Phylogeny of the Cecropieae (Urticaceae) and the Evolution of an Ant-Plant Mutualism

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Abstract—Ant-plant mutualisms are abundant in the tropics and are popular models for ecological study, but investigating the origin and evolution of such systems requires a phylogenetic framework. A common ant-plant mutualism in the Neotropics involves the genus *Cecropia*, a group of fast-growing pioneer trees that are important in forest regeneration. Relationships between genera in the tribe Cecropieae (Urticaceae), including *Cecropia*, *Coussapoa*, *Musanga*, *Myrianthus*, and *Pourouma*, are unknown and are necessary to investigate the evolutionary history of the *Cecropia*-ant mutualism. Bayesian phylogenetic analyses of the NADH dehydrogenase (*ndhF*) chloroplast gene region, the 26S region of nuclear ribosomal DNA, and an exon-primed intron-crossing DNA region support the position of non-myrmecophytic African *Musanga* within a paraphyletic *Cecropia*. Neotropical *Pourouma* and *Coussapoa* are supported as sister taxa with African *Myrianthus* as their closest relative. Although it remains uncertain whether myrmecophytism was the ancestral condition of the *Cecropia* clade, a close relationship between non-myrmecophytic *Cecropia sciadophylla* and *Musanga* suggests that the loss of ant associations did not accompany African colonization.

Keywords—ancestral state reconstruction, *Cecropia*, EPIC markers.

Ant plants (myrmecophytes) are found in many terrestrial ecosystems and are especially abundant in the tropics. They have served as models for the study of mutualism in general (Trager et al. 2010), contributing significantly to current understanding of the nature of reciprocal benefits, how benefits vary among partners, and what costs are associated with partnerships among species (Bronstein 1998). Ecological studies of ant plants have suggested that fitness advantages and the specificity of interactions might have played roles in plant diversification (Davidson and McKey 1993; Lengyel et al. 2009; Weber and Agrawal 2014). However, inferred histories of myrmecophytism are often limited by the extent of systematic knowledge. Phylogenetic information is needed to identify the origins of myrmecophytism and to infer how ant-plant mutualisms might have changed over evolutionary time. If hypotheses about ancient species interactions and diversification rates are to be tested, it is especially important to resolve phylogenetic relationships among ant plants and related non-myrmecophytes at taxonomic levels above the species (Weiblen and Treiber 2015).

A common ant-plant mutualism in the Neotropics involves trees of *Cecropia* Loefl. and ants of the genus *Azteca* Forel. *Cecropia* are fast growing pioneers that play an important role in tropical forest regeneration after disturbance through-out Central and South America. *Cecropia* provide nesting sites (domatia) and nutritious Müllerian bodies for their ant inhabitants (Berg and Franco-Rosselli 2005; Dejean et al. 2012). Host trees may benefit from the presence of aggressive ants that prey on insect herbivores, prune competing vegetation, and deposit nitrogen-rich debris (Sagers et al. 2000; Bronstein et al. 2006). Ecological studies have speculated about the origin and adaptive significance of this mutualism (Latteman et al. 2014) and, although *Cecropia* was revised by Berg and Franco-Rosselli (2005), a sister group has yet to be identified. *Cecropia* is a member of the tribe Cecropieae (Urticaceae),

which also includes *Coussapoa* Aubl., *Musanga* R. Br., *Myrianthus* P. Beauv., *Poikilospermum* Zipp. ex Miq., and *Pourouma* Aubl. Recent molecular phylogenetic studies have shown that Cecropieae is not monophyletic and that *Poikilospermum* should be transferred to Urticaceae Juss. (Monro 2006; Hadiah et al. 2008; Wu et al. 2013). Our study was motivated by the need to clarify phylogenetic relationships among the remaining five genera as a basis for investigating the origin of myrmecophily in Cecropieae.

The tribe has a long and complex taxonomic history owing to patterns of morphological intermediacy between Moraceae Gaudich. and Urticaceae. The first reference to Cecropieae is attributed to Dumortier (1829), who included *Cecropia* and *Coussapoa* within the family Artocarpideae Dumort. Gaudichaud (1830) included Cecropieae in his "Urticées vraies" (true Urticaceae) based on the orthropous orientation of the ovule, along with the tribes Boehmerieae Gaudich., Elatostemateae Gaudich., Forsskaoleeae Gaudich., Parietarieae Gaudich., and Urereae Gaudich. *Pourouma* was assigned to a monotypic Pouroumeae Gaudich. in a different group of "Urticées," whereas Trécul (1847) included *Pourouma* with *Cecropia* and *Coussapoa* in the family Artocarpeae. Engler (1889) placed Conocephaleae Trécul including *Cecropia*, *Coussapoa*, *Pourouma*, *Myrianthus*, and *Poikilospermum* in the subfamily Conocephaloideae (Moraceae). Corner (1962) suggested the transfer of Conocephaloideae from Moraceae to Urticaceae, based on the basal placentation of the orthropous ovules. However, Berg (1978) proposed a new family Cecropiaceae on the grounds that straight stamens in bud and basal, sub-basal, or (sub)orthropous ovules were diagnostic. Romaniuc-Neto (1999) proposed that all genera of Cecropiaceae sensu Berg (1978) be regarded as a subfamily of Moraceae except for *Poikilospermum* which he placed in the Urticaceae. Molecular phylogenetic studies supported the placement of Cecropieae in the Urticaceae (Datwyler and

Weiblen 2004; Zerega et al. 2005; Monro 2006; Hadiah et al. 2008; Clement and Weiblen 2009; Wu et al. 2013) and the most recent review of Urticaceae nomenclature validated Cecropieae Gaudich. as the name for the tribe (Conn and Hadiah 2009).

Disagreement among taxonomists over the phylogenetic position of Cecropieae can be attributed to conflicting patterns of morphological similarity in Moraceae and Urticaceae. The tribe includes dioecious trees, shrubs, and hemiepiphytes with aerial or stilt roots, a reduced system of clear latex-bearing canals, spiral phyllotaxis, amplexicaul stipules, terminal inflorescences in either cymes, fascicles, spikes, or globose heads and staminate flowers with straight filaments (Table 1, Fig. 1). None of the recent molecular studies included all genera of Cecropieae in a comprehensive analysis of the tribe nor has morphology of the tribe been examined in a phylogenetic framework.

