

## ON THE ORIGIN OF THE FIG: PHYLOGENETIC RELATIONSHIPS OF MORACEAE FROM *NDHF* SEQUENCES<sup>1</sup>

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The majority of species in the mulberry family (Moraceae) are figs (*Ficus*), marked by a specialized inflorescence (syconium) and an obligate mutualism with pollinating fig wasps. Because of the unique morphology of the syconium, it has been difficult to investigate the evolutionary position of the fig. We sequenced the chloroplast gene *ndhF* to examine relationships in Moraceae and to elucidate shifts in reproductive traits. The reclassification of tribes is warranted, and the limits of Artocarpeae, Moreae, and Castilleae are revised to reflect evolutionary relationships. The results point to ancestral dioecy in Moraceae and multiple origins of monoecy, androdioecy, and gynodioecy. Ancestral wind pollination gave way to insect pollination at least twice. Strong support for the sister-group relationship of a revised Castilleae with *Ficus* suggests that entomophily and involucre bracts encircling the flowers preceded the evolution of the syconium. Bracts surround flowers in Castilleae only during early development, but in *Ficus* the involucre and the receptacle enclose the fruit as well. Molecular dating suggests that fig pollination is at least 80–90 million years old. The diversity of *Ficus* relative to its sister group is a likely consequence of ancient specialization and cospeciation with pollinating fig wasps.

**Key words:** breeding systems; *Ficus*; molecular dating; Moraceae; phylogenetic classification; pollination.

The 37 genera of Moraceae have a striking array of inflorescence forms, pollination syndromes, and breeding systems (Fig. 1). Most of the 1100 species are figs (*Ficus*), known for their unique inflorescence and obligate pollination mutualism with fig wasps (Agaonidae). The fig inflorescence (syconium) is an urn-shaped receptacle lined with unisexual flowers and enclosed at the apex by involucre bracts (ostiole). Pollinating fig wasps negotiate these tightly arranged bracts to access the interior of the syconium where some of the flowers are galled and others develop into fruits. Ultimately, mutualism centers on the exchange of pollination services for the rearing of pollinator offspring. The remarkable specificity and complexity of the fig/pollinator interaction is widely appreciated and has often served as a model for the study of coevolution (reviewed in Weiblen, 2002; Cook and Rasplus, 2003; Joussetin et al., 2003). However, the origin of the fig inflorescence has remained a mystery because its unusual morphology is not easily related to other Moraceae inflorescences (Fig. 1). Different evolutionary pathways to the syconium have been suggested based on the diversity of inflorescences in the family. Corner (1978) speculated that the fig evolved from an urn-shaped receptacle resembling *Antiaropsis* or *Sparattosyce* (Fig. 1), while Berg (1989) hypothesized a cymose ancestor. Recent molecular studies suggested a close relationship with *Poulsenia* (Herre et al., 1996) or *Castilla* (Sytsma et al., 2002). However,

taxon sampling in these studies was not sufficient to resolve the sister group to the figs. Investigating the fig origin requires a detailed phylogenetic hypothesis that has been lacking for the family until now.

Molecular studies have demonstrated that Moraceae are part of the Rosidae and are closely allied to the Urticalean rosids, including Cannabaceae, Celtidaceae, Urticaceae, Cecropiaceae, and Ulmaceae (Zavada and Kim, 1996; Wiegrefe et al., 1998; Sytsma et al., 2002). The Urticalean rosids differ from most other rosids in the presence of solitary ovules, lactifers, cystoliths, paired inflorescences in leaf axils, and unisexual flowers. Urticaceae plus Cecropiaceae are sister to Moraceae, distinguished from the latter in having lactifers only in the bark, clear latex, and orthotropous ovules (Sytsma et al., 2002).

Moraceae are characterized by milky latex in all parenchymatous tissue, unisexual flowers, anatropous ovules, and aggregated drupes or achenes. Growth forms include trees, shrubs, hemiepiphytes, climbers, and herbs. Flowers are reduced and, when present, the perianth is four- or five-merous, tepaloid, and often membranous. Filaments are either inflexed in bud or straight. Inflexed stamens, often referred to as “urticaceous,” are associated with a pistillode against which the anthers are appressed in bud. These stamens, springing outward at anthesis to release their pollen, are indicative of wind pollination (Corner, 1962; Berg, 2001). Straight filaments are often but not always associated with pollination involving insects. The perianths of carpellate flowers are often connate or adnate to the receptacle (Berg, 2001), a condition hypothesized to protect flowers against phytophagous insects (Berg, 1989, 1990).

Moraceae have been divided into five tribes (Table 1; Rohwer, 1993). Ficeae are monotypic with a pantropical distribution and ~750 species. Plants can be either monoecious with bisexual inflorescences or gynodioecious but functionally dioecious (Weiblen, 2000). Artocarpeae are represented by 12 genera and 87 species, including the economically important *Artocarpus* (jackfruit, breadfruit). Species are either monoe-

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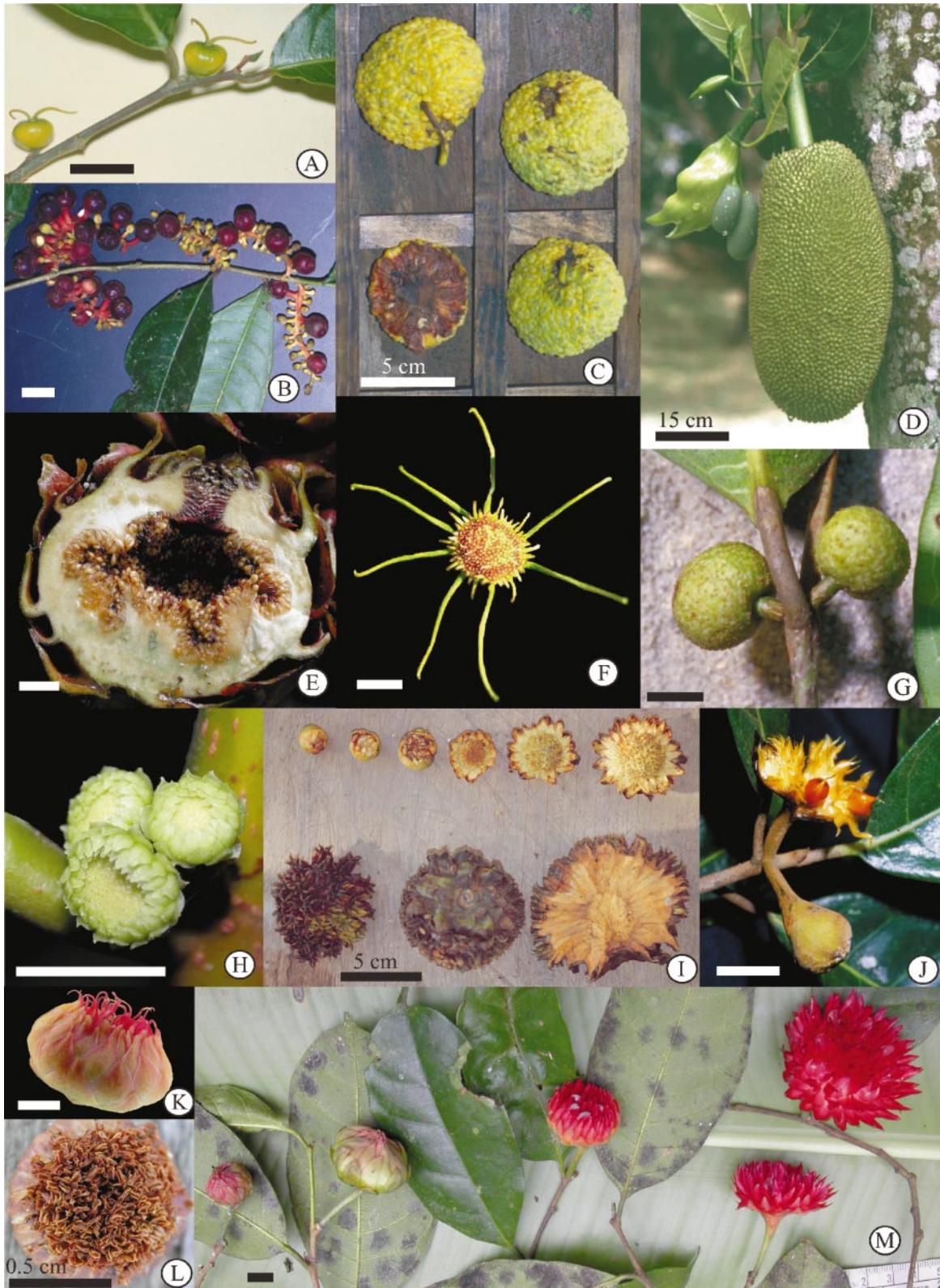


