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Source: Systematic Botany, 36(3):690-701. 2011.
Published By: The American Society of Plant Taxonomists
URL: http://www.bioone.org/doi/full/10.1600/036364411X583655

Systematic Botany (2011), 36(3): pp. 690–701

DOI 10.1600/036364411X583655

Phylogenetic Analyses of *Eriotheca* and Related Genera (Bombacoideae, Malvaceae)

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Communicating Editor: Andrew Hipp

***Abstract*—**Molecular and morphological data have shown that Bombacoideae and Malvoideae together form a well-supported Malvatheca clade. Phylogenetic relationships in Bombacoideae have been studied, but some genera in *Bombax* s. l. have not been adequately sampled for sufficiently variable molecular markers. The relationships of *Eriotheca*, for example, have yet to be resolved. Here, nuclear (ITS) and chloroplast (*trnL-F* and *matK*) sequence data from 50 exemplars of Bombacoideae and seven additional taxa from other genera of Malvatheca were used to test monophyly of *Eriotheca* and its relationships with related genera of *Bombax* s. l. Parsimony and Bayesian analyses of individual and combined sequence data suggest that *Eriotheca* is not monophyletic as currently circumscribed but forms a paraphyletic grade containing *Pachira* s. l. The newly discovered *Eriotheca* + *Pachira* clade has a probable synapomorphy of striate seeds. In addition, two other moderately supported clades emerged within the core Bombacoideae: *Pseudobombax* + *Ceiba* s. l. and *Bombax* + *Spirotheca* + *Pachira quinata*. These three clades, and the African *Rhodognaphalon* together constitute the major clade of core Bombacoideae, whereas *Adansonia* appears to be more closely related to *Catostemma*, *Scleronema,* and *Cavanillesia*. The phylogenetic results imply three independent migrations from the New to Old World and homoplasy in staminal morphology.

***Keywords*—**Bombacoideae, *Bombax* s. l., ITS, *Malvatheca*, *matK*, *Pachira*, *trnL–F*.

Molecular and morphological data have shown that the formerly recognized Bombacaceae, Malvaceae, Tiliaceae, and Sterculiaceae are better treated as a single monophyletic family, Malvaceae s. l., which consists of nine subfamilies (Judd and Manchester 1997; Alverson et al. 1999; Bayer et al. 1999). The subfamilies Bombacoideae and Malvoideae together form the Malvatheca clade, which is well-supported by plastid DNA sequences (Alverson et al. 1999; Bayer et al. 1999; Nyffeler et al. 2005). Malvatheca is also united by the possession of modified anther morphology (von Balthazar et al. 2006; Janka et al. 2008). Within Malvatheca, clade Bombacoideae corresponds to the bulk of the traditional family Bombacaceae (Alverson et al. 1999; Bayer et al. 1999; Nyffeler et al. 2005). Although Baum et al. (2004) elucidated the phylogenetic relationships in Malvatheca, some significant genera were not sampled, especially *Eriotheca* and other elements of *Bombax* s. l.

The taxonomic revision of *Bombax* s. l. by Robyns (1963) is the most comprehensive and relevant study of *Eriotheca* and related genera. Robyns recognized five Neotropical genera: *Pseudobombax* Dugand, *Bombacopsis* Pittier, *Rhodognaphalopsis* A. Robyns, *Eriotheca* Schott & Endl., and *Pachira* Aubl., and two paleotropical genera: *Bombax* L. and *Rhodognaphalon* (Ulbrich) Roberty emend. A. Robyns. In Robyns' treatment, only *Eriotheca* (small flowers reaching 6.5 cm and 18–170 stamens) and *Pseudobombax* (inarticulate leaflets) were separated based on macromorphological characters. The other Neotropical genera, *Pachira, Bombacopsis,* and *Rhodognapholopsis*, were differentiated instead by palynological characters.

Steyermark and Stevens (1988) and Alverson (1994) identified problems with Robyns' distinctions among *Bombacopsis*, *Rhodognaphalopsis,* and *Pachira* s.s., including overlapping floral and fruit characters. Additionally, Steyermark and Stevens (1988) showed that palynological features that had been used to diagnose *Rhodognaphalopsis* were encompassed in the pollen variation of *Bombacopsis*, whereas Alverson (1994) showed that if *Bombacopsis* is considered in the broader sense (i.e. with *Rhodognaphalopsis*), its pollen grades into *Pachira* s.s. Based on this evidence, Alverson (1994) and Alverson and Steyermark (1997) have synonymized *Bombacopsis* and *Rhodognaphalopsis* in *Pachira* s. l. This treatment has been followed by most floristic treatments, including Fernández-Alonso (1998, 2003), and is adopted here.

Eriotheca was created (Schott and Endlicher 1832) based on two species originally described in *Bombax*. Schumann (1886) and van den Brink (1924) rejected *Eriotheca* and treated it as a synonym of *Bombax* or as a section of *Bombax*, respectively. It was not until Robyns (1963), that *Eriotheca* was recognized as a distinct genus again. Alverson (1994) emphasized the importance of molecular studies to clarify the relationships between *Pachira* s. l. and *Eriotheca*, considering the morphological differences between *Eriotheca* (flowers 2.5–6.5 cm and with usually fewer than 120 stamens) and *Pachira* s. l. (flowers 7–35 cm and usually 150 stamens, sometimes as many as 1,000) to be subtle. However, *Eriotheca* has been excluded from most broad-scale molecular analyses of Bombacoideae (e.g. Bayer et al. 1999; Baum et al. 2004). Whereas Alverson et al. (1999) included *Eriotheca*, the markers studied provided no resolution within core Bombacoideae.

In this study, our goal was to conduct a phylogenetic analysis of *Eriotheca* and related genera using sequences of nuclear (ITS) and rapidly evolving plastid DNA regions (*matK* and *trnL-F*) to elucidate the relationships among the genera of Bombacoideae, determine if *Eriotheca* and other elements of *Bombax* s. l. are monophyletic, and help make sense of the morphological diversity found in the group.

Materials and Methods

***Taxon Sampling*—**Taxon sampling included 50 representatives from Bombacoideae and additional taxa of Malvatheca: *Chiranthodendron*, *Fremontodendron*, *Camptostemon*, *Matisia*, *Pentaplaris*, *Gossypium,* and *Abutilon*. Fremontodendreae (*Chiranthodendron* and *Fremontodendron*) was

specified as the outgroup, based on the results of Nyffeler et al. (2005). Sources of DNA material are summarized in the Appendix.

DNA Extraction, Amplification and Sequencing—Total DNA was extracted from silica-dried or herbarium leaf tissue using DNeasy plant mini kits (QIAgen, Valencia, California) according to the manufacture's protocol. The ITS regions were amplified using primers described in Baum et al. (1998) and the *trnL-F* region and *trnK/matK* genes were amplified using primers as described in Taberlet et al. (1991) and Nyffeler et al. (2005), respectively. Polymerase chain reaction (PCR) amplification of ITS was performed with 25 µl reactions containing 5 µl PCR Buffer (5×), 2.5 µL $MgCl_2$, 0.5 µL of each primer (10 pmol/µ), 1 µL dNTPs (2 mM each), 1.5–2.5 µL BSA, 1–1.5 µL DMSO, 0.2 µL Taq DNA polymerase and 1–2 µL of DNA template. Amplification of both plastid regions was performed in 25 µl reactions containing 5 µl PCR Buffer (5×), 2.5 µL $MgCl_2$, 0.5 µL of each primer (10 pmol/µ), 0.5 µL dNTPs (2 mM each); 0.2 µL BSA, 0.2 µL Taq DNA polymerase and 1 µL of DNA template. The amplifications began with 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 90 sec, with a final extension at 72°C for 5 min. Products were cleaned using AMPure beads (Agencourt Bioscience Corp., Beverley, Massachusetts) and cycle sequenced (Big Dye v.3.1, Applied Biosystems Corp., Foster City, California) following the manufacturer's instructions. Sequencing was performed at the Biotechnology Center, University of Wisconsin, Madison, Wisconsin. Sequences were edited and assembled in Sequencher 4.7 (Gene Codes Corp., Ann Arbor, Michigan). Plastid DNA was aligned manually in MacClade 4.08 (Maddison and Maddison 2005). The ITS sequences were aligned initially with Clustal W (Thompson et al. 1994) through the Cipres Portal (Miller et al. 2009), with subsequent manual adjustment in MEGA4.0 (Tamura et al. 2007).