Cecropia, Coussapoa, and *Pourouma* are distributed in the Neotropics with approximately 80% of the species occurring in the Amazon region. *Musanga* and *Myrianthus* are Afrotropical with most species in the rainforests of the western coast. *Cecropia* and *Musanga* are similar morphologically and ecologically, but *Musanga* lacks the domatia and Müllerian bodies that are common among the *Cecropia* species engaged in mutualism with ants. Ants are associated with most but not all *Cecropia* species, whereas only a few species of *Coussapoa* (O'Dowd 1982; Berg et al. 1990) and *Pourouma* (Bonsen 1990) are reported to be myrmecophytes. *Myrianthus* has morphological traits similar to *Pourouma*, but ants are entirely absent from *Myrianthus* and *Musanga*.

Understanding the history of myrmecophytism in Cecropieae requires knowledge of relationships among five genera that until now have not been included in a comprehensive phylogenetic analysis. We estimated a Cecropieae phylogeny using both nuclear and chloroplast DNA regions and compared our findings to a phylogeny based on morphology. Our phylogenetic approach provides a framework for more thoroughly investigating the biogeographic history of myrmecophytism in this tribe.

Materials and Methods

Taxon Sampling—We examined 24 samples representing the five genera included in the core Cecropieae (*Cecropia, Coussapoa, Musanga, Myrianthus*, and *Pourouma*) and five outgroup taxa representing four of the five other Urticaceae tribes (Boehmerieae, Elatostemateae, Parietarieae, and Urticeae Lam. & DC) (Appendix 1). Among ingroup taxa, the sampling intensity was scaled with the size of the genus (i.e. the greatest number of samples was analyzed for *Cecropia*, the largest genus in the tribe).

Sequencing—Three regions were sequenced, including the *ndhF* cpDNA region (Zerega et al. 2005), the 26S nuclear rDNA region (Olmstead and Sweere 1994), and the nuclear exon-primed intron-crossing (EPIC) marker A16170b, which was developed for Moraceae (Yao et al. 2013). These regions were chosen to include a slowly evolving locus (26S) for resolving phylogentic relationships at higher taxonomic levels, as well as variable and more rapidly evolving loci (*ndhF* and EPIC), which are more useful at lower levels. DNA was extracted using the Qiagen DNeasy plant mini kit (Valencia, California) with 20 mg of silica gel preserved leaf fragments or herbarium specimens. The PCR amplification of the *ndhF* chloroplast region was performed in two separate reactions using primer combinations *ndhF8f–ndhF1318r* and *ndhF972f–ndhF2110r* (Olmstead and Sweere 1994). Amplification conditions followed those outlined in Zerega et al. (2005) and thermal cycling conditions were 94°C for 1 min followed by 35 cycles of 95°C for 30 s, 46°C for 1 min, and 68°C for 1 min 30 s with a final extension of 72°C for 7 min. The 26S region was amplified in a single fragment using forward and reverse primers previously developed for Moraceae (Zerega et al. 2005). The PCR amplification of the EPIC marker was performed using primers and conditions outlined in Yao et al. (2013). PCR products for all regions were cleaned by ethanol precipitation and quantified using a NanoDrop 2000c (Thermo Scientific Inc., Waltham, Massachusetts). Sequencing was performed in 10 μL reactions using Big Dye sequencing reagents and protocols (Applied Biosystems, Foster City, California), and data were collected using an ABI Prism 3730xl DNA analyzer (Applied Biosystems). Previously sequenced samples from Zerega et al. (2005) were obtained from Genbank for *ndhF* (AY289253, AY289254, AY289256, AY289257, AY289259–AY289264, and AY289266) and 26S (AY686767–AY686772, AY6868774, AY6868776, AY686780, AY6868782, and AY6868835). Sequences were edited and aligned in Geneious v6.1.7 (Kearse et al. 2012), with manual adjustments in Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) when necessary.

Morphology—We examined morphological features that varied at or below the tribal level and have been used to distinguish groups in the literature (Table 1, Supplemental Appendix S1). Hadiah and Conn (2009) showed that morphology of Urticaceae provides phylogenetic information in some groups. We scored a matrix of 40 discrete characters for the taxa listed in Appendix 1. We collected data from both field observations (A. L. Gaglioti) and the systematic literature. Information on vegetative morphology was gathered from Guérin (1923), Metcalfe and Chalk (1950), Hickey (1973), Radford et al. (1974), Sorsa and Huttunen (1975), Barth (1976), Bonsen and Welle (1983), Barth (1984), Humphries and Blackmore (1989), Bonsen (1990), Welle et al. (1992), Romaniuc-Neto (1999), Clement (2008), and Clement and Weiblen (2009). Anatomical literature informed our scoring of glandular trichome characters (Metcalfe and Chalk 1950; Gangadhera and Inamdar 1977; Kachroo and Bhat 1981; Setochi et al. 1993). Reproductive characters and states were based on taxonomic literature (Gaudichaud 1830; Font Quer 1985: multiple characters; Chew 1963; Ruiter 1976; Berg 1978; Berg et al. 1990; Chen et al. 2003; Berg and Franco-Rosselli 2005). Taxa were scored at the species level and if the morphological state was either unknown, inconclusive based on the literature, or inapplicable, the character was coded as missing.

Phylogenetic Analysis—Bayesian phylogenetic analysis of morphology was conducted using Mr. Bayes v.3.2.2 (Huelsenbeck and Ronquist 2001;

TABLE 1. Classification, species richness, distribution and distinguishing features of Cecropieae (Urticaceae).

Genus	Geographical distribution	Species richness	Diagnostic features
Cecropia	Neotropical	~70	tree; leaves peltate, spirals, with incisions and venation radial; cystoliths absent; petiole mostly with trichilia; stipules fully amplexicaul, stipule scars horizontal; pistillate and staminate flowers in spikes, spathe covered; fruit less than 5 mm long
Coussapoa	Neotropical	~55	hemiepiphytic or tree, leaves entire, not peltate, spirals; cystoliths absent; stipules fully amplexicaul, stipule scars usually ascending; pistillate and staminate flowers in globose heads; staminate flower with filaments connate; pistillate flowers sessile; fruit less than 5 mm long
Musanga	Afrotropical	2	tree; leaves peltate, with incisions and venation radial; cystoliths absent; stipules fully amplexicaul, stipule scars horizontal; pistillate flower in spikes, staminate flower in globose heads, both inflorescences without spathe; fruit, less than 5 mm long
Myrianthus	Afrotropical	7	tree, shrub or liana; leaves entire or palmate; cystoliths absent; stipules fully amplexicaul, stipule scars horizontal; pistillate flowers and staminate flower sessile, in globose or cylindrical heads; fruit greater than 10 mm long
Pourouma	Neotropical	~43	tree; leaves entire or palmate; cystoliths absent; stipules fully amplexicaul, stipule scars horizontal; pistillate flowers in cymes and staminate flower in facicles; large fruit, greater than 10 mm long