Fig. 1. Inflorescences and infructescences of Moraceae. (A) *Clarisia biflora*; solitary carpellate flowers. (B) *Sorocea affinis*, racemose infructescence. (C) *Maclura brasilensis* infructescences. (D) *Artocarpus heterophyllus*, carpellate (right) and staminate (left) inflorescences. (E) *Ficus dammaropsis* Diels, cross-section through syconium showing the involucre bracts (ostiole). (F) *Dorstenia hildebrandtii*, bisexual inflorescence with marginal bracts. (G) *Brosimum rubescens*, bisexual inflorescences. (H) *Perebea guianensis*, staminate inflorescences with involucre bracts surrounding developing flowers. (I) *Naucleopsis krukovii* developmental series from carpellate inflorescence (top left) to infructescence (bottom right). (J) *Sparattosyce dioica*; carpellate inflorescence (bottom) and infructescence (top). (K) *Antiaropsis decipiens*; carpellate inflorescence with involucre bracts surrounding carpels. (L) *Antiaropsis decipiens*; staminate inflorescence, top view. (M) *Antiaropsis decipiens*, infructescence developmental series. Scale bars are 1 cm unless noted.

TABLE 1. Classification, species richness, and distribution of Moraceae according to Rohwer (1993).

Tribe	Genus	Species	Distribution	
Artocarpeae R.Br.	<i>Antiaropsis</i> K.Schum.	1	New Guinea	
	<i>Artocarpus</i> J.R.Forst. & G.Forst.	~50	Asia & Indo-Pacific	
	<i>Bagassa</i> Aubl.	1	Neotropics	
	<i>Batocarpus</i> H.Karst.	4	Neotropics	
	<i>Clarisia</i> Ruiz & Pav.	3	Neotropics	
	<i>Hullettia</i> King ex Hook.f.	2	SE Asia	
	<i>Parartocarpus</i> Baill.	3	Indo-Pacific	
	<i>Poulsenia</i> Eggers	1	Neotropics	
	<i>Prainea</i> King	4	Indo-Pacific	
	<i>Sorocea</i> St. Hil.	14	Neotropics	
	<i>Sparattosyce</i> Bur.	1	New Caledonia	
	<i>Treculia</i> Decne. ex Trécul	3	Afrotropics	
	Castilleae C.C.Berg	<i>Antiaris</i> Lesch.	1	Paleotropics
		<i>Castilla</i> Sessé in Cerv.	3	Neotropics
<i>Helicostylis</i> Trécul		7	Neotropics	
<i>Maquira</i> Aubl.		5	Neotropics	
<i>Mesogyne</i> Engl.		1	Afrotropics	
<i>Naucleopsis</i> Miq.		~20	Neotropics	
<i>Perebea</i> Aubl.		9	Neotropics	
<i>Pseudolmedia</i> Trécul		~9	Neotropics	
Dorstenieae Gaudich.		<i>Bosqueiopsis</i> Wildem. & Th.Dur.	1	Afrotropics
	<i>Brosimum</i> Sw.	13	Neotropics	
	<i>Dorstenia</i> L.	~105	Afrotropics & Neotropics	
	<i>Helianthostylis</i> Baill.	2	Neotropics	
	<i>Scyphosyce</i> Baill.	2	Afrotropics	
	<i>Trilepisium</i> Thouars	1	Afrotropics	
	<i>Trymatococcus</i> Poepp. & Endl.	3	Neotropics	
	<i>Utsetela</i> Pellegr.	1	Afrotropics	
	Ficeae Gaudich.	<i>Ficus</i> L.	~750	Pantropical
Moreae Gaudich.	<i>Bleekrodea</i> Blume	3	Madagascar & SE Asia	
	<i>Broussonetia</i> L'Hér. ex Vent.	8	Madagascar & SE Asia	
	<i>Fatoua</i> Gaudich.	3	Asia, Australia & Madagascar	
	<i>Maclura</i> Nutt.	11	Pantropics & North America	
	<i>Milicia</i> Sim	2	Afrotropics	
	<i>Morus</i> L.	~12	Cosmopolitan	
	<i>Streblus</i> Lour.	~25	SE Asia & Africa	
	<i>Trophis</i> P.Browne	9	Neotropics & SE Asia	

cious or dioecious, with unisexual inflorescences of variable architecture including racemes, spikes, capitula, globes, discs, and solitary flowers (Jarrett, 1959; Berg, 1988). Berg (1988, 2001) recognized that the Artocarpeae lack the homogeneity of other Moraceae tribes and suggested that it might be subdivided into three subtribes on account of the morphological variation.

Castilleae include eight genera (Table 1) distributed throughout the neotropics and to a limited extent in tropical Africa. The tribe has unisexual, discoid to urceolate receptacles with involucrate bracts in most species, the presence of septate wood fibers, and self-pruning branches (Berg, 1972, 1977a, 2001). Species are monoecious, dioecious, or androdioecious (Berg, 1972, 1977a, 2001).