The ITS sequences were analyzed with MFold (Zuker 2003) to estimate their thermodynamic stability (folding energy) in an effort to identify possible nonfunctional paralogs (pseudogenes). MFold predicts nucleic acid folding and hybridization by free energy minimization using empirically derived thermodynamic parameters (Zuker 2003).

Phylogenetic Analyses—Maximum parsimony (MP) analyses were performed in PAUP* 4.0b10. Maximum parsimony heuristic searches used 10,000 random taxon addition replicates (holding 20 tree at each step) and TBR branch swapping. All characters were equally weighted, and gaps were treated as missing data. To estimate clade support, we obtained bootstrap percentages (BS) for each clade using 1,000 replicates with simple taxon addition (holding 20 trees at each step) and TBR branch swapping.

Bayesian phylogenetic analyses were implemented with MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001). The best available substitution model was determined in MrModeltest 2.2 (Nylander 2004). Three independent MCMC runs were conducted, each composed of four linked chains that ran for 3,000,000 generations with sampling every 1,000 generations. The burn-in period was estimated by visual examination of a likelihood-by-generation plot. After discarding the trees from the burn-in period, a 50% majority rule consensus tree was constructed from the remaining trees and the three posterior distributions were pooled to obtain the best estimates of clade posterior probabilities (PP).

Incongruence was estimated with incongruence length (ILD) tests (Farris et al. 1994), implemented in PAUP*4.0b10 (Swofford 2002). The ILD test was conducted using the parsimony optimality criterion, using 100–1,000 partition homogeneity replicates, simple taxon addition, tree bisection- reconnection (TBR) and holding one tree at each step. In some tests MAXTREES was set to 100 to speed up analysis.

Results

We obtained 48 new ITS, 56 *trnL-F* and 39 *matK* sequences, which were combined with 16 *matK* sequences previously published (Baum et al. 2004, Nyffeler et al. 2005), creating a data set for 57 taxa, within which all taxa were represented by data from at least two of the three regions. It was not possible to obtain satisfactory ITS sequences from *Eriotheca gracilipes, E. pentaphylla, E. roseorum, E. ruizii, Neobuchia paulinae,* and *Pachira* (*Rhodognaphalopsis*) *minor*. Likewise, our data matrix lacked *trnL-F* sequences from *P.* (*Rhodognaphalopsis*) *brevipes* and *matK* sequences from *E. obcordata* and *E. squamigera*. These DNA isolations were obtained from older herbarium collections and presumably degraded. Nonetheless, these exemplars were included in the combined analysis with the missing portions coded as such.

Calculation of the folding energies for each ITS sequence identified a subset of sequences with notably lower folding energies (Supplemental figure S1). Considering the range of folding energies obtained from all ITS sequences, we chose to exclude all sequences with a folding energy less than –157.00 as possible pseudogenes. The ITS sequences from *E. discolor*, *E. squamigera,* and *P. (Bombacopsis) quinata* were identified as probable pseudogenes. The resulting data matrix was submitted to TreeBASE (study number S11080)

Phylogenetic Analyses of Individual Data Sets—The ITS data set had an aligned length of 808, with 332 informative characters. Parsimony analyses retained 20 trees with length 1,711, CI = 0.4880 and RI = 0.6458. The *matK* and *trnL-F* data sets had aligned lengths of 2,701 and 1,375, with 187 and 96 informative characters respectively. MP analyses retained 80 trees with length 732, CI = 0.7923 and RI = 0.7419 for the *matK* data set and retained 60 trees with length 362, CI = 0.8343 and RI = 0.7872 for *trnL-F* data set.

MrModeltest indicated that the model nst = 6 rates = invgamma is preferred for ITS, *matK,* and *trnL-F* data sets according to AIC model selection (AIC weight = 0.9990, 0.9886, and 0.4962, respectively). The three Bayesian MCMC runs for each data set were composed of 9,003 post burn-in trees each. Comparison among the independent runs showed that all had converged on the same posterior distribution, suggesting that they had mixed adequately. Stability was reached by approximately 50,000 generations, which was designated as burnin. The single-gene posterior distributions were summarized using 50% majority-rule consensus trees (Figs. 1–3).

All three genes supported a monophyletic bombacoid clade. The deep relationships within this clade were unresolved for the plastid genes, but ITS supported the existence of a *Bernoullia-Gyranthera-Huberodendron* clade as sister to a core Bombacoideae clade, which contains all the palmately-compound-leaved species, and just one simple-leaved taxon, *Cavanillesia*. This topology is consistent with patterns of staminal evolution (von Balthazar et al. 2006).

Within core Bombacoideae, both ITS and *trnL-F* support a clade that includes *Ceiba* s. l. (*Ceiba* and *Neobuchia*), *Spirotheca*, and all of *Bombax* s. l. except *Rhodognaphalon* (i.e. *Bombax, Eriotheca, Pachira* s. l., and *Pseudobombax*). The position of *Rhodognaphalon* varies between these genes. The ITS data set supports *Rhodognaphalon* as in or sister to the *Ceiba* s. l. - *Bombax* s.l clade. The *trnL-F* data set, in contrast, places this taxon with *Adansonia* as sister to the *Ceiba* s. l. - *Bombax* s.l clade.

All individual analyses showed that *Eriotheca* forms a moderately to strongly supported clade with all species of *Pachira* except *Pachira quinata*. Resolution within the *Pachira/Eriotheca* clade varied among the genes and was generally weakly supported. The *matK* data supported a monophyletic *Eriotheca* (PP = 0.98, BS = 52%) sister to *Pachira* clade, with weak support (PP = 0.68, BS ≤ 50%; Fig. 3).

Both *Pseudobombax* and *Ceiba* s. l. consistently emerged as monophyletic groups that were sister to one another in both the *trnL-F* and ITS phylogenies. The relationships among *Bombax* s.s., *Spirotheca,* the *Pachira-Eriotheca* clade, and *Pseudobombax-Ceiba* s. l. clade varied greatly among the three data sets (Figs. 1–3)

Evaluation of Discordance Between the Data Sets—An ILD test found marginally significant heterogeneity between the two plastid regions, *matK* and *trnL-F* (p = 0.04–0.06).