FIG. 1. *Cecropia peltata*: A. Leafy twig with stipule, pistillate inflorescences with spathe and pistillate inflorescences. B. Pistillate flower. C. Detail of the pistillate inflorescence. D. Achene. *Cecropia palmata*: E. Leafy twig with stipule, staminate inflorescences with spathe and staminate inflorescences. F. Staminate inflorescence transverse section. G. Staminate flower. H. Pistillate flower. *Coussapa microcarpa*: I. Leafy twig with stipule and pistillate inflorescences. J. Staminate flower. K. Infrutescence. *Musanga cecropioides*: L. Leafy twig with stipule and pistillate inflorescences. M. Pistillate flowers and pistillate flower in frontal section. *Myrianthus arboreus*: N. Leafy twig with stipule and staminate inflorescences. O. Infrutescence. P. Staminate flower. *Pourouma myrmecophila*: Q. Leafy twig with stipule, pistillate inflorescences and infrutescences. V. Pistillate flower. *Pourouma guianensis*: R. Leafy twig with stipule and infrutescences. S. Pistillate flower, frontal section. T. Fruiting perianth and achene. U. Staminate flower. [A–D: from *Aubréville 23* (P); E–G: from *Gaglioti et al. 118* (SP); H: from *Cuatrecasas 26658* (P); I, K: from *Gaglioti et al. 102* (SP); J: from *Proença et al. 73* (SP); L–M: from *Jansen 2138* (P); N, P: from *Kami 1242 ter* (SP); O: from *Kami 1242 bis* (SP); Q, V: from *Gaglioti et al. 168* (SP); R, T: from *Gaglioti et al. 163* (SP); S: from *Carauta et al. 6303* (RB); U: from *Furlan et al. 1037* (SP)].

Ronquist et al. 2012) on the CIPRES Science Gateway (Miller et al. 2010) with a Markov *k* model and a gamma distribution. Four (one cold and three hot) Markov chain Monte Carlo (MCMC) simulations, swapping at default settings, were run for five million generations while sampling every 500 generations until the average deviation of split frequencies fell below 0.01. The posterior distribution of trees was summarized by >50% majority rule consensus tree after discarding the first 25% of the sample as burn-in.

Phylogenetic analyses of DNA sequences were also performed using Mr. Bayes on the CIPRES Science Gateway (Miller et al. 2010), but with a GTR substitution model, gamma-distributed rate variation across sites, and a parameter for the proportion of invariable sites. Four (one cold and three hot) MCMC chains were run for five million generations while sampling every 500 generations and until the average deviation of split frequencies fell below 0.01. Once more, the posterior distribution of trees was summarized by >50% majority rule consensus tree after discarding the first 25% of the sample as burn-in. Analyses examined each DNA region alone, (1) ndhF, (2) 26S, and (3) EPIC, plus (4) a concatenated dataset including all three regions and all taxa listed in Appendix 1, and (5) a concatenated dataset of all three regions but excluding taxa for which any of the three DNA regions were unavailable.

Ancestral State Reconstruction—Ancestral state reconstruction of myrmecophytism was performed in Mesquite v. 3.02 (Maddison and Maddison 2015) on the posterior distribution of trees from the concatenated dataset including all three regions and all taxa. Trees were drawn from the posterior distribution and ancestral states were estimated onto each of 400 trees using maximum likelihood with a Mk1 model of character evolution. Uncertainty associated with unresolved nodes in the majority rule consensus tree was examined by filtering trees in *Mesquite* to separate trees with *C. sciadophylla* and *Musanga* sister to the rest of *Cecropia* from the posterior trees with trees where *C. sciadophylla* and *Musanga* were arranged otherwise. Ancestral states were estimated as above and the probability of a myrmecophytic ancestor of the *Cecropia* clade was recorded for a random sample of 200 trees for each posterior tree type.

Results

Morphological Phylogeny—The Bayesian consensus tree largely supported prior taxonomic groupings (Fig. 2, Appendix 1). Three genera of Cecropieae were supported as monophyletic. A highly supported clade included *Musanga* and monophyletic *Cecropia*. There was less support for a clade including *Myrianthus* and monophyletic *Pourouma*. *Coussapoa* had low clade support and appeared to be sister to the rest of the core of Cecropieae, while *Poikilospermum* was positioned between the outgroup and the ingroup. The equivocal position of *Poikilospermum* is consistent with conflicting morphology, in which the dioecious breeding system and hemi-epiphytic habit are reminiscent of Cecropieae, while other characteristics such as unlignified vessel elements, dimorphic wood fibers, stipules not fully amplexicaul, and stamens with inflexed filaments are strikingly similar to Urticeae.

Molecular Phylogeny—The aligned ndhF dataset including all 29 samples was 2,046 base pairs (bp) in length, contained 182 variable positions, and 69 phylogenetically informative positions among the ingroup taxa (3%). For three of four *Myrianthus* samples (*Mwangoka 3151, Birnbaum 913*, and *Birnbaum 917*), complete sequences could not be obtained from the herbarium material and sequences were considerably shorter (325–731 bp). The 26S alignment of all 29 samples was 1,001 bp in length with 49 variable and 30 phylogenetically informative characters among the ingroup (3%). The EPIC dataset was smaller than the other two because sequences could not be obtained from 13 of 29 samples despite repeated attempts at PCR optimization. The EPIC alignment of 371 bp had 46 variable and 17 phylogenetically informative characters among the ingroup (4.6%).

Bayesian results for ndhF and 26S strongly supported the exclusion of *Poikilospermum* from Cecropieae despite the DNA regions yielding different branching order among *Poikilospermum* and the outgroups *Laportea* (Urticeae) and *Pilea* (Elatostemateae). Limited outgroup sampling and substantial sequence divergence among these taxa are likely to account for branching order among these three taxa being the only highly supported difference between the ndhF and 26S topologies (Soltis and Soltis 2004; Bergsten 2005). Bayesian consensus trees from ndhF and 26S each supported the monophyly of *Coussapoa*, *Myrianthus*, and *Pourouma*, whereas *Cecropia* was not monophyletic due to the embedded position of *Musanga* (Figs. 3, 4). The EPIC phylogeny was otherwise poorly resolved and yielded no highly supported conflicts with the ndhF or 26S phylogenies apart from a long branch uniting divergent sequences from *Coussapoa nymphaeifolia* and *Pourouma tomentosa*.