The eight African and neotropical genera of Dorstenieae are dominated by ~100 species of *Dorstenia*. The tribe is defined by discoid or globose bisexual inflorescences with the carpelate flowers often embedded within the receptacle, peltate interfloral bracts, and uncinat hairs in many species. Growth form varies from arborescent in most genera to herbaceous or suffrutescent in *Dorstenia* (Berg and Hijman, 1999). Although most species are monoecious, dioecy and androdioecy also occur (Berg, 1988).

The eight genera of Moreae include approximately 70 species. Species are mostly dioecious, although a few species are monoecious with bisexual inflorescences. Inflorescences are

relatively simple racemes, spikes, or globose heads. Most species have urticaceous stamens, apparently related to anemophily. However, there is a great deal of variation in vegetative and floral morphology, and generic delimitation is somewhat uncertain (Berg, 2001). Up to 40 generic names have been recognized at one time or another in this group (Berg, 2001). Several genera have been enlarged in an attempt to devise a more satisfactory and cohesive classification (Corner, 1962, 1975; Berg, 1982, 1986, 1988, 2001). It has been suggested that taxonomic uncertainty within Moreae stems from the retention of plesiomorphic features (Berg, 1989, 2001).

We examined Moraceae phylogenetic relationships, reproductive character evolution, and the origin of the fig pollination mutualism. We employed sequences from the chloroplast gene *ndhF* because it has proven useful for examining ordinal and familial relationships among plants (Olmstead et al., 2001; Sytsma et al., 2002). Moraceae chloroplast DNA phylogeny provided insights into (1) tribal relationships, including the sister group to the figs, (2) ancestral changes in breeding systems and pollination syndromes, (3) ancestral traits associated with the origin of the syconium, and (4) molecular divergence time estimates for the origin of fig pollination.

#### MATERIALS AND METHODS

Eighteen outgroups in four families and 83 taxa representing 33 genera of Moraceae were sampled (see Supplemental Data accompanying the online

version of this article). Although 89% of Moraceae genera were sampled, *Bosquiopsis*, *Hullettia*, *Scyphosyce*, and *Treulia* were not included because fresh material was unavailable and DNA extraction from herbarium material was unsuccessful. DNA extraction was performed using the Qiagen DNeasy plant extraction kit (Valencia, California, USA) from 10–15 mg of silica-gel preserved leaf fragments or herbarium specimens. Polymerase chain reaction (PCR) amplification of a 2100 bp fragment was performed in two reactions of ~1200 bp each using primers from Olmstead and Sweere (1994) including *ndhF8f*, *ndhF972*, *ndhF1318r*, and *ndhF2110r*. Amplification conditions were as follows: ~20 ng genomic DNA, 1× *TaKaRa Ex Taq* buffer (2 mmol/L MgCl<sub>2</sub>), 160 nmol/L each primer, 0.2 mmol/L each dNTP, 1.25 unit *TaKaRa Ex Taq* DNA polymerase (Otsu, Shiga, Japan). Thermal cycling was performed in 25 cycles of 94°C for 30 s, 48°C for 60 s, 68°C for 90 s, and a final extension at 72°C for 7 min. The PCR products were cleaned using the QIAquick or MinElute PCR purification spin columns (Qiagen, Valencia, California, USA). Clean PCR products were quantified using Hoechst 33258 fluorescent dye (Acros Organics, Morris Plains, New Jersey, USA) in a Turner Quantech fluorometer (Barnstead-ThermoLyne, Dubuque, Iowa, USA). Ten-microliter sequencing reactions were performed with Big Dye sequencing reagents and protocols (versions 2, 3; Applied Biosystems, Foster City, California, USA) and data were collected using an ABI 377 Automated DNA sequencer (Applied Biosystems). Between seven and nine sequencing primers were used for each taxon, including the aforementioned primers, *ndhF972r*, *ndhF1318*, and *ndhF1603r*. In addition, we designed two primers for Urticales, *ndhF84f* (5'-TCT TCG CCG TAT AGT GGG TTT TTC C-3'), and *ndhF713r* (5'-ATC RGG TAA CCA TAC ATG AAG RGG-3'). Sequences were edited using Sequencher version 3.0 (Gene Codes, Ann Arbor, Michigan, USA). Sequence alignment was approximated with ClustalX (Thompson et al., 1997), followed by manual alignment to maintain open reading frames for the entire portion of the gene.

**Parsimony analyses**—Tree searches were performed using PAUP\* version 4.0b10 (Swofford, 2002) with the tree-bisection-reconnection (TBR) branch-swapping algorithm and 10000 random addition sequence replicates. Maxtrees was set to increase without limit. Support was assessed with 1000 bootstrap replicates (10 addition sequence replicates per bootstrap replicate) and maxtrees set at 10000. The decay index (Bremer, 1988, 1994) was assessed using TreeRot version 2 (Sorenson, 1999) with 20 random addition sequences per replicate and maxtrees set at 10000. All clade support and tree length calculations were conducted with uninformative characters excluded.

**Likelihood analyses**—Modeltest (Posada and Crandall, 1998) was used to identify the best fitting model of sequence evolution with the fewest additional parameters. Tree-bisection-reconnection branch swapping was performed under the likelihood criterion using PAUP\* 4.0b10 on a starting tree generated by neighbor joining with the parameters obtained from Modeltest. After the search located the tree with the highest likelihood, model parameters were estimated for that tree. These parameters set a new round of branch swapping on the maximum likelihood (ML) tree. Branch swapping and parameter estimation were iterated until analyses converged on the same likelihood score and model parameters.

**Parametric bootstrapping**—Particular phylogenetic hypotheses were also evaluated by parametric bootstrapping (Huelsenbeck and Hillis, 1996). We focused on support for the monophyly of Ficeae plus Castilleae. Parsimony searches were performed in which Ficeae plus Castilleae were constrained to be non-monophyletic. Likelihood parameters and branch lengths were estimated from one of the parsimony trees resulting from the reverse constraint search. The tree postulating the non-monophyly of Ficeae plus Castilleae and its likelihood parameters were used to simulate 100 replicate data sets using SEQ-GEN (Rambaut and Grassly, 1997). Maximum parsimony trees were inferred for each simulated data set, and  $-\ln L$  scores given the aforementioned parameters were compared between an MP tree and the tree used to simulate the data. The log-likelihood difference of the constrained (null) and unconstrained (best) topologies provided a distribution of the test statistic under the null hypothesis that systematic error accounted for the monophyly

of Ficeae + Castilleae. The log likelihood difference of the empirical data yielded the probability of obtaining this clade in error.