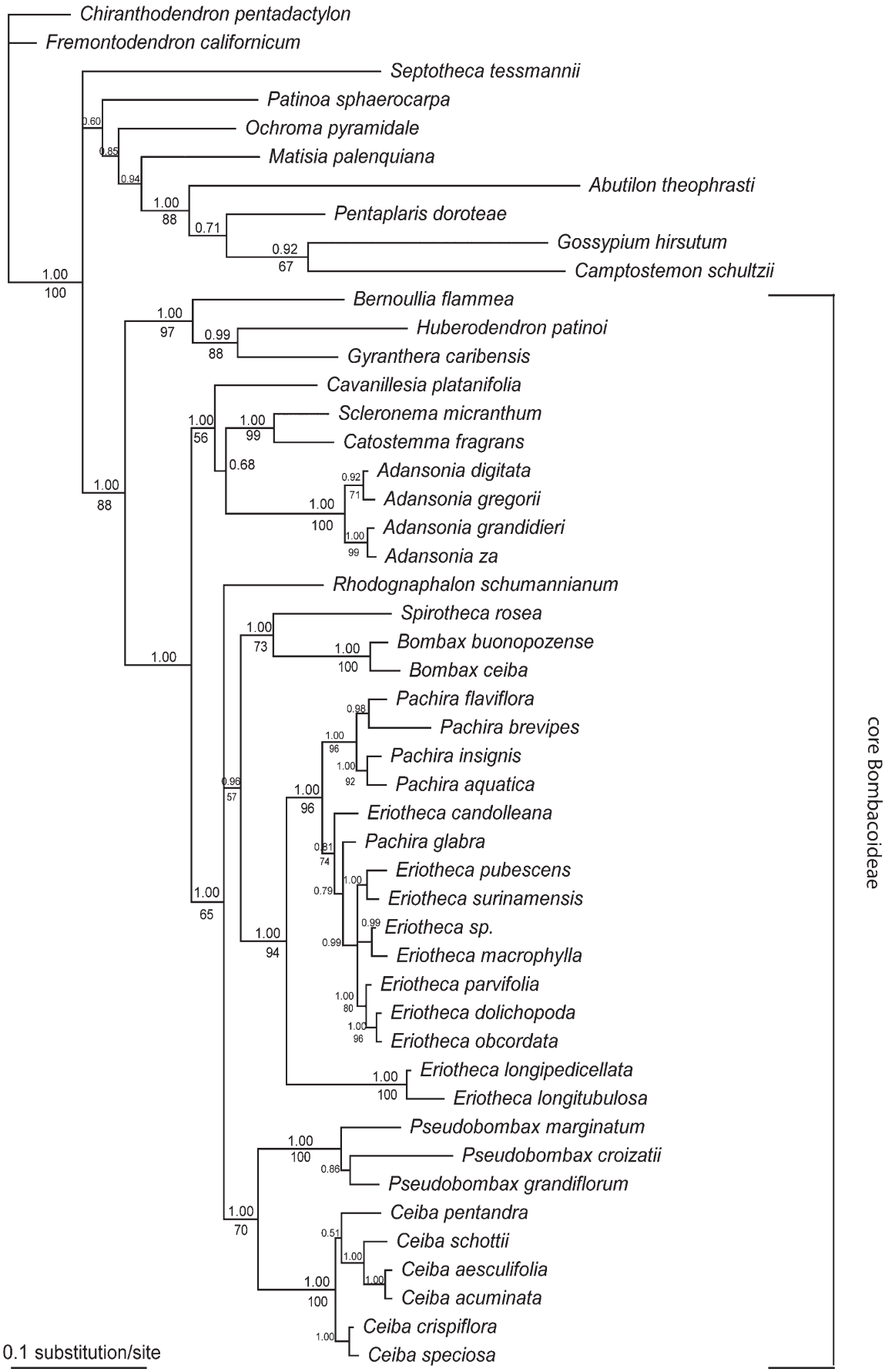

FIG. 1. Phylogram of Bayesian analysis of ITS data. Numbers above the branches represent posterior probability (PP) and numbers below these branches are bootstrap values (BS).

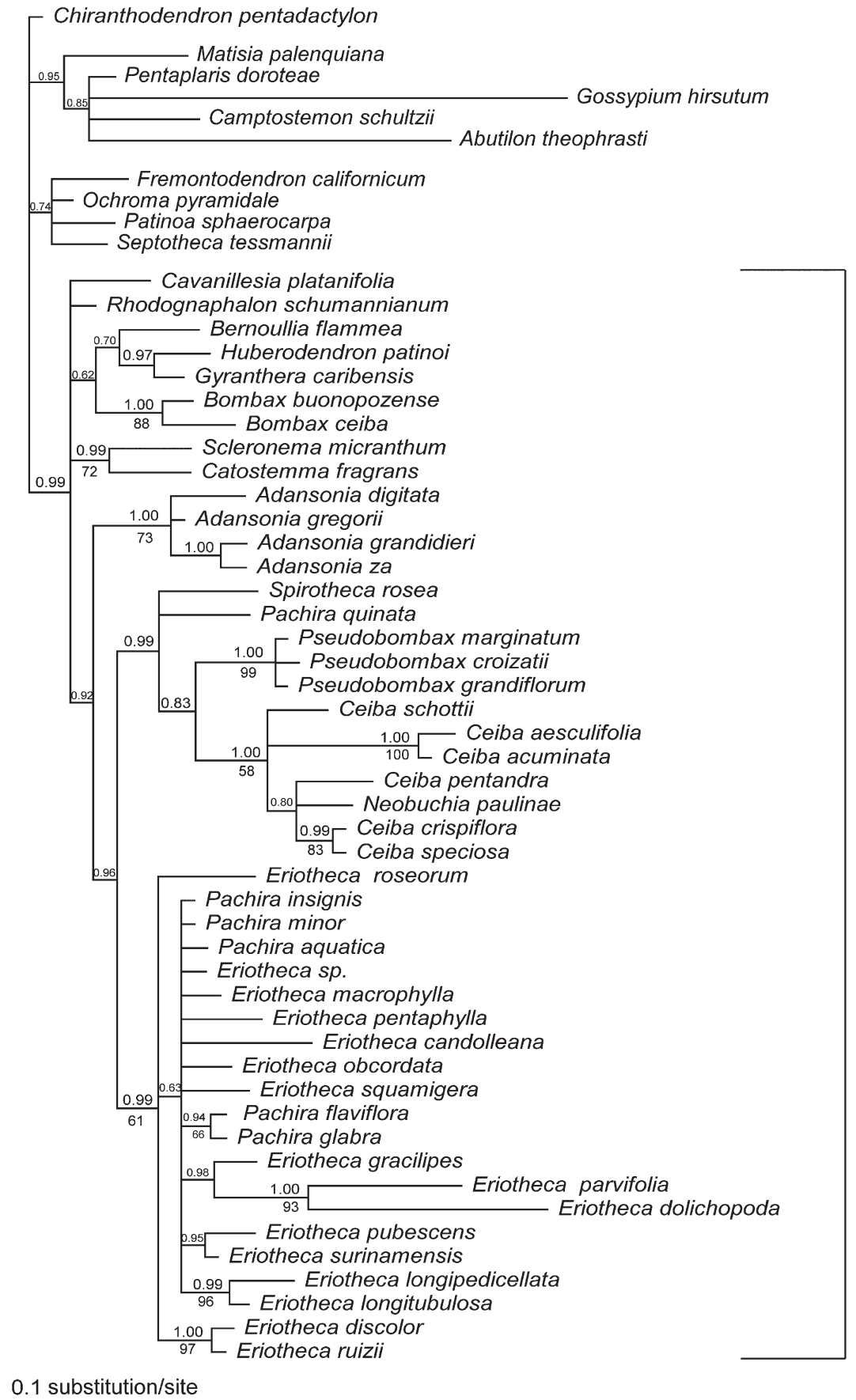

FIG. 2. Phylogram of Bayesian analysis of *trnL–F* data. Numbers above the branches represent posterior probability (PP) and numbers below these branches are bootstrap values (BS).