The combined analysis of the three DNA regions strongly supported the monophyly of each Cecropieae genus except for *Cecropia*. There was also strong support for a *Cecropia/Musanga* clade (hereafter *Cecropia* sensu lato) and the position of non-myrmecophytic *Cecropia sciadophylla* as sister to *Musanga* (Fig. 5). The Bayesian consensus further suggested that *C. sciadophylla* plus *Musanga* might be sister to the rest of *Cecropia*. Sister to *Cecropia* s. l. was a clade comprising African *Myrianthus* and Neotropical *Coussapoa* plus *Pourouma*. Simultaneous analysis of a subset of 16 taxa with complete sequences for all three DNA regions also strongly supported the monophyly of *Cecropia* s. l. and the position of *C. sciadophylla* and *Musanga* sister to the rest of the clade. There was low support for *Myrianthus* as the genus sister to the *Cecropia/Musanga* clade. Finally, *Coussapoa* and *Pourouma* were highly supported as sister to the rest of the tribe.

Origin and Loss of Myrmecophytism—Maximum likelihood estimates for the ancestral condition of *Cecropia* s. l. varied depending on which trees were sampled from the Bayesian posterior distribution. The probability of a myrmecophytic common ancestor for the group ranged from 0.01–0.99 according to the position of non-myrmecophytic *Cecropia* and *Musanga* in the tree (Fig. 6). In the random sample of trees with *C. sciadophylla* and *Musanga* sister to *Cecropia*, the majority of ancestral reconstructions had probabilities of a myrmecophytic ancestor of *Cecropia* between 0.30 and 0.45. The distribution was approximately normal with the mean at a probability of 0.40 (Fig. 7). When *C. sciadophylla* and *Musanga* were arranged differently on the posterior trees, the majority of the probabilities of a myrmecophytic ancestor of *Cecropia* were between 0 and 0.25 with a mean probability of 0.35. The distribution was skewed to the left with a long tail (Fig. 7).

