A. from the Maastrichtian (69–65 mya), in northern South America from the Paleocene (65–55 mya) and in Africa and Australia from the early Eocene (55–50 mya; Wolfe 1975; Muller 1981). Fossil pollen that can be assigned to Malvaceae is more recent, and has been found in South America from the late Eocene (44–39 mya), in Australia from the late Eocene—early Oligocene boundary (around 37–35 mya), and in Africa and Europe from the Oligocene (39–22 mya; Muller 1981; MacPhail and Truswell 1989). While the oldest malvaceous fossil pollen appears to be South American, differences in authors' uses of epoch dates coupled with the general difficulties in making reliable age estimates from stratigraphic inferences (Hill et al. 1999) necessitates that these dates be considered tentative. Therefore, we feel that both the South American (late Eocene) and Australian (late Eocene—early Oligocene boundary) fossil pollen could be considered candidates for representing the earliest Malvaceae pollen so far found.

Based on fossil pollen ages, Malvaceae is at least 39–44 million years old. The family is almost certainly older, as this age is of *Hibiscus*-type pollen, which is more derived than the pollen type from the earliest diverging malvaceous lineages (*Camptostemon* and *Radyera*). The latter genera possess pollen whose apertures are equatorially distributed and few in number (3–11), rather than scattered and usually more numerous (some >100) in most *Hibiscus*. It is not entirely clear from Muller (1981), but it seems that the pollen type considered by him to belong to Malvaceae (and reported as such in his review of fossil pollen literature) does not show the equatorial apertures present in *Camptostemon* and *Radyera*.

The current distributions of malvaceous taxa suggests that the family may have had its origin in that part of Gondwana that is now Australia and/or Antarctica. The sister group to Malvaceae found here (*Matisia*, *Phragmotheca*, and *Quararibea*) is Central and South American, while early divergence events in the family have produced several lineages that are restricted in distribution to Australasia (*Camptostemon*, *Howittia*, *Lagunaria*, and *Radyera* - the last also in southern Africa), the more recently derived of these lineages (*Howittia* + *Lagunaria*) being sister to the much more widely distributed remainder of the family (Fig. 4). The connection from the South American through the Antarctic to Australian portions of Gondwana is thought to have severed more recently by the opening of the Tasman and Drake Passage seaways (late Eocene—early Oligocene, c. 40–30 mya; Dingle and Lavelle 2000) than the age of the family as suggested by fossil pollen information (at least 39–44 my old). Therefore, a widely distributed common ancestor to the *Ma-*

tisia, *Phragmotheca*, and *Quararibea* clade together with Malvaceae probably diverged into these two lineages following the Gondwanan breakup. Further divergences in Australia produced some resident lineages, while one lineage dispersed and radiated extensively throughout the tropics. The warmer and wetter Eocene climate experienced by the southern Australian portion of Gondwana, despite its higher latitude, supported lowland rainforest communities (Hill et al. 1999), and thus may have allowed a pan-Gondwanan distribution of the ancestor of these present-day mostly tropical taxa.

An alternative explanation is that Malvaceae originated in South America after the Gondwanan breakup, where (arguably) the oldest pollen fossils have been found. However, as these fossils are of the more derived *Hibiscus* and *Thespesia* type (Muller 1981) than that possessed by the Australasian genera that diverge early, several long distance dispersal events to Australasia need to be invoked to explain the distribution of extant taxa and fossil pollen. The presence of these possibly older pollen records from South America and not Australia could simply be an artifact of the fossil record. On the balance of evidence available, the hypothesis that Malvaceae had an origin in the Australian and/or Antarctic portions of Gondwana is more plausible.