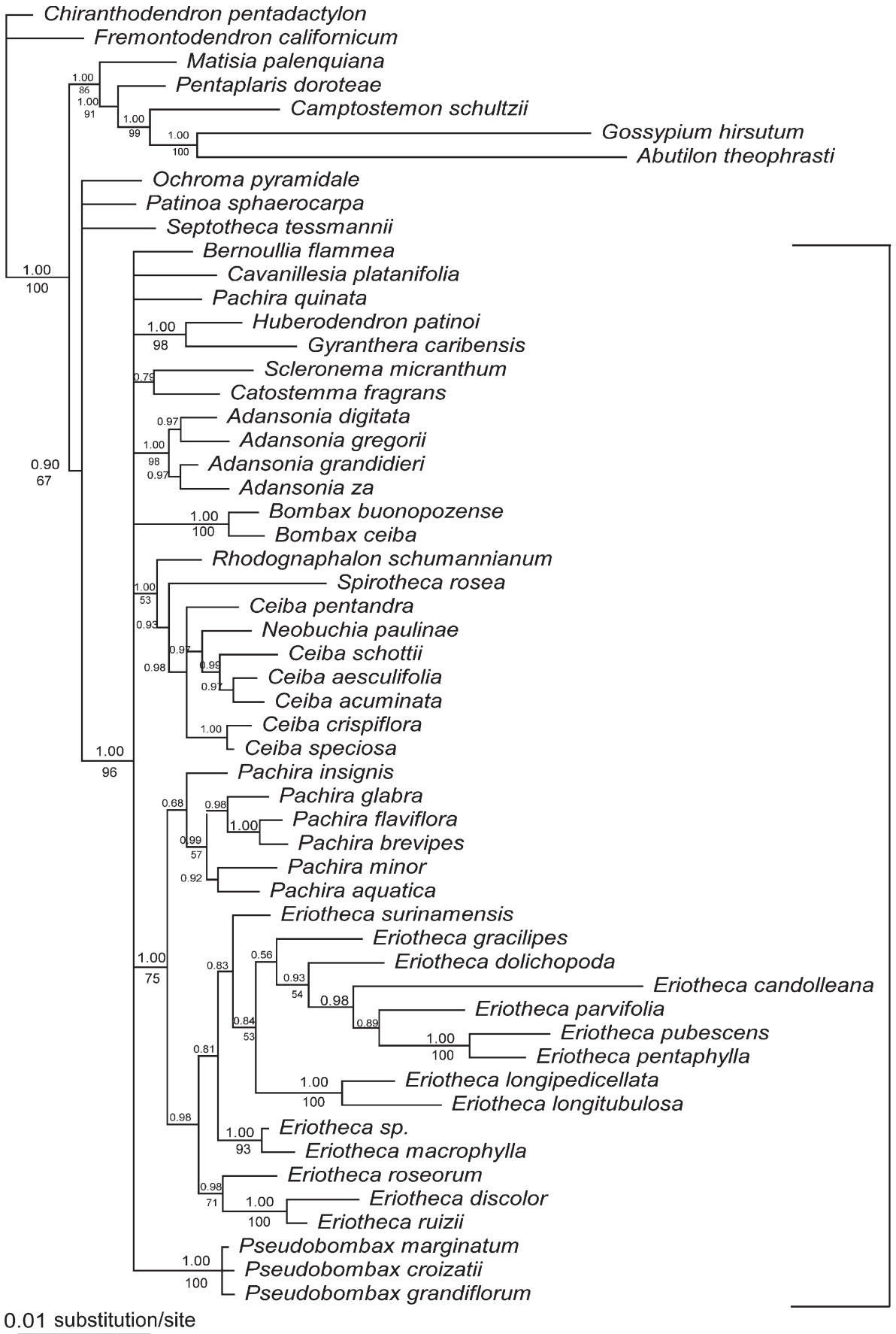

FIG. 3. Phylogram of Bayesian analysis of *matK* data. Numbers above the branches represent posterior probability (PP) and numbers below these branches are bootstrap values (BS).

Exploration of possible sources of incongruence were conducted by selectively deleting taxa and rerunning the ILD test. The ILD test showed the existence of significant conflict between ITS and plastid regions ($p = 0.013$). This result is not due just to the placement of *Ochroma*, *Patinoa* and *Septotheca* since the ILD test is significant when these taxa are deleted ($p = 0.003$). Significant ILD test p values should not be taken as a conclusive demonstration that analyzing independent data partitions in combination will produce misleading phylogenies (Hipp et al. 2004). The ILD test has only limited power to detect incongruence caused by differences in tree topology, except when numerous characters are present and the substitution rate is homogeneous from site to site (Darlu and Lecointre 2002). There is no strongly-supported topological difference among trees inferred from the different data partitions. Therefore, we will treat the combined data matrix as the best estimate of the group's phylogeny.

Combined Phylogenetic Analyses—The combined data set has an aligned length of 4,884, with 617 informative characters. Parsimony analyses retained 140 trees with length = 2,854, CI = 0.6030 and RI = 0.6609. MrModeltest indicated that the model nst = 6 rates = invgamma is preferred for combined data set according to AIC model selection (AIC weight = 1.00). The Bayesian MCMC runs contained 9,003 post burn-in trees each. Comparison among the independent runs showed that all had mixed adequately. Stability was reached by approximately 50,000 generations. The combined posterior distribution was summarized using a 50% majority-rule consensus tree of all the postburnin trees (Fig. 4).

The combined analysis most closely resembles the ITS phylogeny, but contains elements of all three genes' topologies and has higher measures of support for many clades. For example, the *Eriotheca-Pachira* clade is well supported (PP = 1.00, BS = 99%), confirming the individual analyses. Likewise, the monophyly of *Pseudobombax* (PP = 1.00, BS = 100%) and *Ceiba* s. l. (PP = 1.0, BS = 79%) and their sister-group relationship (PP = 0.95, BS = 50%) was confirmed in the combined analysis.

The core bombacoid clade found by Baum et al. (2004) is supported (PP = 1.00, BS = 100%). The only taxon in this clade that has palmately-lobed, simple leaves is *Cavanillesia*. Examination of the posterior distribution did not yield any trees in which *Cavanillesia* is sister to the rest of the core Bombacoideae, which would be needed to invoke a single, unreversed origin of palmately compound leaves. Furthermore, a Templeton test comparing the most-parsimonious tree with the optimal tree found under the constraint of a palmately compound clade (all core bombacoideae except *Cavanillesia*) was significant ($p < 0.006$). This shows that these data are explained significantly better on trees that require homoplasy in leaf evolution, relative to trees that allow for a single, unreversed origin of compound leaves.

An unexpected result of our analyses was the paraphyly of *Eriotheca.* The combined analyses suggested that *Pachira* s. l. is embedded within an *Eriotheca* grade. This result is largely due to the ITS partition, which strongly contradicts *Eriotheca* monophyly by virtue of a small clade, *E. longipedicellata* and *E. longitubulosa*, as sister to a larger clade that includes *Pachira* s. l. and the remainder of *Eriotheca* (PP = 1.00, BS = 96%). On the other hand, *matK* supports *Eriotheca* monophyly (PP = 0.98, BS ≤ 50%), which may explain why the combined analysis does not provide overwhelming support for *Eriotheca* paraphyly: the branches contradicting monophyly have bootstrap scores of 78% or less. The shortest tree for the combined data under the constraint of *Eriotheca* monophyly is nine steps longer than the overall optimal tree. This cost is not judged significant using a Templeton test ($p = 0.20$).

Discussion

Phylogenetic Relationships and Character Evolution—We consider the combined analysis to be the best estimate of the evolutionary relationships in Bombacoideae (Fig. 4) and will use this as a basis for further discussion. The overall relationships are similar to those reported by Baum et al. (2004) based on *ndhF* and *matK.* In the present study, we confirmed the monophyly of core Bombacoideae (PP = 1.00, BS = 100%). This group is characterized by compound leaves (1–9-foliolate; Fig. 5A, E, G, N), except for *Cavanillesia*, which has simple leaves (Fig. 5I). Given this tree, equally-weighted parsimony would imply one origin of compound leaves and a reversal to simple leaves in *Cavanillesia.*

The placement of *Ochroma* and *Patinoa* regarding Bombacoideae was discussed by Alverson et al. (1999), Baum et al. (2004) and Nyffeler et al. (2005). In our analysis, both genera emerged in Malvoideae (Figs. 1, 4), differing from the results obtained by Baum et al. (2004), where the genera fell as a sister-group to Malvatheca (Bombacoideae and Malvoideae). Our combined analysis did, however, agree with Baum et al. (2004) in placing *Septotheca* as sister to core Bombacoideae. However, in both studies, the resolution of *Ochroma*, *Patinoa*, and *Septotheca* is uncertain. Phylogenetic resolution is likely to depend upon the development of more informative nuclear markers.

The use of rapidly evolving genes and the inclusion of *Neobuchia*, *Bernoullia*, *Cavanillesia*, *Rhodognaphalon*, *Spirotheca*, *Pseudobombax,* and *Eriotheca*, for the first time, allows our study to clarify the phylogenetic relationships among these genera and other taxa of core Bombacoideae.

In the combined phylogeny, as well as in ITS, we can distinguish three major clades (Fig. 4). Clade 1, sister to the remainder of core Bombacoideae and composed of *Huberodendron*, *Gyranthera,* and *Bernoullia*, was strongly supported (PP = 1.00, BS = 98%). All the exemplars of clade 1 present indehiscent fruits (Fig. 5H) and staminal filaments completely fused into a tube, with the sessile, polythecate anthers positioned near the apex of the staminal tube (Fig. 5J) (von Balthazar et al. 2006). Indehiscent fruits are also present in *Adansonia*, *Cavanillesia,* and *Scleronema* (clade 2), suggesting that loculicidal dehiscence might be a synapomorphy of clade 3, albeit one showing homoplasy (e.g. *Catostemma* in clade 2 has a dehiscent fruit).

The sessile stamens found in clade 1 resemble those found in the outgroups, *Ochroma*, *Patinoa*, *Septotheca,* and *Matisia*, whereas clades 2–3 generally have stalked, monothecate units that extend from the staminal tube either individually or in phalanges (Fig. 5B, L). Von Balthazar et al. (2006) hypothesized that the origin of stalked, monothecate anthers might have been driven by a transition from mammal to insect pollination. The exception to this pattern is *Ceiba* s. l. (including *Neobuchia*), which appears to have reverted to sessile anthers, as illustrated by *Ceiba speciosa* (Fig. 5O).