Discussion

A comprehensive phylogenetic analysis of Cecropieae genera supports the emerging consensus based on molecular data that the tribe is monophyletic and that *Poikilospermum* belongs elsewhere in Urticaceae (Romaniuc-Neto 1999; Hadiah et al. 2008; Wu et al. 2013). Results of sampling three gene regions and all genera in the tribe agree with earlier studies in suggesting that the morphological similarities of *Poikilospermum* that led taxonomists to place it near Cecropieae are homoplasious. Morphology alone supported a sister relationship of *Poikilospermum* and Cecropieae (Fig. 2) whereas nuclear and chloroplast DNA sequences of *Poikilospermum* are more closely related to members of Urticeae (Figs. 3–5).
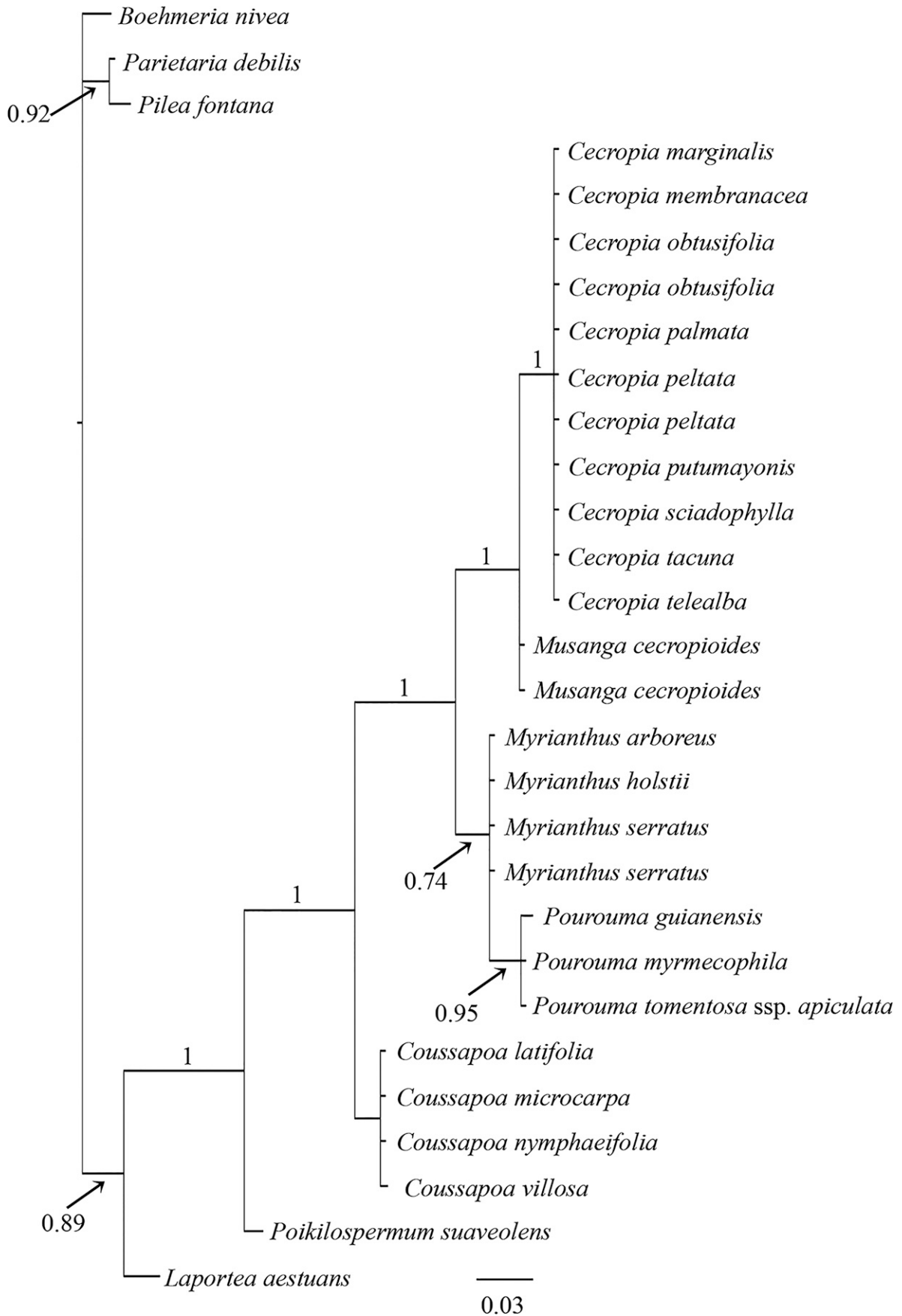
FIG. 2. Bayesian (> 50%) majority consensus rule tree for Cecropieae based on 40 morphological characters. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. Posterior probabilities greater than 0.75 are noted on respective branches.
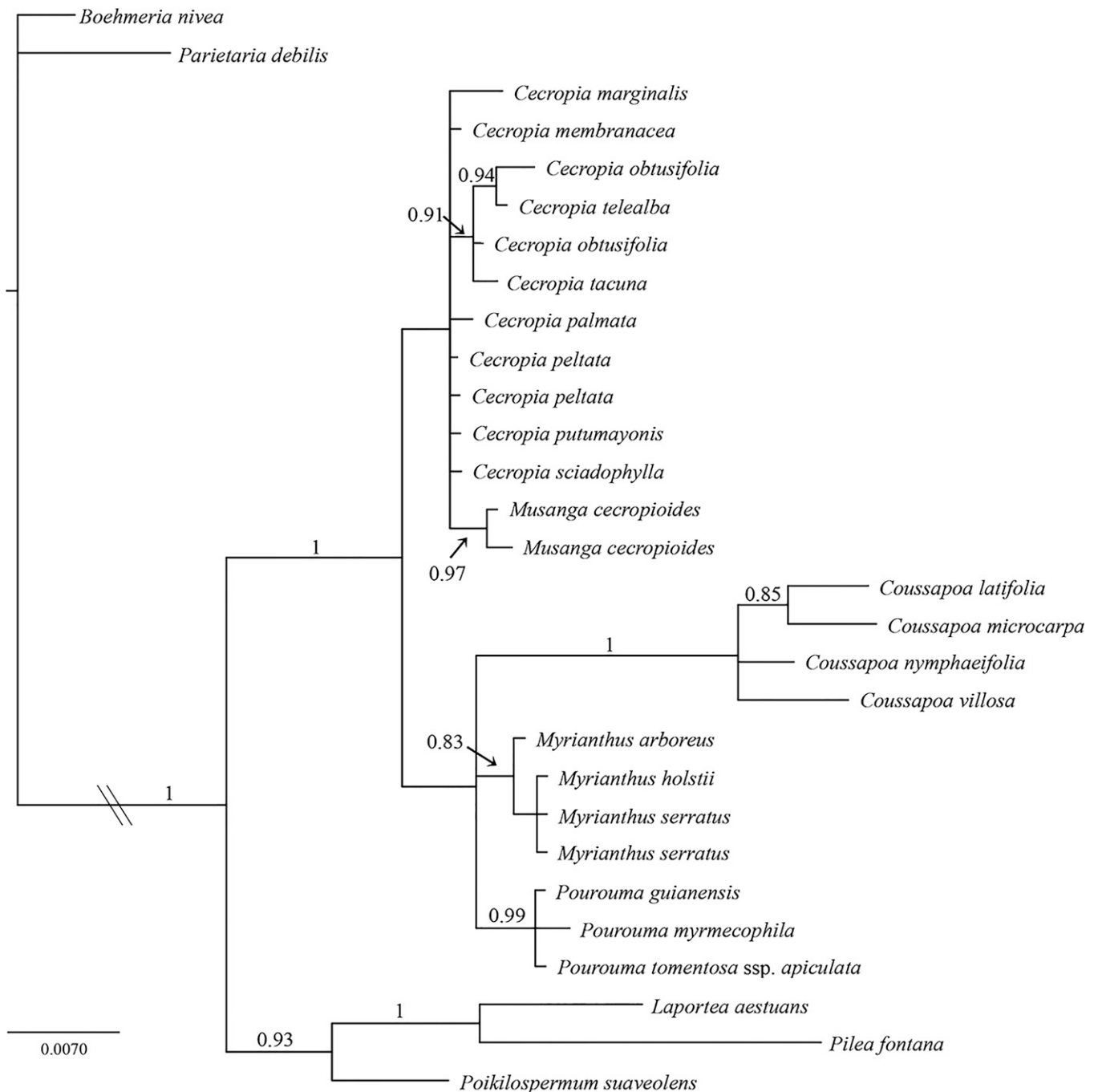
FIG. 3. Bayesian (> 50%) majority consensus rule tree for Cecropieae based on *ndhF* DNA sequence data. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. The branch bearing double hatch marks indicates that it has been truncated and is not proportional to the rest. The original length of the truncated branch was approximately 0.04. Posterior probabilities greater than 0.75 are noted on respective branches.