***Hibiscus* is Paraphyletic and Contains Other More Derived Genera.** A clade containing all species of *Hibiscus* thus far sampled is monophyletic; however, this clade also includes species from four other genera in Hibisceae, as well as four genera from other tribes (three from Malvaceae and one from Decaschiaceae). The nesting of other genera within *Hibiscus* is consistent with the presence of many features shared with *Hibiscus*, but the monophyly of the clade was unexpected. There are no clear morphological synapomorphies known for members of this clade, although possibly convergent gains of a) teeth atop the staminal column (shared with Gossypieae) and b) branching styles (shared with Malveae) may serve to delimit the group. With either of these tribes as sister to the *Hibiscus* clade, one or the other of these characters must exhibit homoplasy. Therefore no unique synapomorphy for the *Hibiscus* clade is known at this time. These findings show that the current tribal classification does not adequately reflect their evolutionary relationships. Furthermore, *Hibiscus* has been made paraphyletic by the segregation of several genera.

***Schizocarpus* has Evolved Twice.** Previous workers who advocated placing both capsular- and schizocarpic-fruited tribes in the one family did not explicitly suggest that the schizocarpic-fruited tribes were sister taxa. However, one taxonomist who restricted Malvaceae to the schizocarpic-fruited tribes (placing the capsular-fruited genera in Bombacaceae) did just that, im-

plying that schizocarpy was a synapomorphy (Edlin 1935). This hypothesis is not supported by cpDNA and morphological evidence presented here (e.g., Fig. 4). Our evidence shows that schizocarpy (if considered simply) must have arisen at least twice from within a clade of capsules.

Decaschistia. *Decaschistia* is a genus of c. 17 species, which extends from tropical Asia to Australia (Paul 1993). In our analyses, one Australian representative (*D. occidentalis*) is placed within a clade containing all of the species sampled from *Hibiscus* section *Furcaria* (Fig. 2). The Indian species appear to have 3-nerved calyx lobes, some prominently so (Sivarajan and Pradeep 1996), a feature that appears to link them with *H. sect. Furcaria*. However, none of these species is reported to have nectaries on the calyx mid-vein or bifurcate epicalyx lobes, one or the other feature being present in many species of *H. sect. Furcaria* (e.g., Wilson 1999). If the placement of this single species does reflect accurately the origin of *Decaschistia*, then the genus should not be maintained as a separate tribe, nor possibly as a separate genus, unless several parts of *H. sect. Furcaria* are also raised to generic rank. This is suggested on the assumption that taxon names in the Linnaean ranked classification contain most information when only monophyletic taxa are named—a point under debate. Further sampling of taxa and further investigation into the morphology of this genus is desirable to clarify this finding.

Hibiscus Section Furcaria. The genomes of *H. sect. Furcaria* have been intensively investigated, primarily by Menzel and Wilson over several decades (summarised in Wilson 1994). These authors (and their colleagues) designated chromosomal groupings of many diploid and polyploid taxa based on chromosome pairing (Wilson 1994). Where known, these groupings are indicated in Figure 2. The maternal origin of some allopolyploid taxa can be assigned based on the cpDNA phylogeny (Fig. 2). For example, *Hibiscus radiatus* Cav. is an allotetraploid that combines the A and B diploid genomes. However, the chloroplasts of this species are closely related to *H. surattensis* L., a B-genome diploid. Therefore, it seems highly likely that the *H. radiatus* clade arose from the hybridisation of a maternal B-genome species with an A-genome pollen donor.

Alyogyne cravenii Fryxell. The placement of *Alyogyne cravenii* in a clade with members of *Hibiscus* section *Bombicella* (Fig. 3) would appear to indicate that the species is generically misplaced. However, the possibility remains that this species may have acquired a *H. sect. Bombicella*-like chloroplast from a hybridisation event. Upon examination of several morphological features (Table 5) it was found that this species shares numerous features with other *H. sect. Bombicella* species (branching styles, capitate stigmata, staminal column teeth, reduced endosperm) that are not shared

with other species of *Alyogyne* sampled here, confirming the placement of this species in *Hibiscus*.