Clade 2, including *Adansonia*, *Catostemma*, *Cavanillesia,* and *Scleronema*, has only moderate support (PP = 1.00; BS = 65%). It is resolved as sister to clade 3, but this result is weak, as judged by bootstrap analysis (PP = 1.00, BS ≤ 50%).

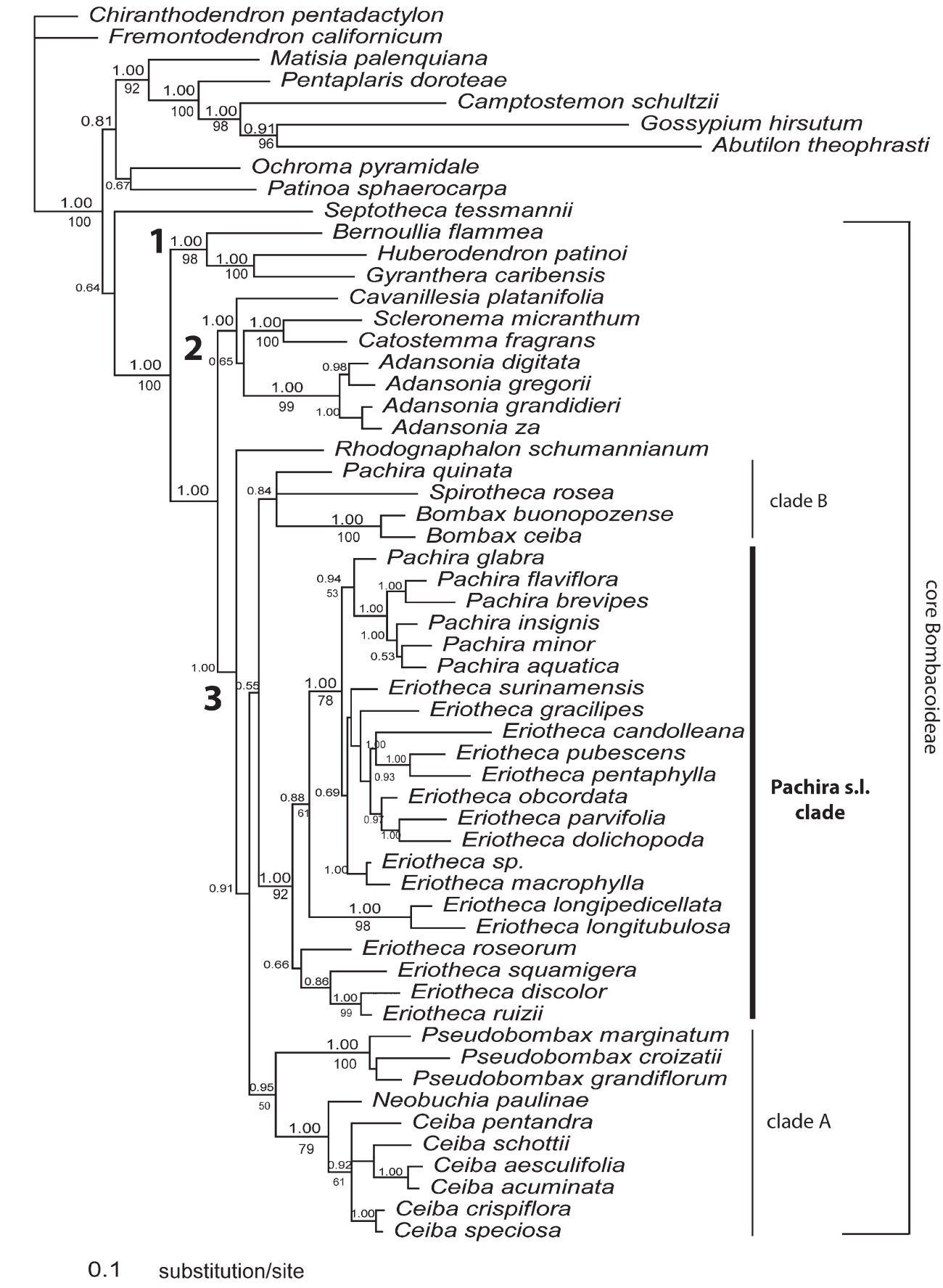

FIG. 4. Phylogram of combined ITS, *trnL–F* and *matK* data. Numbers above the branches represent posterior probability (PP) and numbers below these branches are bootstrap values (BS).

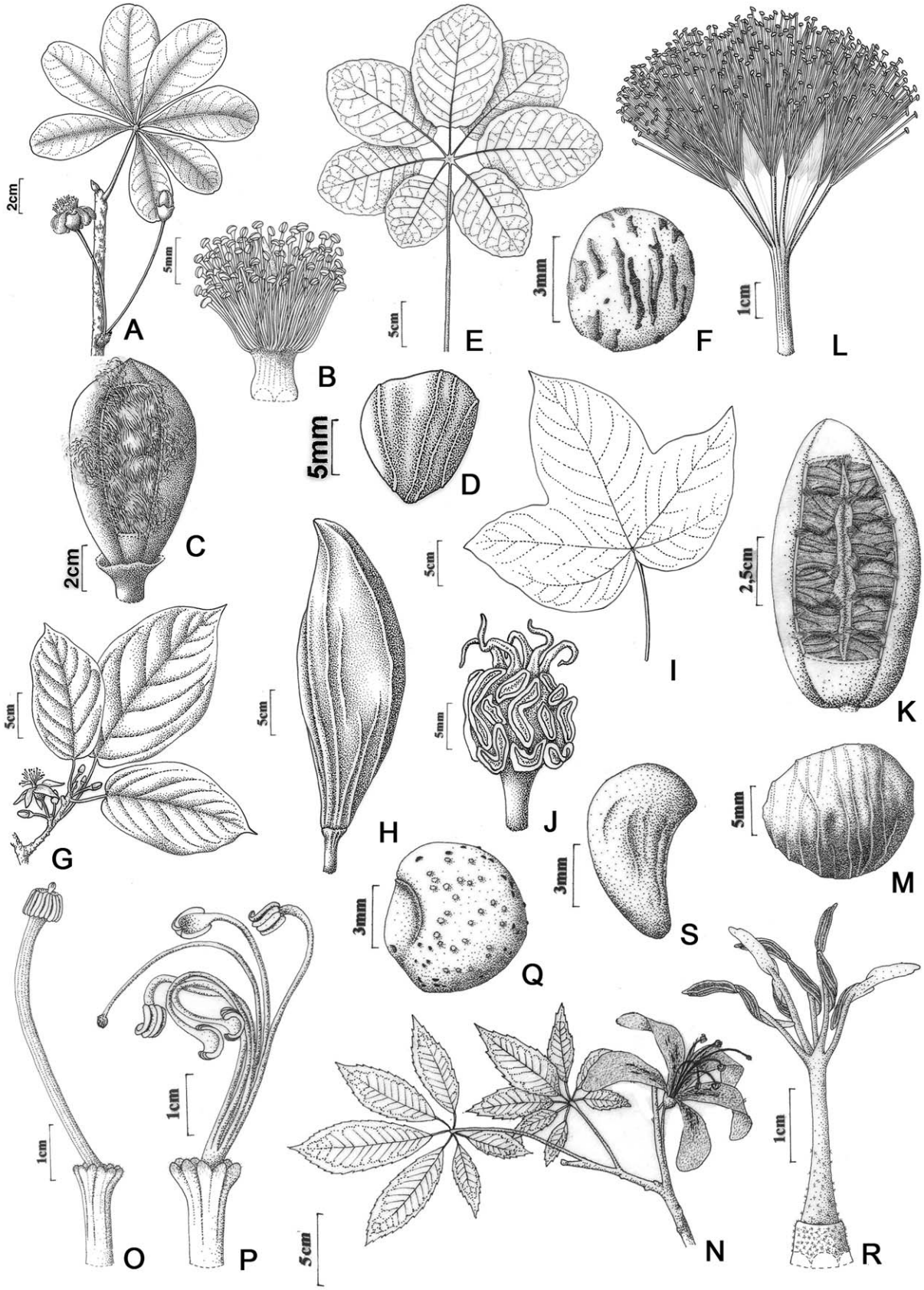

FIG. 5. A – S. Vegetative and floral structures of the genera of Bombacoideae. A – D: *Eriotheca*. A. Flowering branch. B. Staminal tube. C. Opened fruit, showing kapok. D. Seed. E –F: *Pseudobombax*. E. Leaf. F. Seed. G-H: *Scleronema*. G. Flowering branch. H. Fruit. I. *Cavanillesia*, leaf. J. *Huberodendron*, apical portion of staminal tube with anthers. K – M: *Pachira*. K. Opened fruit, showing seeds involved by kapok. L. Staminal tube. M. Seed. N – Q: *Ceiba*, N. Flowering branch. O and P. Staminal tubes. Q. Seed. R – S: *Spirotheca*. R. Staminal tube. S. Seed (figs. A-D: extracted from Duarte and Esteves, in prep.; E-F, K-S: extracted from Duarte 2006; G: modified from Schumann 1886; H: extracted from Esteves 2005; I: modified from Robyns 1964; J: extracted from Alverson and Mori 2002).