We now turn our attention to understanding relationships among the remaining members of Cecropieae and their bearing on the evolution of myrmecophytism in this clade.

Molecular data supported the monophyly of three genera (*Coussapoa*, *Myrianthus*, and *Pourouma*), whereas *Cecropia* was rendered paraphyletic by the strongly supported position of *Musanga* (Fig. 5). This finding is not surprising given the morphological similarities of *Cecropia* and *Musanga* (Figs. 1, 2). Synonymizing *Musanga*, the smallest genus in the tribe, with the largest would restore the monophyly of *Cecropia* but *Musanga leo-errerae* Hauman & Léonard, has yet to be sequenced. Alternatively, *C. sciadophylla* could be transferred to *Musanga* but more complete sampling of *Cecropia* is needed to identify the most appropriate taxonomic change. DNA isolated from herbarium specimens has proven too degraded for analysis and it has not been possible to obtain new collections of the montane *M. leo-errerae* from East Zaire and Uganda. We predict that further study will support the synonmy of *Musanga* and the recognition of a more distributed *Cecropia* sensu lato encompassing both the Afrotropics and the Neotropics.

Musanga and *Cecropia* are ecologically similar light-demanding pioneer trees in lowland forest succession with
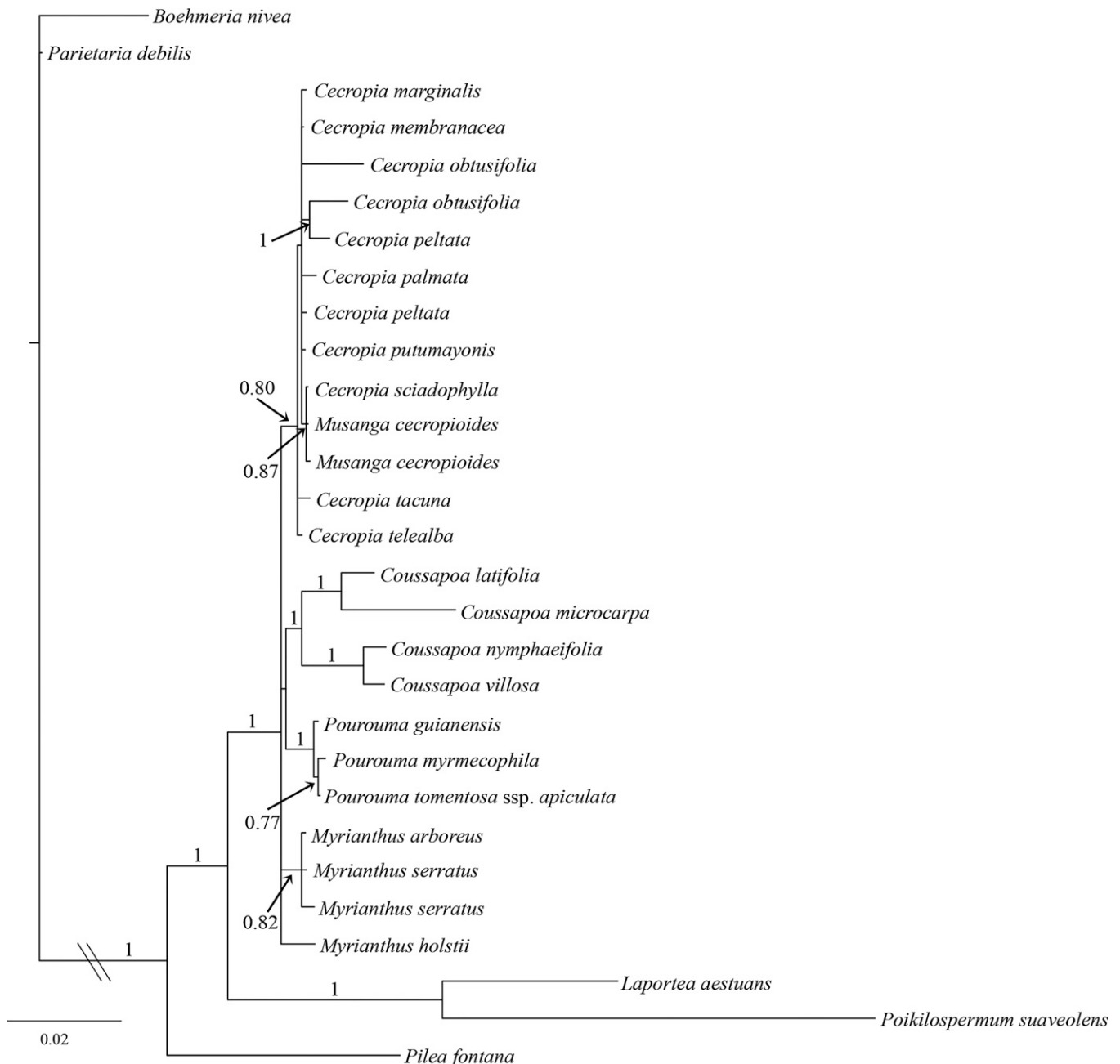
FIG. 4.   Bayesian (> 50%) majority consensus rule tree for Cecropieae based on 26S DNA sequence data. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. The branch bearing double hatch marks indicates that it has been truncated and is not proportional to the rest. The original length of the truncated branch was approximately 0.028. Posterior probabilities greater than 0.75 are noted on respective branches.

highly similar vegetative and reproductive characteristics (Table 1). Among the only distinguishing features are ant-associated traits such as Müllerian bodies and trichillia that are present in most *Cecropia* species but are absent in *Musanga*. It is noteworthy that the only strongly supported intergeneric relationship in the three-gene phylogenetic analysis involved non-myrmecophytic *C. sciadophylla* and *M. cecropioides*. Most non-myrmecophytic *Cecropia* species are Andean high-altitude specialists occupying habitats where ants are either rare or absent (Latteman et al. 2014), whereas *C. sciadophylla* is a lowland species that often occurs in sympatry with other ant-associated *Cecropia* species.

Another feature distinguishing *Musanga* from all but one *Cecropia* species is the absence of a spathe enclosing the infloresences. *Cecropia hololeuca* is a non-myrmecophyte that shares with *Musanga* a reduced and caducous bract in place of the spathe. These characters suggest that *C. hololeuca* could belong to the same non-myrmecophytic clade as *C. sciadophylla* and *M. cecropioides*. Janzen and McKey (1977) suggested that *Musanga* lost the ant association during migration from the Neotropics to Africa. However, considering that *C. sciadophylla* and *M. cecropioides* lack trichilia and Müllerian bodies, our results suggest that they shared a non-myrmecophytic common ancestor whose descendants
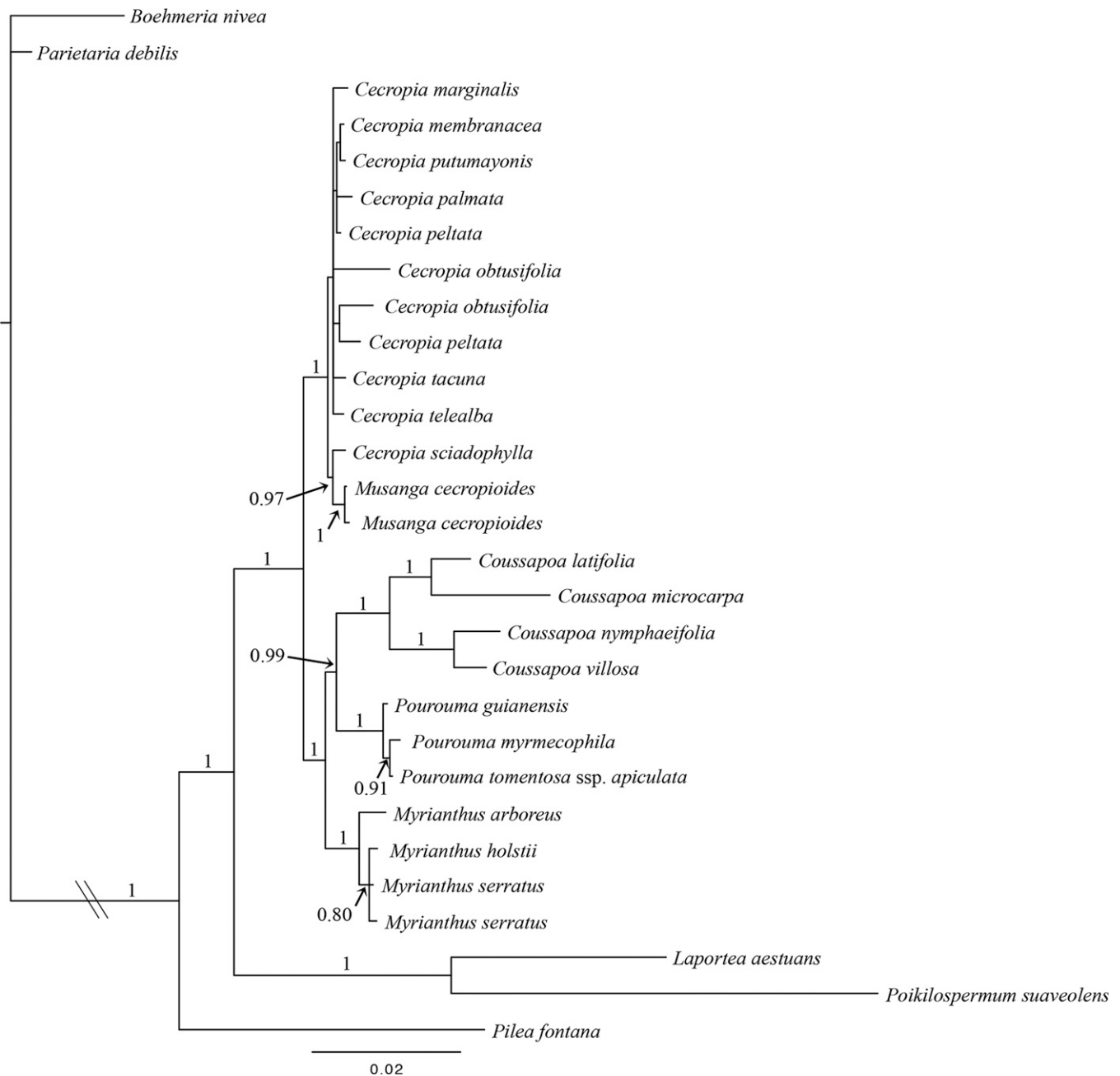
Fig. 5. Bayesian (> 50%) majority consensus rule tree for Cecropieae based on 26S, *ndhF*, and EPIC DNA regions. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes are used to root the tree. The branches bearing double hatch marks indicates that they have been truncated and are not proportional to the rest. Values for nodes are noted when posterior probabilities are greater than 0.75.