**Character evolution**—The following four reproductive traits were scored for each taxon based on information from the taxonomic literature, herbarium specimens, and observations in the field. Pollination was scored as (0) anemophilous or (1) entomophilous. Taxa with “urticeous” stamens were scored for wind pollination because no species with this morphology are known to be insect pollinated. Carpellate inflorescences were scored as (0) lacking an involucre of bracts around the carpels or (1) with an involucre of bracts surrounding carpels. Breeding system was scored as (0) monoecious, (1) dioecious, (2) androdioecious, or (3) gynodioecious. Monoecious, androdioecious, and gynodioecious plants were further scored for (0) unisexual inflorescences or (1) bisexual inflorescences. Ancestral state reconstruction was performed on the maximum likelihood tree using MacClade version 3.0 (Maddison and Maddison, 2000).

Correlated change in pollination syndrome, breeding system, and the presence of an involucre of bracts around the carpels on a randomly resolved ML tree was examined using the concentrated changes test (Maddison, 1990). Correlated evolution was also examined in a likelihood framework using Discrete (Pagel, 1994, 1997). Omnibus tests evaluated the hypothesis of correlated evolution in two characters by examining the fit of an independent model of character evolution to a dependent model. Likelihood tests were conducted on a pruned data set of only species for which pollination syndrome is known (63 of 102 taxa) because pollination syndromes are unknown for many species. Branch lengths were calculated using likelihood parameters estimated on the ML tree.

**Molecular dating**—A likelihood ratio (LR) test compared the likelihood of the data with and without the assumption of a molecular clock (Felsenstein, 1988), where  $LR = 2 (\ln L_{\text{clock}} - \ln L_{\text{no clock}})$  was assumed to be  $\chi^2$  distributed with the degrees of freedom equal to  $n$  taxa minus two. Because rates of evolution varied greatly across Moraceae, we performed dating procedures using a penalized likelihood approach (Sanderson, 2002) as implemented in the program r8s version 1.6 (Sanderson, 2003). Penalized likelihood is a semiparametric method that allows substitution rates to vary among lineages according to a smoothing parameter. The optimal smoothing parameter was chosen by means of a data-driven cross validation procedure in which taxa were sequentially pruned from the tree. The r8s program calculated the parameter estimate that best predicted the removed data (i.e., minimized the  $\chi^2$  error). Cross-validation and initial dating were performed with the age of the root node fixed at one. Nodes in the resulting ultrametric tree were constrained with the minimum ages of Moraceae fossils including *Ficus* achenes aged at 60 mya, *Morus* leaves dated at 40 mya, and Moraceae fruits dated at 90 mya (Collinson, 1989). The root node of the tree was also set to a maximum age of 135 mya based on the oldest known angiosperms fossil (Magallon et al., 1999).

## RESULTS

The aligned data set included 2089 base pairs (bp) of which 652 positions were parsimony-informative. Parsimony recovered 11 057 trees of 2003 steps (CI = 0.518; RI = 0.844). There was strong support for a Moraceae clade in the bootstrap consensus trees (Fig. 2). Cecropiaceae plus Urticaceae clade was strongly supported, but the Cecropiaceae were paraphyletic due to the position of *Poikilospermum*. Moreae comprised a paraphyletic grade, with a lineage including *Morus*, *Milicia*, *Streblus*, and *Trophis* that was sister to Artocarpeae, while the remaining Moreae were more closely related to Dorstenieae. The sister-group relationship of the Artocarpeae plus Moreae sensu stricto had relatively weak support (59% bootstrap). *Trophis* and *Streblus* were polyphyletic, with some species more closely related to Artocarpeae and others more closely related to Dorstenieae. The position of *Maclura* and *Streblus smithii* was not resolved.

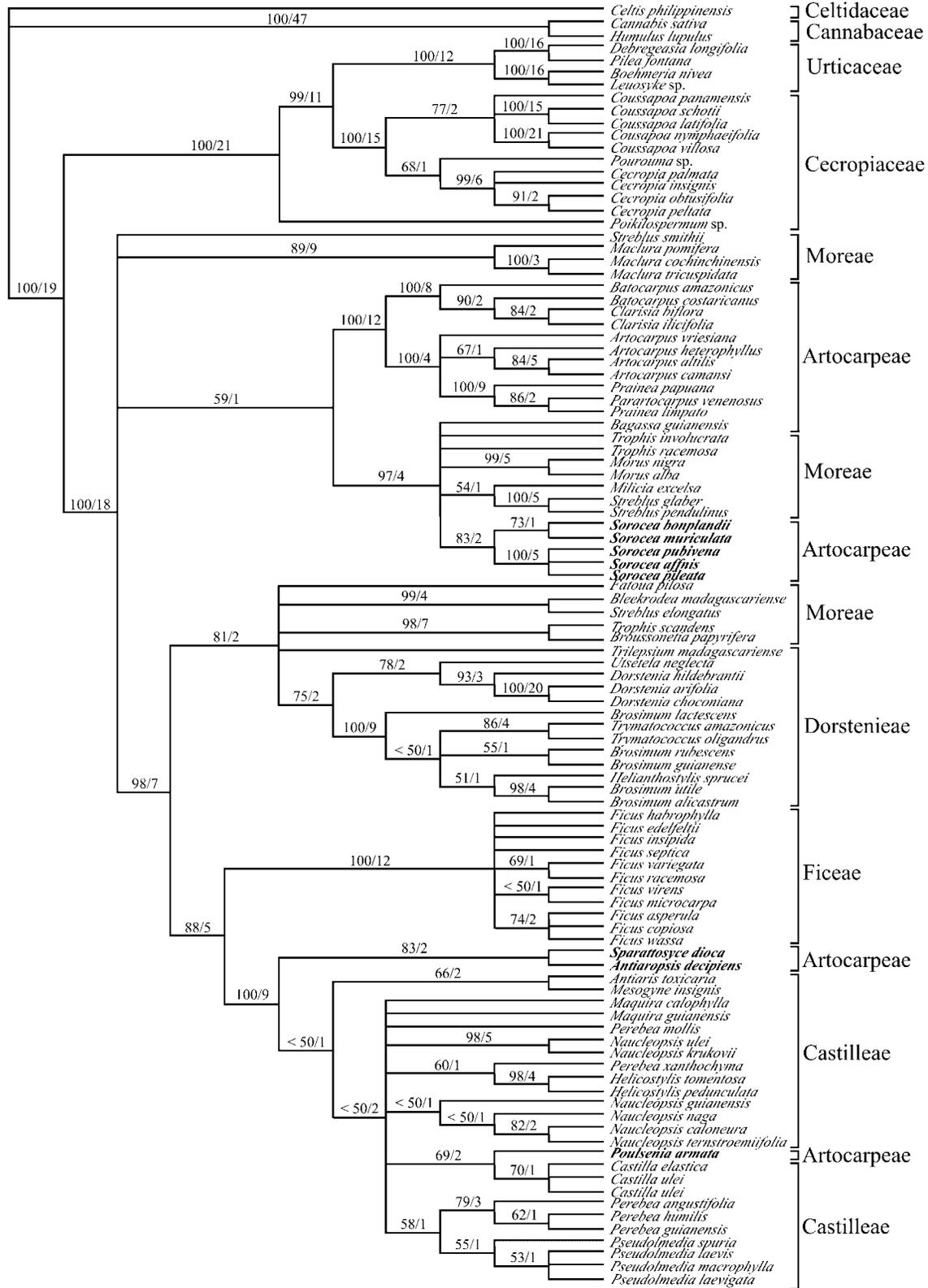


Fig. 2. Strict consensus of 11 057 most parsimonious trees. Numbers above branches refer to bootstrap percentage/decay index. Classification follows Rohwer (1993) with outgroup families and ingroup tribes indicated in brackets. Taxa in boldface type indicate genera for which tribal placement is revised.