A relationship between *Scleronema* and *Catostemma* has been noted previously (e.g. Alverson et al. 1999), and an affinity between these two and *Cavanillesia* is implied by certain taxonomic schemes. However a close relationship between these three Neotropical genera and the paleotropical genus *Adansonia* has not previously been hypothesized. *Adansonia*, *Cavanillesia,* and *Scleronema* share an indehiscent fruit, but this may be a plesiomorphic trait. Additionally, these taxa have a campanulate, 5-lobed calyx, which contrasts with taxa in clade 3, which usually have a truncate to 5-apiculate, cupulate to tubular calyx. However, a campanulate calyx is also likely to be plesiomorphic and is hard to compare across clade 2 because of the great differences in flower size between *Scleronema*/*Catostemma* (typical less than 1.5 cm) and *Adansonia* (10–30 cm). Further work is needed to confirm the reality of clade 2 and to identify possible synapomorphies.

Clade 3, comprising *Rhodognaphalon*, *Spirotheca*, *Bombax*, *Pachira-Eriotheca*, *Pseudobombax*, *Ceiba,* and *Neobuchia*, is characterized by stalked, monothecate staminal units, like clade 2 (except for *Ceiba speciosa*, which has sessile staminal units). Clade 3 is characterized by dehiscent fruit with kapok (Fig. 5C, K). This is composed of long hairs derived from the fruit wall, which contrasts with cotton fibers, which derive from the seed coat (Marzinec and Mourão 2003).

The African genus *Rhodognaphalon* is resolved as sister to the rest of clade 3 (PP = 0.91; BS < 50%). This genus is characterized by porate or pororate pollen grains with a spinulose sexine (Robyns 1971, Nilsson and Robyns 1986), whereas other members of *Bombax* s. l. have colpate to colporate pollen with a reticulate sexine. However, pollen traits cannot easily be mapped onto the broader Bombacoid relationships. Somewhat tringular, colpate/colporate pollen, such as that found in *Bombax* s. l., is also found in *Bernoullia*, *Cavanillesia*, and *Catostemma* (Nilsson and Robyns, 1986). More spherical porate pollen types resembling *Rhodognaphalon* also occur in *Adansonia*, *Gyranthera*, and *Huberodendron*, making it impossible to account for pollen evolution on these trees without homoplasy.

The Neotropical genera *Ceiba*, *Pseudobombax,* and *Neobuchia* constitute clade 3A (PP = 0.95, BS = 50%), with *Pseudobombax* and *Ceiba* each supported as monophyletic, and with the monotypic genus *Neobuchia* as sister to *Ceiba*. The monophyly of *Pseudobombax* was found by Carvalho-Sobrinho (2006) based on morphological data, and is supported by a synapomorphy of inarticulate leaflets and dilated and disciform petioles (Fig. 5E). The placement of *Neobuchia* with *Ceiba* in all analyses is plausible. Both genera are the only members of clade 3 to share crenate to serrate folioles and to have staminal filaments partially united into a tube with just five free or sessile stamens (Fig. 5N,P). However, the reduction to five stamens is also seen in *Spirotheca* (Fig. 5R). The relationship between *Ceiba/Neobuchia* and *Pseudobombax* has not previously been proposed, and we can identify no potential synapomorphies.

Clade 3B contains three heterogeneous elements: *Bombax*, *Spirotheca* and *Pachira quinata* (Jacq.) W. S. Alverson. These three taxa share spiny trunks, but this trait also occurs in Clade 3A, and in some members of clade 2 (e.g. *Cavanillesia*). *Spirotheca* and *P. quinata* are both Neotropical and have a persistent calyx (the norm in Bombacoideae) and dotted seeds (Fig. 5S). The paleotropical genus *Bombax*, has a caducuous calyx (which apparently evolved independently within *Adansonia;* Baum, 1995) and unmarked seeds.

The position of *Pachira quinata* in clade 3B invalidates recognition of this taxon within *Pachira.* Excluding this species will make *Pachira* a more cohesive group in terms of morphology, because no other species in the group has dotted seeds or aculeate trunks and branches.

The *Pachira* clade (PP = 1.00, BS = 92%) comprises *Eriotheca* and all members of *Pachira* s. l. except *P. quinata*. This grouping is supported in most analyses and is consistent with a probable synapomorphy: the presence of striations on the seeds (Fig. 5D, M). Although the seed coat has not generally been utilized, it appears to show useful variation within Bombacoideae. Duarte (2006) and Duarte et al. (2007) showed that the seeds are maculate in *Pseudobombax*, verrucose in *Ceiba*, dotted in *Spirotheca* (Fig. 5F, Q, S) and striate in *Eriotheca* and *Pachira* (Fig. 5D, M), whereas other genera (*Bombax*, *Gyranthera*, *Cavanilesia*, *Huberodendron*, *Catostema*, *Scleronema*, *Adansonia*, *Septotheca,* and *Bernoullia*) have unmarked seeds.

Our sampling of *Pachira* s. l., while perhaps less extensive than is ideal, includes representatives of all three segregate genera recognized by Robyns (1963), namely *Pachira* s.s. (*P. aquatica* and *P. insignis*), *Bombacopsis* (*P. glabra)*, and *Rhodognaphalopsis* (*P. flaviflora*, *P. minor* and *P. brevipes*) (Figs. 1–4). While resolution is weak, neither segregate genus with more than one accession appeared to be monophyletic. Our results, thus, tend to corroborate the proposed synonymy of these three genera in *Pachira* s. l. (Alverson 1994; Alverson and Steyermark 1997; Fernández-Alonso 1998, 2003). Furthermore, *Pachira* s. l. appears to be monophyletic in the combined and *matK* analyses.

An unexpected result of our research was the paraphyly of *Eriotheca.* The ITS, *trnL-F* and combined analyses all suggested that *Pachira* s. l. is embedded within an *Eriotheca* grade. On the other hand *matK* supports *Eriotheca* monophyly, and the combined data are not sufficient to rule out *Eriotheca* monophyly, as judged in a parsimony framework, using a Templeton test. Thus, while paraphyly of *Eriotheca* is the best-supported hypothesis based on the available data, it would be premature to combine *Eriotheca* and *Pachira* on this basis alone.

Robyns (1963) distinguished *Pachira* s. l. and *Eriotheca* based on the size of flowers, number of stamens, and number of androecial whorls: *Eriotheca* with small flowers, 18–170 stamens and one androecial whorl and *Pachira* with large flowers, 90–1,000 stamens and two androecial whorls. However, in terms of both flower size and number of stamens per flower the range seen in the two genera overlaps (Duarte and Esteves in prep.). Although developmental analysis shows that *Eriotheca* androecia initially form two stamen whorls which later merge into a single whorl (Janka et al. 2008), the distinction in adult form can be used to separate *Eriotheca* and *Pachira*.