Macrostelia grandifolia Fryxell. *Macrostelia* is a genus of four species, three of which are endemic to Madagascar, whilst the fourth (which was most recently described; Fryxell 1974) is endemic to Australia. The Australian species, *M. grandifolia* (including samples from several morphologically distinct Australian populations), is unusual in its placement within *H. sect. Bombicella* (Figs. 2, 3). However, it is not known how the Madagascan species are related. The Australian species may not be congeneric with those of Madagascar (Fryxell also pointed out this possibility), but the testing of this hypothesis awaits further sampling.

Abelmoschus and Fioria. These two genera have previously been placed within *Hibiscus* (Hochreutiner 1900), although more recent treatments have considered them generically distinct (e.g., Sivarajan and Pradeep 1996). However, both genera are nested within a clade containing *Hibiscus* and *Malvaceae* (Fig. 3). If this position is substantiated by other evidence, these genera cannot be maintained unless *Hibiscus* is also subdivided into several genera.

In conclusion, the cpDNA evidence presented here shows that *Hibiscus* appears to be a paraphyletic group, containing elements of other genera in *Hibiscaceae*, as well as members of two other tribes, namely *Malvaceae* and *Decaschistieae*. Some of the sections of *Hibiscus* appear to form monophyletic groups, while others do not. The tribe *Hibiscaceae* was also found to be a paraphyletic group. Several genera that have usually been placed in this tribe instead diverge early from the remainder of the family. This result was supported by several morphological characters. Taken together, these lines of evidence show that the tribal classification does not adequately represent the evolutionary relationships of these genera.

There are several possible options for the amendment of the tribal classification. One solution would be to create a 'super' genus *Hibiscus* to accommodate the two tribes nested within it (incorporating c. 280 additional species). Alternatively, several (perhaps more than 10) new genera could be segregated, if the current generic limits in *Malvaceae* were accepted. Either situation would involve numerous name changes of well-known and commonly cultivated plants.