Artocarpeae were polyphyletic. A core group included *Artocarpus*, *Prainea*, *Parartocarpus*, *Clarisia*, and *Batocarpus*, while *Sorocea* and *Bagassa* grouped with the Moreae sensu stricto. Three genera previously attributed to Artocarpeae (*Sparattosyce*, *Antiaropsis*, *Poulsenia*) were embedded in a paraphyletic Castilleae. With the inclusion of these genera in a broadened Castilleae, the tribe was sister to *Ficus* with 88% bootstrap support (Fig. 2). Parametric bootstrapping indicated that the probability of recovering the Castilleae plus *Ficus* clade in error was less than 0.01. Not surprisingly, *Ficus* was monophyletic with 100% bootstrap support.

Modeltest identified a model with equal substitution rates for transitions and unequal rates for transversions and a parameter for heterogeneity in rates of substitution across sites (TVM +  $\Gamma$ ; Posada and Crandall, 1998) as the most appropriate model of sequence evolution with the fewest additional parameters. After three iterations, the analysis converged on the same likelihood tree and parameter estimates. Branch swapping yielded two equally likely trees with a score of  $-\ln L$  17928.474. The rate matrix for the tree shown in Fig. 3 was 1.69381, 2.02679, 0.39974, 1.17943, 2.06549, with  $\alpha = 0.688340$ . Base frequencies were A = 0.303690, C = 0.142979, G = 0.159156, and T = 0.394175. The topology of the two trees differed only with respect to the position of the *Bleekrodea madagascariense* plus *Streblus elongatus* clade.

**Character evolution**—Equally weighted ancestral state reconstruction indicated that dioecy is ancestral in Moraceae, with between two and four shifts to monoecy (Fig. 4). Within the Dorstenieae, *Helianthostylis* represented a single origin of androdioecy. *Ficus* showed at least one origin of gynodioecy. In *Ficus* and Dorstenieae, monoecious species have bisexual inflorescences, whereas in Artocarpeae and Castilleae, monoecious species have unisexual inflorescences.

Wind pollination was likely the ancestral condition of Urticales (Fig. 4). Although the pollination biology of many Moraceae is not known, there appear to have been at least two independent origins of entomophily, in the paleotropical Artocarpeae and in the Castilleae plus *Ficus* clade. Many genera have morphologies suggestive of insect pollination, but observations of floral visitors are lacking, particularly in Dorstenieae and *Sorocea*. Ancestral state reconstruction suggests that insect pollination preceded the divergence of Castilleae and *Ficus* (Fig. 4).

An involucre of bracts surrounding the developing carpels also occurs in Castilleae and *Ficus* (Fig. 4). The involucre encircles the developing flowers in Castilleae, and the bracts are pushed apart as the receptacle expands. By contrast, the involucre in *Ficus* forms an ostiole that limits access to the flowers, traps most pollinators inside of the syconium, and seals the infructescence as well. Outside of the Castilleae plus *Ficus* clade, involucre bracts surrounding the carpels are present only in *Trophis caucana* (Pittier) C.C.Berg. A few Dorstenieae and Artocarpeae have small bracts associated with the receptacle but these do not enclose the flowers at any stage of development.

The evolution of involucre bracts was not significantly concentrated in insect-pollinated lineages ( $P = 0.50$ ), despite the perfect correlation of entomophily and bracts in the Castilleae plus Ficeae clade. The concentrated changes test is sensitive to limited numbers of evolutionary events, in this case, a single origin of involucre bracts. By contrast, the correlation be-

tween bracts and insect pollination was significant according to the omnibus test (LR = 17.0; df = 4;  $P < 0.05$ ).

The concentrated changes test indicated that changes to monoecy were not significantly concentrated in wind-pollinated lineages ( $P = 0.11$ ), when breeding system was considered to be dependent on pollination syndrome. Likewise, changes to wind pollination were not significantly concentrated in monoecious lineages ( $P = 1.00$ ). The omnibus test indicated an overall association between breeding system and pollination syndrome (LR = 14.66; df = 4;  $P < 0.05$ ). However, models restricting the probability of change in pollination syndrome to be dependent on the state of the breeding system (LR = 0.4; df = 1), and vice versa (LR = 0.6; df = 1), were not significantly more likely than the independent model.

**Molecular dating**—Even with a parameter-rich model of nucleotide substitution, the data strongly rejected the assumption of a molecular clock (LR = 709.42; df = 100;  $P < 0.001$ ). Therefore, a penalized likelihood analysis assuming nonclock-like evolution was performed. Cross-validation analysis estimated the optimal smoothing parameter at 1.12. Based on a maximum age constraint of 135 mya for the root of Urticalean rosids and minimum age constraints for *Ficus* (60 mya), *Morus* (40 mya), and Moraceae (90 mya), the minimum divergence for the stem lineage of Ficeae plus Castilleae was ~83 mya. These estimates indicated that Moraceae diverged at least 99.4 mya while the Cecropiaceae plus Urticaceae diverged at least 98 mya, which is consistent with fossil Urticaceae fruits dated at 90 mya (Collinson, 1989).

## DISCUSSION

Phylogenetic analyses based on *ndhF* sequence data support the monophyly of Moraceae with the exclusion of Cecropiaceae. Moraceae *ndhF* sequences also confirm that fig pollination evolved only once, as suggested by other chloroplast genes and mitochondrial DNA from fig pollinators (Herre et al., 1996; Machado et al., 2001). Previous studies based on limited sampling of Moraceae suggested that some members of Castilleae are close relatives of the figs (Herre et al., 1996; Sytsma et al., 2002). We demonstrate for the first time that the entire Castilleae plus some Artocarpeae are sister to *Ficus*, and we propose taxonomic changes in accord with these findings.