In general, the relationships among the exemplars within the *Eriotheca* grade corroborate the groups proposed by Robyns (1963) based on the morphology of the staminal tube. The *E. roseorum*-*E. ruizii* clade, sister-group to *Pachira* s. l., is weakly supported and apparently lacks morphological synapomorphies. However, the relationships within this clade correlate with differences in androecium morphology. *E. roseorum* (Fig. 6A) has 20–25 stamens and an obconical staminal tube with a constriction in the basal portion, a structure that is found in no other genus of Bombacoideae. *Eriotheca squamigera*, *E. discolor,* and *E. ruizii*, in contrast all have about 100 stamens. Whereas, *E. squamigera* has a staminal tube that is expanded and thickened at the point of origination of the free filaments

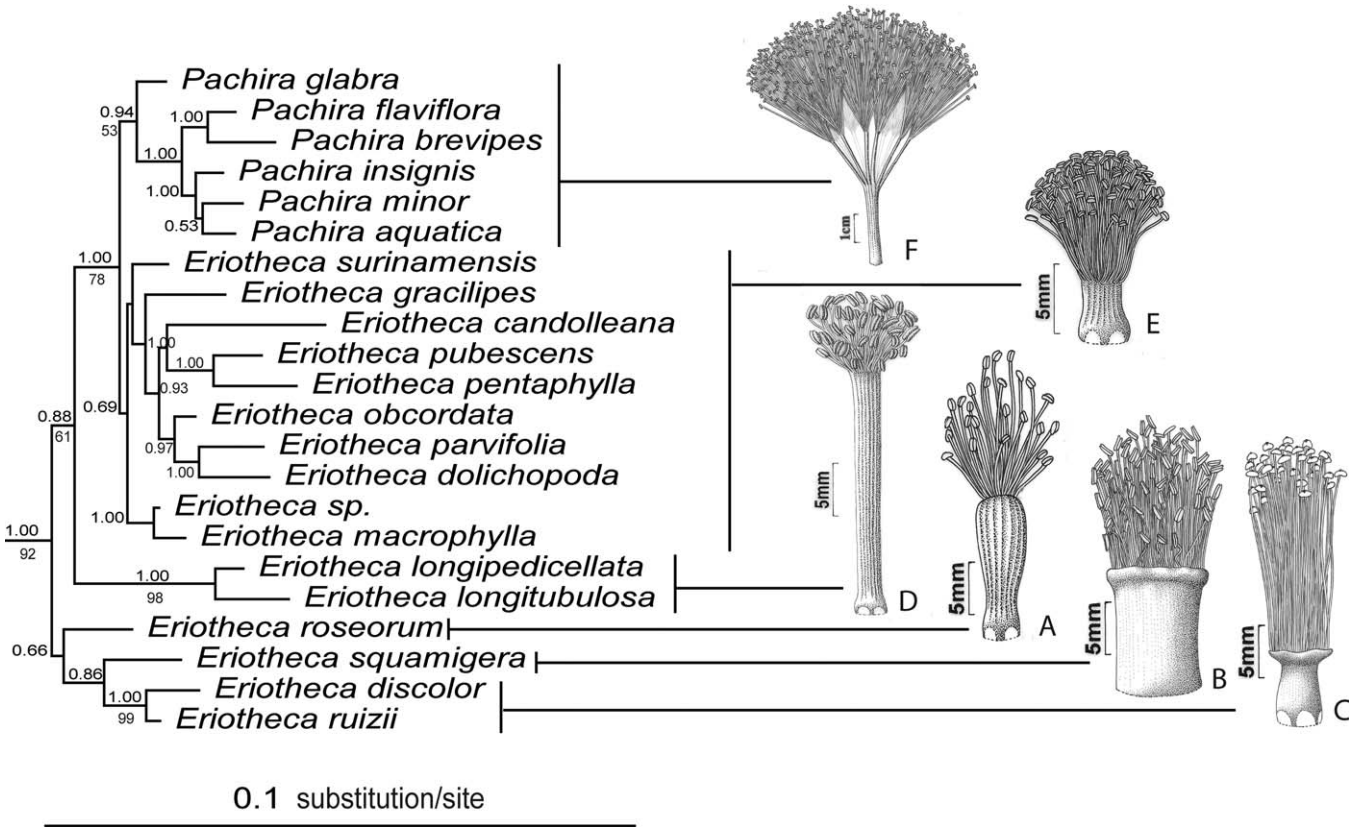

FIG. 6. Phylogram of *Pachira* clade, and staminal tube of species of *Eriotheca* (A–E) and *Pachira* (F).

(Fig. 6B), *E. discolor* and *E. ruizii* have staminal tubes with a 5-lobed apical portion (Fig. 6C). The latter two species are also both deciduous trees that inhabit dry forests in high altitudes in Ecuador and Peru.

A clade comprising *E. longipedicellata* and *E. longitubulosa* is strongly supported by molecular data and by their androecium morphology. Both species have only 20–60 stamens and have a cylindrical and elongated staminal tube, whose length is greater than the length of the free filaments (Fig. 6D). MacFarlane et al. (2003) reported hawkmoths as possible pollinators of *E. longipedicellata* and *E. longitubulosa*, associating this kind of pollination to the small number of stamens and shape of flowers (not as a brush). This morphology resembles *Adansonia perrieri*, another hawkmoth-pollinated bombacoid (Baum 1995).

Members of the *Eriotheca surinamensis* clade produce oblong to obovoid flower buds, have flowers reaching only 6.5 cm, and are characterized by an androecium with free filaments that emerge from the apex of the staminal tube (Fig. 6E). Members of the *Eriotheca surinamensis* clade are distributed predominantly in Brazil (including the Amazonian forests, central Cerrado, and northeast and southeast Atlantic forest). Further work is needed to identify possible macromorphological synapomorphies within the *E. surinamensis* clade.

The *Pachira* s. l. clade is characterized by linear flower buds, flowers that are seven to 35 cm in length, and an androecium with two rings of stamens, the outer ones clustered into phalanges (Fig. 6F). The species of *Pachira* s. l. are concentrated in the forests of northern South America, except *P. glabra*, which has a cosmopolitan distribution.

Directions for Future Work—Many of the major conclusions we have reported were based largely on ITS sequences. However, this region has been shown to pose particular problems for phylogenetic inference due to ancient or recent tandem duplication events and the potential for pseudogenes or divergent sequences to persist in some genomes in various states of decay. These phenomena create paralogous sequence relationships that can potentially confound phylogenetic reconstruction. Additionally, homoplasy has been shown to be higher in ITS than in other DNA sequence data sets, most likely because of orthology/paralogy conflation, compensatory base changes, and problems in alignment due to frequent indels (Álvarez and Wendel 2003). Therefore, future phylogenetic work in Bombacoideae might benefit from focusing on low-copy nuclear markers.

With an improved data set it would be possible to test some of the less expected results of our analysis, for example clade 2, clade 3B, and *Eriotheca* paraphyly. Likewise, a future study could confirm our inferences of three independent migrations from the New World to the Old World and could use molecular dating to assess whether these might have occurred at a time when migration via the Boreotropical route was possible.

Our study also suggests promising avenues for future morphological work. For example, we found that seed characters provided several valuable morphological characters for diagnosing the major clades. It appears that fruit dehiscence may also be a useful trait, although this will require more analysis of modes of fruit development and dehiscence. We believe that other organ systems, for example scales and leaf venation patterns, show promise for diagnosing species and clades. Through such additional morphological work and the addition of new molecular markers we can hope eventually to fully resolve relationships in Bombacoideae, to identify visible synapomorphies for all genera, and to use this knowledge

to achieve a clearer understanding of the group's morphological and ecological evolution.

Acknowledgments. This work was funded by the National Science Foundation (NSF), grant DEB-0416096. The present work was also funding by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): grant 485614/2007-3, Ph. D scholarship for the first author and Productivity Research grant for G. L. Esteves. The first author also thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for international interchange scholarship and the International Association for Plant Taxonomists (IAPT) for research grant. We thank Stephanie McFarlane, Rebecca Oldham-Haltom, and Stacey Smith for technical assistance and sequencing and Bil Alverson for expert advice and DNA material.

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Appendix 1. Taxa, GenBank accession numbers for the regions and vouchers of plant material form which DNA was extracted for sequecing. Sequences generated in previous studies are referenced with GenBank accession numbers. Taxa are listed alphabetically by genus and species. (—= sequence not obtained). Data are presented in the order of taxon, ITS, *trnL–F, matK,* and voucher.