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LITERATURE CITED

- ALEFELD, F. G. C. 1861. Über die Stellung der Gattung *Gossypium* und mehrer Andrer. *Botanische Zeitung* 19: 299–301.
- ALVERSON, W. S. 1989. *Quararibea* (Bombacaceae): five new species from moist and wet forests of Costa Rica and Panama. *Brittonia* 41: 61–74.
- . 1991. A synopsis of *Phragmothea* (Bombacaceae), with two new species and a new subspecies. *Brittonia* 43: 73–87.
- , K. G. KAROL, D. A. BAUM, M. W. CHASE, S. M. SWENSEN, R. MCCOURT, and K. J. SYTSMAN. 1998. Circumscription of the Malvales and relationships to other Rosidae: evidence from *rbcL* sequence data. *American Journal of Botany* 85: 876–887.
- , B. A. WHITLOCK, R. NYFFELER, C. BAYER, and D. A. BAUM. 1999. Phylogeny of the core Malvales: evidence from *ndhF* sequence data. *American Journal of Botany* 86: 1474–1486.
- BAUM, D. A., R. L. SMALL, and J. F. WENDEL. 1998. Biogeography and floral evolution of Baobabs (*Adansonia*, Bombacaceae) as inferred from multiple data sets. *Systematic Biology* 47: 181–207.
- BAYER, C., M. F. FAY, A. Y. DE BRULIN, V. SAVOLAINEN, C. M. MORTON, K. KUBITZKI, W. S. ALVERSON, and M. W. CHASE. 1999. Support for an expanded family concept of Malvaceae within a recircumscribed order Malvales: a combined analysis of plastid *atpB* and *rbcL* DNA sequences. *Botanical Journal of the Linnean Society* 129: 267–303.
- BENTHAM, G. 1863. *Flora Australiensis* 1. London: Lovell Reeve.
- BORSSUM WAALKES, J. VAN 1966. Malesian Malvaceae revised. *Blumea* 14: 1–251.
- CHRISTENSEN, P. B. 1986. Pollen morphological studies in the Malvaceae. *Grana* 25: 95–117.
- CORRELL, D. S. and M. C. JOHNSTON. 1970. *Manual of the vascular plants of Texas*. Renner: Texas Research Foundation.
- DE CANDOLLE, A. P. 1824. *Prodromus Systematis Naturalis Regni Vegetabilis* 1. Paris: Sumptibus Sociorum Treuttel et Wurtz.
- DINGLE, R. V. and M. LAVELLE. 2000. Antarctic peninsula late Cretaceous—early Cenozoic palaeoenvironments and Gondwana palaeogeographies. *Journal of African Earth Sciences* 31: 91–105.
- DOYLE, J. J. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. *Systematic Botany* 17: 144–163.
- EDLIN, H. L. 1935. A critical revision of certain taxonomic groups of the Malvales Part II. *New Phytologist* 34: 122–143.
- ERDTMAN, G. 1969. *Handbook of palynology*. Copenhagen: Munksgaard.
- FAITH, D. P. 1991. Cladistic permutation tests for monophyly and nonmonophyly. *Systematic Zoology* 40: 366–375.
- FELSENSTEIN, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- FRYXELL, P. A. 1968. A redefinition of the tribe Gossypieae. *Botanical Gazette* 129: 296–308.
- . 1974. New species of *Gossypium*, *Decaschistia* and *Macrostelia* (Malvaceae) from Australia. *Australian Journal of Botany* 22: 183–193.
- . 1975. Generic relationships of *Decaschistia* (Malvaceae) and the description of a new tribe, Decaschistieae. *American Journal of Botany* 62: 172–175.
- . 1988. Malvaceae of Mexico. *Systematic Botany Monographs* 25.
- , and S. H. HASHMI. 1971. The segregation of *Radyera* from *Hibiscus* (Malvaceae). *Botanical Gazette* 132: 57–62.
- GARKE, A. 1849. Kritische Bemerkungen zu der Familie der Malvaceen, nebst Beschreibung neuer Arten aus derselben. *Botanische Zeitung* (Berlin) 7: 817–825, 833–841, 849–855.
- HEEL, W. A. VAN 1966. Morphology of the androecium in Malvales. *Blumea* 13: 177–394.
- HENNIG, W. 1966. *Phylogenetic systematics*. Urbana: Illinois Natural History Survey.
- HILL, R. S., E. M. TRUSWELL, S. MCLOUGHLIN, and M. E. DETTMANN. 1999. Evolution of the Australian flora: fossil evidence. Pp. 251–320 in *Flora of Australia* 2nd edn. 1, ed. A. E. Orchard. Melbourne: ABR/CSIRO Australia.
- HOCHREUTINER, B. P. G. 1900. Revision du genre *Hibiscus*. *Conservatoire et Jardin Botaniques Genève. Annuaire* 4: 23–191.
- . 1955. Malvacées. Pp. 1–170 in *Flore de Madagascar et des Comores* 129, ed. H. Humbert. Paris: Firmin-Didot.
- HUTCHINSON, J. 1967. *The genera of flowering plants (Angiospermae)*. Oxford: Clarendon.
- INSTITUTO DE BOTÁNICA DARWINION 1999. *Catálogo de las Plantas Vasculares de la Argentina*, ed. V. C. Hollowell. St Louis: Missouri Botanical Garden Press.
- JORDAN, W. C., M. W. COURTNEY, and J. E. NEIGEL. 1996. Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North American duckweeds (Lemnaceae). *American Journal of Botany* 83: 430–439.
- JUDD, W. S. and S. R. MANCHESTER. 1997. Circumscription of Malvaceae (Malvales) as determined by a preliminary cladistic analysis of morphological, anatomical, palynological, and chemical characters. *Brittonia* 49: 384–405.
- KELCHNER, S. A. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanic Garden* 87: 482–498.
- KELCHNER, S. A. and L. G. CLARK. 1997. Molecular evolution and phylogenetic utility of the chloroplast *rpl16* intron in *Chusquea* and the Bambusoideae (Poaceae). *Molecular Phylogenetics and Evolution* 8: 385–397.
- LA DUKE, J. C. and J. DOEBLEY. 1995. A chloroplast DNA based phylogeny of the Malvaceae. *Systematic Botany* 20: 259–271.
- LINNAEUS, C. 1753. *Species plantarum*. 1st edition facsimilie. London: Ray Society.
- . 1754. *Genera plantarum*. 5th edition facsimilie. Weinheim: Engelmann (Cramer) and Wheldon & Wesley, Ltd.
- MACPHAIL, M. K. and E. M. TRUSWELL. 1989. Palynostratigraphy of the central west Murray Basin. *BMR Journal of Australian Geology and Geophysics* 11: 301–331.
- MICHEL, F., K. UMESONO, and H. OZEKI. 1989. Comparative and functional anatomy of group II catalytic introns—a review. *Gene* 82: 5–30.
- MULLER, J. 1981. Fossil pollen records of extant angiosperms. *The Botanical Review* 47: 1–142.
- NILSSON, S. and A. ROBNS. 1974. Pollen morphology and taxonomy of the genus *Quararibea* s.l. (Bombacaceae). *Bulletin de Jardin Botanique National de Belgique* 44: 77–99.
- OLMSTEAD, R. G. and J. A. SWEERE. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Systematic Biology* 43: 467–481.
- , P. A. REEVES, and A. C. YEN. 1998. Patterns of sequence evolution and implications for parsimony analysis of chloroplast DNA. Pp. 164–187 in *Molecular systematics of plants II*, eds. D. E. Soltis, P. S. Soltis and J. J. Doyle. Boston: Kluwer Academic Publishers.
- PATERSON, A. H., C. L. BRUBAKER, and J. F. WENDEL. 1993. A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Molecular Biology Reporter* 11: 122–127.
- PAUL, T. K. 1993. Malvaceae. Pp. 257–394 in *Flora of India* 3, eds. B. D. Sharma and M. Sanjappa. Calcutta: Botanical Survey of India.
- REICHENBACH, H. T. L. 1828. *Conspectus Regni Vegetabilis*. Lipsiae: Carolus Cnobloch.