**Taxonomic implications**—We present a new classification of Moraceae to reflect the evolutionary relationships of the tribes (Appendix; see Supplemental Data accompanying the online version of this article). Minor revisions in the placement of particular genera result in four monophyletic tribes. Moreae remain paraphyletic in our classification pending more detailed sampling of the group, which encompasses extreme morphological heterogeneity and is recognized on the basis of pleiomorphic features of Urticalean rosids, including inflexed stamens and wind pollination (Sytsma et al., 2002). Our results point to a Moreae sensu stricto (Figs. 3–4) but further study of Moreae genera is warranted. *Trophis* and *Streblus* are especially problematic and have been divided into many monotypic or oligotypic genera by various authors. These genera display tremendous variation in growth form, breeding system, and inflorescence morphology. Berg (1988) broadly circumscribed these genera based on the presence of free tepals in *Streblus* vs. connate tepals in *Trophis*. *Streblus* is further divided into five sections, two of which are monotypic. We sam-

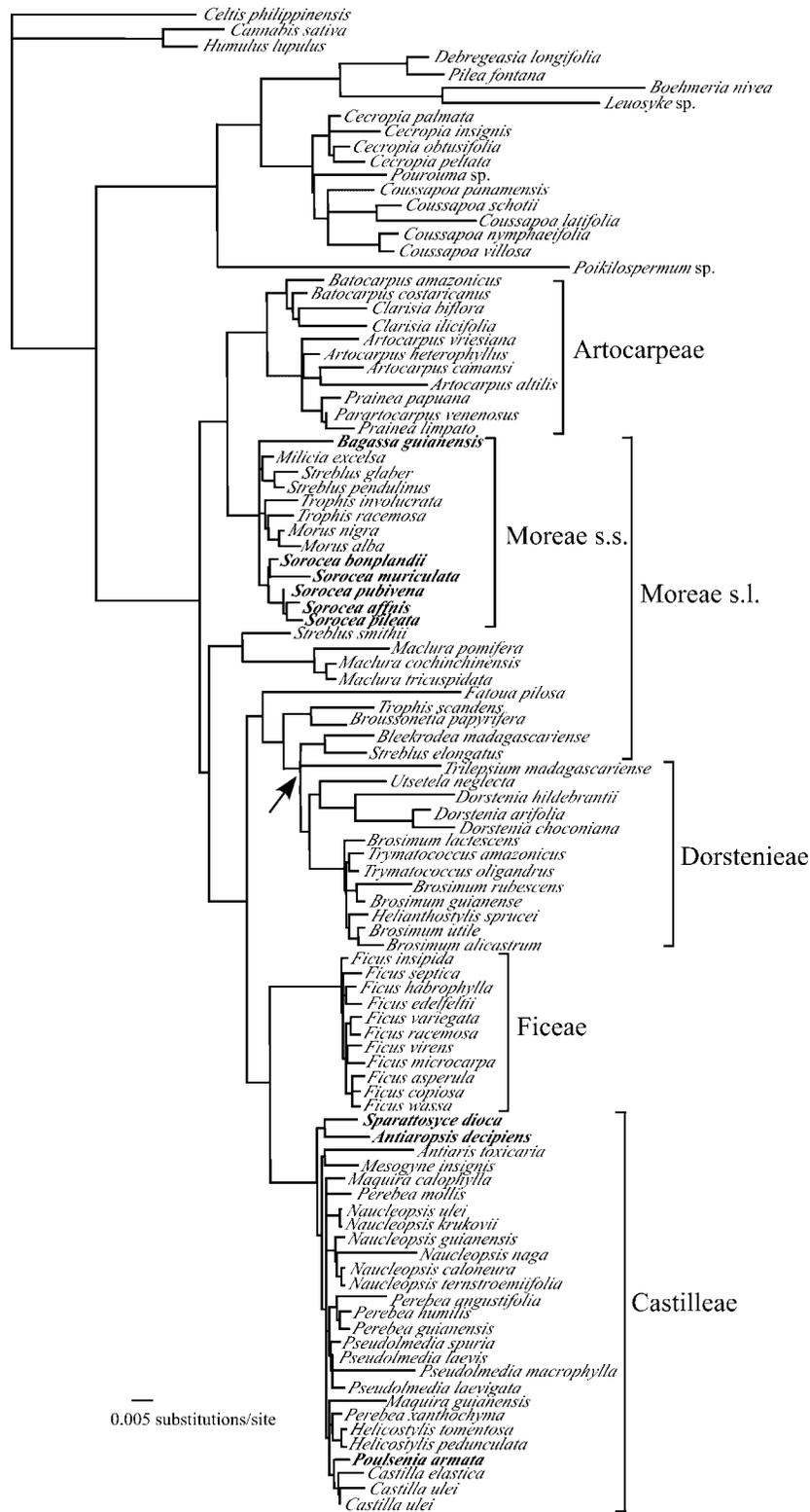


Fig. 3. One of two trees equaling  $-\ln L$  17928.48. The revised tribal classification of Moraceae is indicated in brackets. The arrow indicates the difference between the two equally likely trees. The tree not shown places *Bleekrodea madagascariense* plus *Streblus elongatus* as the sister group to Dorstenieae.