Abutilon theophrasti Medik., HQ658361, HQ696727, HQ696683, *R. Small 315* (WIS); *Adansonia digitata* L., HQ658372, HQ696738, AY321168 (Nyffeler et al. 2005), Pac. Trop. Bot. Gard. Acc.no. 770032002, Kenya (#23); *Adansonia grandidieri* Baill., HQ658373, HQ696739, HQ696687, *Baum 345* (MO); *Adansonia gregorii* F. Muell., HQ658374, HQ696740, HQ696688, *Wendel s. n.* (ISC); *Adansonia za* Baill., HQ658375, HQ696741, HQ696689, *Baum 357* (MO); *Bernoullia flammea* Oliv., HQ658366, HQ696732, HQ696685, *Cochrane s. n.* (WIS); *Bombax buonopozense* P. Beauv., HQ658376, HQ696742, AY321171 (Nyffeler et al. 2005), Pac. Trop. Bot. Gard. Acc. No 770474001,

Nigeria; *Bombax ceiba* L., HQ658377, HQ696743, HQ69669, *Alverson s. n.* (WIS); *Camptostemon schultzii* Mast., HQ658360, HQ696726, AY321162 (Nyffeler et al. 2005), *Dunlap s. n.* (WIS); *Catostemma fragrans* Benth., HQ658370, HQ696736, AY589069 (Baum et al. 2004), *Alverson 4030* (WIS); *Cavanillesia platanifolia* (Bonpl.) Kunth., HQ658371, HQ696737, HQ696686, Fairchild Botanical Gardens acc. no. FG83343A; *Ceiba acuminata* (S. Watson) Rose, HQ658385, HQ696752, HQ696700, Fairchild Botanical Gardens acc. no. X-2–206; *Ceiba aesculifolia* (Kunth) Britten & Baker f., HQ658384, HQ696751, HQ696699, Fairchild Botanical Gardens acc. no. 83301; *Ceiba crispiflora (*Kunth) Ravenna, HQ658387, HQ696754, AY321169 (Nyffeler et al. 2005), Pac. Trop. Gard. Acc. No. 750726001; *Ceiba pentandra* (L.) Gaertn., HQ658386, HQ696753, HQ696701, *Alverson s. n.* (WIS); *Ceiba schottii* Britten & Baker f., HQ658389, HQ696756, HQ696703, Fairchild Botanical Gardens acc. no. 83302; *Ceiba speciosa* (A.St.-Hil.) Ravenna, HQ658388, HQ696755, HQ696702, *Alverson s. n.* (WIS); *Chiranthodendron pentadactylon* Larreat., HQ658356, HQ696722, AY321164 (Nyffeler et al. 2005), *Wendt s. n.* (WIS); *Eriotheca candolleana* (K. Schum.) A. Robyns, HQ658394, HQ696772, HQ696718, *Duarte & Siqueira 99* (CVRD, SP); *Eriotheca discolor* (Kunth) A. Robyns, _____, HQ696775, HQ696720, *Campo 6110* (MO); *Eriotheca dolichopoda* A. Robyns, HQ658402, HQ696773, HQ696719, *Duarte et al. 92* (CEPEC); *Eriotheca gracilipes* (K. Schum.) A. Robyns, ____, HQ696762, HQ696708, *Duarte et al. 120* (SP); *Eriotheca longipedicellata* (Ducke) A. Robyns, HQ658395, HQ696770, HQ696716, *Duarte 93* (SP); *Eriotheca longitubulosa* A. Robyns, HQ658396, HQ696771, HQ696717, *Duarte & Pereira* 96 (SP); *Eriotheca macrophylla* (K. Schum.) A. Robyns, HQ658399, HQ696767, HQ696713, *Duarte 106* (SP); *Eriotheca obcordata* A. Robyns, HQ658403, HQ696774, ____, *Silva 107* (HUEFS); *Eriotheca parvifolia* (Mart & Zucc.) Schott & Endl., HQ658401, HQ696764, HQ696710, *Duarte et al. 109* (SP); *Eriotheca pentaphylla* (Vell. emend. K. Schum.) A. Robyns, ____, HQ696768, HQ696714, *Duarte 75* (SP); *Eriotheca pubescens* (Mart & Zucc.) Schott & Endl., HQ658397, HQ696763, HQ696709, *Duarte 115* (SP); *Eriotheca roseorum* (Cuatrec.) A. Robyns, ____, HQ696765, HQ696711, *Fuentes 1167* (MO); *Eriotheca ruizii* (K. Schum.) A. Robyns, ____, HQ696777, HQ696721, *Peterson & Judziewicz 9487* (US); *Eriotheca sp.*, HQ658398, HQ696766, HQ696712, *Duarte 89* (CEPEC); *Eriotheca squamigera* (Cuatrec.) Fern. Alonso, ____, HQ696776, _____, *Neill 12522* (MO); *Eriotheca surinamensis* (Uittien) A. Robyns, HQ658400, HQ696769, HQ696715, *Duarte 97 (SP); Fremontodendron californicum* (Torr.) Coville, HQ658357, HQ696723, AY321165 (Nyffeler et al. 2005), Ex. Rancho Santa Ana Bot. Garden, Prop. No. 5996, Herb. No 12343; *Gossypium hirsutum* L., HQ658359, HQ696725, AY321158 (Nyffeler et al. 2005), *Alverson s. n.* (WIS); *Gyranthera caribensis* Pittier, HQ658368, HQ696734, AY589071 (Baum et al. 2004), *Iltis et al. s. n.* (WIS); *Huberodendron patinoi* Cuatrec., HQ658367, HQ696733, AY589072 (Baum et al. 2004), *Alverson 2201*(WIS); *Matisia palenquiana* (A. Robyns) W. S. Alverson, HQ658362, HQ696728, HQ696684, *Clark & Pallir 5549* (MO); *Neobuchia paulinae* Urb., ____, HQ696760, HQ696707, Cult. Jardin Botanico, Santo Domingo, Dominican Republic; *Ochroma pyramidale* (Cav. ex. Lam.) Urb., HQ658363, HQ696729, AY321172 (Nyffeler et al. 2005), *Alverson s. n.* (WIS); *Pachira aquatica* Aubl., HQ658392, HQ696759, AY321170 (Nyffeler et al. 2005), *Alverson s. n.* (WIS); *Pachira brevipes* (A. Robyns) W.S. Alverson, HQ658391, ____, HQ696694, *Paul Fine 1060* (UC);

Pachira flaviflora (Pulle) Fern. Alonso, HQ658379, HQ696746, HQ696693, *K. McGuire 589* (BRG);

Pachira glabra Pasq., HQ658393, HQ696761, HQ696706, *Duarte 70* (SP);

Pachira insignis (Sw.) Sw. ex Savigny, HQ658390, HQ696757, HQ696704, *Paul Fine 1061* (UC);

Pachira minor (Sims) Hemsl., _____, HQ696758, HQ696705, *G. Davidse 4901* (MO);

Pachira quinata (Jacq.) W. S. Alverson, _____, HQ696745, HQ696692, *Alverson & Adler 2174* (WIS);

Patinoa sphaerocarpa Cuatrec., HQ658364, HQ696730, AY589074 (Baum et al. 2004), *Alverson s. n.* (WIS); *Pentaplaris doroteae* L. O. Williams & Standl., HQ658358, HQ696724, AY321163 (Nyffeler et al. 2005), *Hammel et al. 18736* (MO); *Pseudobombax croizatii* A. Robyns, HQ658382, HQ696749, HQ696697, *Oldham s. n.* (WIS); *Pseudobombax grandiflorum* (Cav.) A. Robyns, HQ658383, HQ696750, HQ696698, Fairchild Botanical Gardens acc. no. FG-65–35; *Pseudobombax marginatum* (A. St.-Hil., Juss. & Cambess.) A. Robyns, HQ658381, HQ696748, HQ696696, *R. Small s. n.* (ISC); *Rhodognaphalon schumannianum* A. Robyns, HQ658380, HQ696747, HQ696695, *Mark W. Chase 5973* (K); *Scleronema micranthum* (Ducke) Ducke, HQ658369, HQ696735, AY589070 (Baum et al. 2004), *Alverson s. n.* (WIS); *Septotheca tessmannii* Ulbr., HQ658365, HQ696731, AY589073 (Baum et al. 2004), *Vargas s. n.* (WIS); *Spirotheca rosea* (Seem.) P. E. Gibbs & W. S. Alverson, HQ658378, HQ696744, HQ696691, *Alverson 2185* (WIS);