- RIEDEL, I. 1976. Malvaceae. Pp. 1–86 in *Flora Iranica* 120, ed. K. H. Rechinger. Graz: Akademische Druck.
- ROBYNS, A. 1964. Flora of Panama. Family 116. Bombacaceae. *Annals of the Missouri Botanic Garden* 51: 37–68.
- ROGSTAD, S. H. 1992. Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analyses. *Taxon* 41: 701–708.
- SEELANAN, T., A. SCHNABEL, and J. F. WENDEL. 1997. Congruence and consensus in the cotton tribe (Malvaceae). *Systematic Botany* 22: 259–290.
- , C. L. BRUBAKER, J. MCD. STEWART, L. A. CRAVEN, and J. F. WENDEL. 1999. Molecular systematics of Australian *Gossypium* section *Grandicalyx* (Malvaceae). *Systematic Botany* 24: 183–208.
- SIVARAJAN, V. V. and A. K. PRADEEP. 1996. *Malvaceae of southern peninsular India: a taxonomic monograph*. Delhi: Daya Pub. House.
- SWOFFORD, D. L., G. J. OLSEN, P. J. WADDELL, and D. M. HILLIS. 1996. Phylogenetic inference. Pp 407–514 in *Molecular Systematics*, eds. D. M. Hillis, C. Moritz, and B. K. Mable. Sunderland: Sinauer Associates.
- ULBRICH, E. 1921. Malvaceae. Pp. 368–408 in *Die Pflanzenwelt Afrikas 2*, ed. A. Engler. Leipzig: W. Engelmann.
- WILSON, F. D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. *Genetic Resources and Crop Evolution* 41: 13–25.
- . 1999. Revision of *Hibiscus* section *Furcaria* (Malvaceae) in Africa and Asia. *Bulletin of the Natural History Museum, London (Botany)* 29: 47–79.
- WOLFE, J. A. 1975. Some aspects of plant geography of the late Northern hemisphere during the late Cretaceous and Tertiary. *Annals of the Missouri Botanical Garden* 62: 264–279.