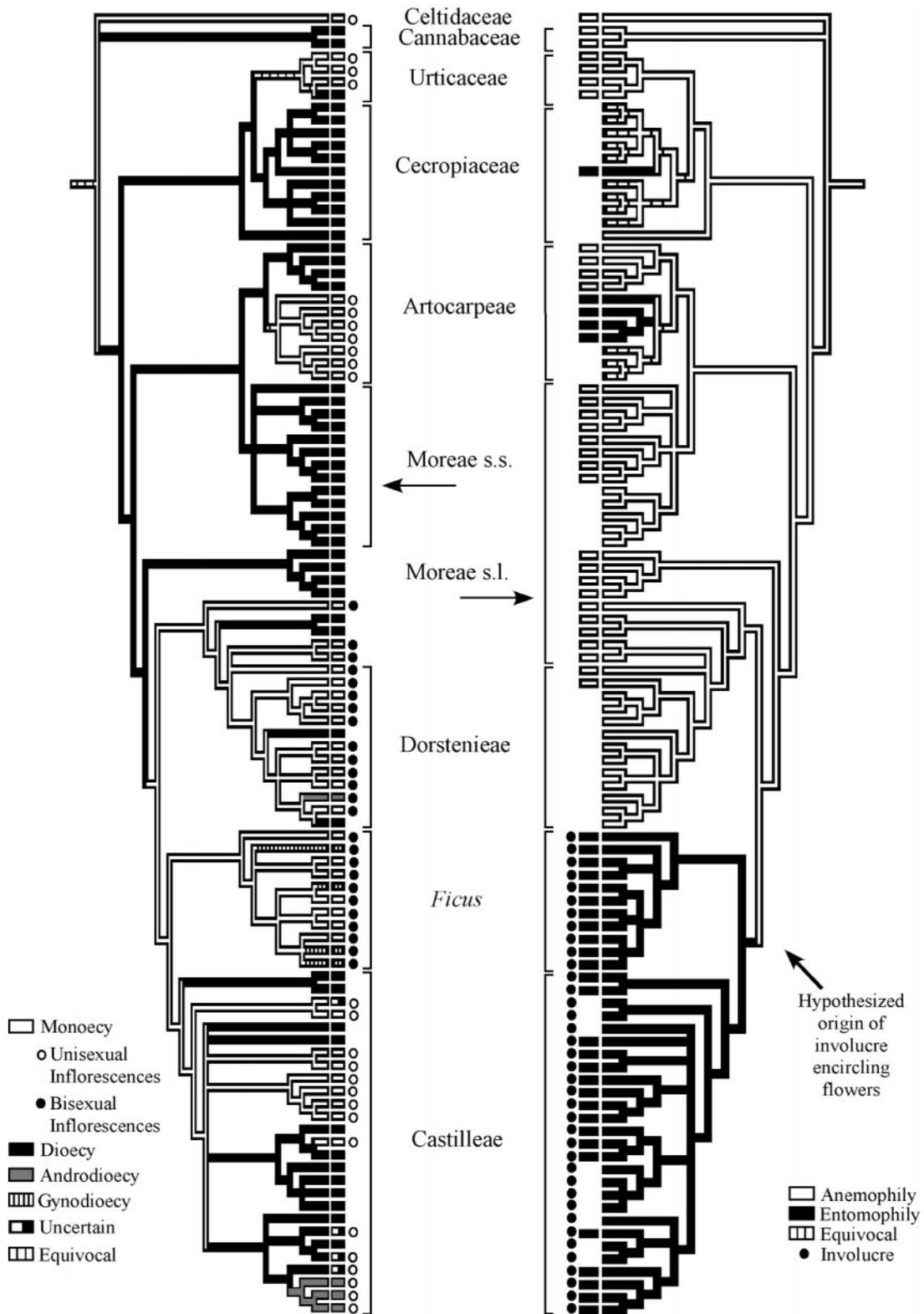


Fig. 4. Breeding system (left) and pollination syndrome (right) mapped onto the ML tree shown in Fig. 3. For breeding system, closed circles and open circles indicate bisexual inflorescences and unisexual inflorescences, respectively, for monoecious, androdioecious, and gynodioecious species. On the pollination syndrome reconstruction, closed circles indicate the presence of involucre bracts surrounding the carpels at anthesis.

pled three representatives of sect. *Paratrophis* and found that *S. pendulinus* and *S. glaber* are sister, while the position of *S. smithii* remains uncertain. In addition, only sect. *Sloetia* was represented in our analysis by *S. elongatus*, which is sister to *Bleekrodea*. Even with limited sampling, our results suggest the disintegration of the genus.

*Trophis* is divided into six sections, four of which are monotypic. *Trophis racemosa* (sect. *Trophis*) and *T. involucrata* (sect. *Echinocarpa*) are a part of the Moreae sensu stricto but *T. scandens* (sect. *Malaisia*) is more closely related to *Broussonetia* and Dorstenieae. As with *Streblus*, the non-monophyly of this genus is not surprising given its morphological complexity, and further investigation is warranted.

Artocarpeae as circumscribed by Rohwer (1993) is polyphyletic, and we recognize a more restricted Artocarpeae by excluding five genera from the tribe. *Antiaropsis*, *Poulsenia*, and *Sparattosyce* are transferred to Castilleae, and *Bagassa* and *Sorocea* are transferred to Moreae sensu stricto (Appendix). This delimitation is strongly supported by *ndhF* (Fig. 2) and nuclear ribosomal DNA (G. D. Weiblen, unpublished data) and is not contradicted by morphology (Fig. 4). All genera in the revised Artocarpeae have a reduction in stamen number (four stamens) and have large seeds that lack endosperm. Although *Hullettia* and *Treculia* were not sampled, we include these genera in the revised Artocarpeae based on morphology.

There is moderate support for the Dorstenieae based on *ndhF* sequences although the position of *Trilepisium* remains uncertain (Fig. 2). African *Scyphosyce* and *Bosqueiopsis* were unavailable, and the inclusion of these genera might influence relationships in the tribe. We expect, however, that they are closely allied with Dorstenieae based on morphology. Many closely allied species of Moreae and Dorstenieae are monoecious with bisexual inflorescences. Dorstenieae are distinguished by the presence of one to several carpellate flowers embedded in the receptacle and surrounded by staminate flowers.

Sequence data support the inclusion of *Sparattosyce*, *Antiaropsis*, and *Poulsenia* in a broadened Castilleae, characterized by self-pruning branches (Berg, 1977a) and unisexual inflorescences with involucre bracts. Interestingly, these three genera were the only members of the Artocarpeae sensu Rohwer (1993) with involucre bracts. Berg (1990) hypothesized that both involucre and interfloral bracts represent adaptations preventing insects from feeding on the developing carpels and stamens. The early development of the involucre prior to floral differentiation is consistent with a protective role, although flowers are not completely enclosed at receptivity or anthesis in many Castilleae. These bracts are free in most species but are fused along most of their length in *Sparattosyce*, resembling an urn-shaped receptacle.

**Breeding system evolution**—Contrary to the traditional view that dioecy evolves from monoecy (Renner and Ricklefs, 1995; Weiblen et al., 2000), our results suggest that dioecy is the ancestral condition in Moraceae and that monoecy has evolved independently between two and four times in the family. However, if gains of dioecy are assigned twice the cost of losses, then monoecy is reconstructed as the ancestral condition under parsimony. Monoecious lineages are neatly divided into those with unisexual inflorescences (Artocarpeae and Castilleae) and those with bisexual inflorescences (Dorstenieae and Ficeae). Based on these differences in inflorescence architecture, monoecy may have evolved four times in the fam-

ily (Fig. 4). Functional dioecy in *Ficus*, resulting from the interaction of gynodioecious inflorescences with pollinating seed eaters, appears to have evolved at least twice with several reversals to monoecy (Weiblen, 2000; Joussetin et al., 2003). Androdioecy, a very rare breeding system in angiosperms (Renner and Ricklefs, 1995), has been documented in *Castilla* (Castilleae) and *Helianthostylis* (Dorstenieae), indicating two origins within Moraceae. Androdioecy and gynodioecy might be more common in the family but incomplete collections make this assessment difficult.

**Pollination syndrome evolution**—An apparently plesiomorphic condition in Moreae is the presence of stamens that are inflexed in bud, generally associated with a pistillode. As the filament elongates, the anthers are oriented along the pistillode in bud such that the stamens spring back explosively and release pollen into the air as the flower opens. This adaptation to wind pollination is common in the Urticaceae. Some members of Moreae have straight filaments in bud, including *Bagassa*, *Sorocea*, and *Maclura* sect. *Cudrania*, suggesting one or two losses of “urticaceous stamens” in the Moreae sensu lato. *Bagassa* has pendant, staminate inflorescences that are often associated with anemophily, but *Sorocea* has no obvious features of either wind- or insect-pollination. Pluricellular trichomes on the carpellate inflorescences exude a nutritious substrate that can serve as a medium for fungal mycelium. This exudate and the associated fungus may serve as a reward for pollinators (Berg, 2001).