dispersed across the Atlantic Ocean in one direction or the other. These alternative biogeographic scenarios for the evolution of *Cecropia* s. l. can be evaluated to some extent in the broader context of Cecropieae phylogeny and the fossil record.

Our study resolved the sister group to *Cecropia* s. l. with strong support from three gene regions for a clade including *Pourouma, Coussapoa,* and *Myrianthus*. The division of this group into a Neotropical clade consisting of *Pourouma* and *Coussapoa* and an Afrotropical clade (*Myrianthus*), together with the paleobotanical record, favors a Neotropical origin for the tribe followed by an ancient migration to Africa in the case of *Myrianthus* and a more recent migration in the case of *Musanga*. A Colombian fossil flora of the Maestrichtian Epoch

with leaves that resemble Cecropieae suggests that the ancestors of the tribe were present in South America at least 65 million years before present (Burnham and Johnson 2004). Macrophyllous fossils from South American deposits dated 10–13 million years before present (Burnham and Graham 1999) have been unambiguously assigned to *Coussapoa* and provide further evidence that Cecropiaeae had already diversified in the Neotropics by at least the mid-Miocene. The alternative scenario, that Cecropiaeae were broadly distributed in Gondwana prior to the rifting of South America from Africa, is inconsistent with the low level of DNA sequence divergence observed in the group and relatively recent fossil-calibrated estimates of divergence time (Zerega et al. 2005).
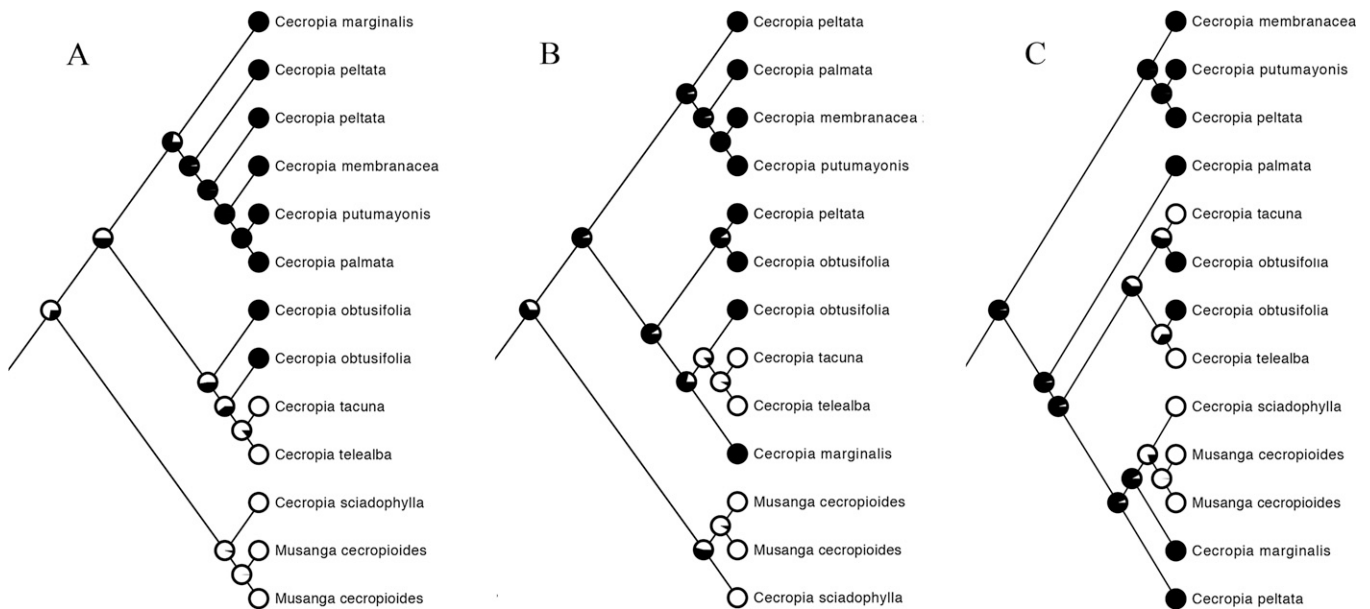
Fig. 6. Ancestral reconstruction of ant associations on sample phylogenies from the Bayesian posterior distribution. Ancestral state reconstructions were done on: (A) a tree similar to the consensus, where *C. sciadophylla* and *Musanga* are sister to the other *Cecropia* with the other antless *Cecropia* branching off earlier; (B) a tree like A, but with other antless *Cecropia* branching embedded in the *Cecropia* clade; and (C) a tree with *C. sciadophylla* and *Musanga* embedded within the *Cecropia* clade.