Many species of Dorstenieae are hypothesized to be insect pollinated based on floral structure and scents, and beetles have been observed visiting some African species (Berg and Hijman, 1999). However, seed set in the absence of pollinators has been recorded in *Dorstenia* (Berg and Hijman, 1999). In *Trilepisium*, staminate inflorescences with a strong odor are known to attract beetles that may breed in the inflorescences (Berg, 1977b). Among the neotropical Dorstenieae, *Brosimum alicastrum* has been reported to produce clouds of pollen from staminate inflorescences suggestive of wind pollination (Berg, 2001). However, visitation by “small diverse insects” has also been reported (Bawa et al., 1985; Kress and Beach, 1994), and other authors suggest that inflorescences of *Brosimum* are adapted for insect pollination (Croat, 1978; Berg, 1990).

In *Artocarpus integer*, pollination appears to be mediated by gall midges feeding on fungal parasites of staminate inflorescences (Sakai et al., 2000). Gall midges feed on an exudate from the mycelium of the fungus and breed in the mycelium. Insects may be attracted to the inflorescences by a strong, sweet smell that is emitted at night (Sakai et al., 2000). In the process of feeding and breeding on staminate inflorescences, insects pick up sticky pollen grains and occasionally transport them to carpellate inflorescences. Gall midges have been observed at low frequency on carpellate inflorescences, possibly attracted by the floral fragrance that is emitted. However, the fungus on which midges feed is not found on carpellate inflorescences, and short visits by gall midges to these inflorescences suggest pollination by deceit. Fungal growth may be limited by the structure of the *Artocarpus* inflorescence. The perianths of adjacent flowers fuse to form an exterior surface through which the stigmas protrude through a small opening at the apex. This structure may limit entry of phytophagous insects to the delicate tissue of the gynoecium (Berg, 1990). We hypothesize that, in contrast to the paleotropical Artocarpeae, the pendulous, catkin-like staminate inflorescences of the

neotropical genera are adapted to wind pollination. On the other hand, some authors allude to wind pollination in *Artocarpus* and nothing is known of syndromes in closely allied *Parartocarpus* and *Prainea* (Jarrett, 1959; Berg, 2001). Field observations are needed to test these predictions based on phylogeny.

Berg (1990) hypothesized that the involucre bracts of Castilleae may be an adaptation to prevent phytophagous insects from feeding on the flowers. Pollination syndrome is unknown for about half of the genera of Castilleae, but thrips pollination has been reported in *Castilla*, *Antiaropsis*, *Naucleopsis*, *Persea*, and *Poulsenia* (Sakai, 2001; G. D. Weiblen, unpublished data). The same species of thrips, *Frankliniella diversa* (Thripidae; Thysanoptera), has been observed visiting *Castilla* and *Poulsenia* (Sakai, 2001). Thrips pollination appears to be associated with insects breeding in staminate inflorescences. Pollen serves as the primary food source for both nymphs and adults. Females also visit carpellate inflorescences, but for shorter periods of time. Similar to the situation in *Artocarpus*, thrips may be attracted to carpellate flowers by a floral scent that mimics the staminate inflorescences (Sakai, 2001). Further investigation of the generality of thrips pollination in the Castilleae is needed, but our results indicate that traits associated with pollination in the Castilleae are critical to understanding the evolution of fig pollination.

**Origin of the fig**—The sister relationship of thrips-pollinated Castilleae and *Ficus* suggests that insect pollination was a feature shared by the common ancestor of these lineages. Maximum likelihood analysis of correlated evolution further detected a significant association between entomophily and the presence of involucre bracts that encircle the floral primordia in *Ficus* and Castilleae (Berg, 1972; Verkerke, 1989). During fig development, the receptacle expands to accommodate the flowers, while the tightly appressed bracts form the ostiole. The involucre bracts of many Castilleae encircle the carpels and form a ring through which the receptive stigmas protrude. Even partial enclosure of the carpels could provide protection from phytophagous insects and play a role in pollinator specificity (Berg, 1990). The staminate inflorescences of Castilleae provide breeding sites for pollen-feeding thrips, which are deceived into visiting the carpellate inflorescences by means of floral scent (Sakai, 2001). In contrast, ostiolar bracts that completely seal the flowers (and the fruit) inside the receptacle serve to trap fig pollinators. We interpret the precondition of the syconium as a partially closed, involucre inflorescence similar to that of the modern Castilleae. The complete enclosure of the fig by the ostiole was likely associated with pollination by specialized, parasitic Hymenoptera and led to the origin of the obligate mutualism. At present, we can only speculate as to how Agaonids became Moraceae specialists. Cospeciation between specialized pollinators and their hosts might account for the extreme species diversity of *Ficus* relative to its sister group (Weiblen and Bush, 2002), and the notion that pollinator specialization was the engine of speciation in *Ficus* can now be explored through the phylogenetic comparison of diversification rates (Sanderson and Donoghue, 1996).

Molecular dating using penalized likelihood suggests that *Ficus* diverged from Castilleae at least 83 mya, close to the date obtained from fig wasp DNA sequences, suggesting that the mutualism originated 87.5 mya (Machado et al., 2001). However, divergence time estimates from fig wasps pointed to the crown group radiation of *Ficus*, while our date refers to

the origin of the stem lineage. It is noteworthy that Machado et al. (2001) based their divergence time estimates on an analysis in which taxa with nonclock-like substitution rates were discarded from a group that demonstrates a great deal of rate heterogeneity. Furthermore, the analysis was calibrated with a fossil fig wasp dated at 18 mya. Dates based on clock-like substitution rates are most accurate near the calibration point and tend to overestimate the timing of ancient events (Arbogast et al., 2002). Although *Ficus* appears to have a Cretaceous origin, the crown radiation of *Ficus* may have occurred more recently than suggested by Machado et al. (2001). Molecular biogeographic studies that integrate knowledge of figs, pollinators, and their relatives are the next logical step in this line of investigation.

**Conclusions**—The origin of the fig has not figured in the extensive literature on coevolutionary complexity of the fig/pollinator interaction (reviewed in Cook and Rasplus, 2003; Weiblen, 2002). Our revised classification of the Moraceae based on *ndhF* sequences provides a phylogenetic framework for understanding the evolution of fig pollination. The sister relationship of Castilleae to *Ficus* provides molecular corroboration of the view that insect pollination and involucre bracts encircling the flowers in a Cretaceous ancestor were likely associated with the origin of the remarkable pollination mutualism. However, other aspects of the fig origin remain a mystery. How did the extreme protogyny evolve that established the synchrony of fig and pollinator life cycles? When and where did the Agaonidae become fig specialists? It is hoped that developmental studies and molecular biogeography may provide further insights into these questions.

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