The relatively large number of myrmecophytic *Cecropia* species (~70) compared to a few species of non-myrmecophytic *Musanga* and *Cecropia* is consistent with the notion that mutualism could be associated with adaptive diversification (Weiblen and Treiber 2015). Mutualism may have enabled *Cecropia* populations to expand or occupy new niches if, for example, contributions of nitrogen from ants allowed *Cecropia* to thrive in nitrogen-limited environments (Sagers et al. 2000). Large population size would reduce extinction risk and niche expansion might have led to speciation. Defensive mutualisms involving ants and plants also appear to be accompanied by accelerated diversification rates (Weber and Agrawal 2014) and plant traits that reward ants have been regarded as key innovations (Lengyel et al. 2009). Understanding whether this is the case in *Cecropia* s. l. will require a more fully resolved phylogeny and more thorough sampling of myrmecophytes and non-myrmecophytes than in our study.

We encountered unexpectedly low levels of DNA sequence variation among *Cecropia* species (e.g. 0.8% and 0.13% phylogenetically informative characters for EPIC and *ndhF*, respectively) such that virtually no relationships within the genus were either resolved or supported in this study. Additional sequencing of *Cecropia* using more variable molecular markers such as the ribosomal internal transcribed spacer region (ITS) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) has yielded the same result (Treiber unpublished data). The difficulty of phylogenetic inference in *Cecropia* might be attributed to either a slow rate of molecular evolution or to a long history of hybridization and introgression (Xu 2000; Machado and Hey 2003). Next generation sequencing techniques can provide the quantity of data necessary to evaluate these alternatives and to resolve recalcitrant clades. Recent phylogenetic studies employing restriction-site associated DNA (RAD) sequence data (Baird et al. 2008; Emerson et al. 2010; Wagner et al. 2012; Eaton and Ree 2013) suggest that this technique holds promise for *Cecropia*. Rubin et al. (2012) found that RAD sequencing for phylogenetic analysis

worked best for diploid species that diverged relatively recently (<60 Mya) which is the case for *Cecropia*.

Preliminary insights on relationships within *Cecropia* s. l. illustrate the challenges associated with ancestral state reconstruction (Fig. 6). The probability of myrmecophytism having originated in the common ancestor of the group varied depending on the position of non-myrmecophytic species relative to myrmecophytes. Most trees drawn from the Bayesian posterior distribution (97%) included the non-myrmecophytic clade (*C. sciadophylla* and *Musanga*) as sister to the rest of *Cecropia* where the probability of a myrmecophytic common ancestor ranged from 0.01–0.95 depending on the position of the other non-myrmecophytic *Cecropia* species (Figs. 6A, 6B, 7). The distribution of probabilities of a myrmecophytic ancestor for the sampled trees, when *C. sciadophylla* and *Musanga* were sister to *Cecropia*, was approximately normal with the mean at 0.40. When non-myrmecophytic *C. sciadophylla* and *Musanga* were embedded elsewhere, as was the case for 3% of the trees in the posterior distribution, the probability of a myrmecophytic common ancestor ranges from 0–0.99 (Figs. 6C, 7). The distribution of probabilities on the trees sampled for the different topologies was skewed towards zero but was fairly flat. Likely this is due to the number of different ways the five non-myrmecophytic samples may be embedded in the clade when *C. sciadophylla* and *Musanga* are not constrained as sister to the rest of the *Cecropia* clade. The ratio of myrmecophytes to non-myrmecophytes in our sample (8:5) compared to *Cecropia* as a whole (32:5) may also influence the ancestral reconstruction of ant association (Salisbury and Kim 2001). Our sample includes three of the eight non-myrmecophytic *Cecropia* species (Berg and Franco-Rosselli 2005) such that our estimates may be biased toward non-myrmecophytism (Fig. 7). Nonetheless, resolving relationships in Cecropieae and identifying the sister group to *Cecropia* are important first steps toward a robust classification of the clade and toward understanding the evolution of myrmecophytism in this group. These systematic findings provide a foundation for future investigation of the
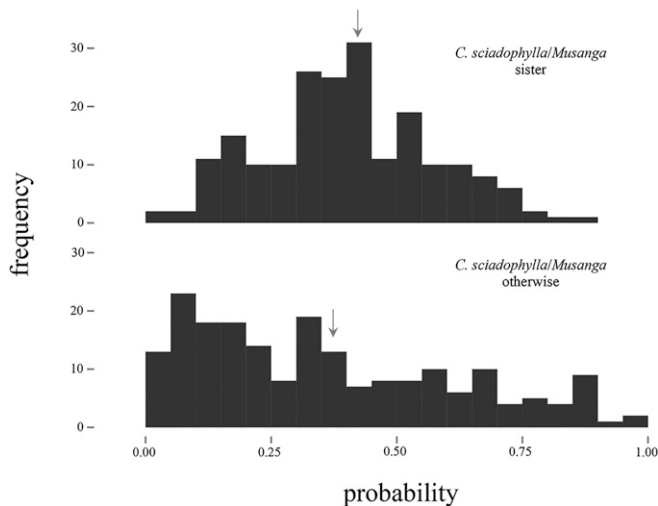
FIG. 7. Probability of a myrmecophytic ancestor for samples from the posterior of a Bayesian analysis. In the upper panel is the probability distribution for 200 random sample trees from a subset of the posterior trees that had topologies with *C. sciadophylla* and *Musanga* sister to the remaining *Cecropia* samples. Approximately normal distribution around a mean of 0.40. The lower panel shows the probability distribution for 200 random sample trees from a subset of the posterior trees with *C. sciadophylla* and *Musanga* embedded otherwise. The distribution is skewed towards 0 with a mean of 0.35. Means are marked with arrows.

role that ant mutualism may have played in the radiation of Cecropieae and plant diversification in general. A phylogeny will also allow for reconstruction of ant-associated traits, such as domatia and Müllerian bodies, and tests for correlation with the origin of myrmecophytism, as well as other biologically interesting traits including the vertebrate-dispersed fruiting syndromes of Cecropieae.

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APPENDIX 1.   Species and specimens examined with collector numbers, localities, and GenBank accession numbers for 26S, EPIC, and ndhF, analy respeictively. An asterisk (*) indicates sequence for the subsequent region was not obtained.

Boehmerieae, *Boehmeria nivea* (L.) Guadich (*Weiblen* 1214), cult. Beal Bot. Gard., AY686767, *, AY289254, Cecropieae, *Cecropia marginalis* Cuatrec. (*Barriga 4*), Ecuador, KP835217, KP835236, KP835254, Cecropieae, *Cecropia membranacea* Trécul (*Barriga 1*), Ecuador, KP835218, KP835237, KP835255, Cecropieae, *Cecropia obtusifolia* Bertol. (*Weiblen 1424*), Costa Rica, AY686774, KP835238, AY289264, Cecropieae, *Cecropia obtusifolia* Bertol. (*Weiblen 1436*), Panama, KP835219, KP835239, KP835256, Cecropieae, *Cecropia palmata* Willd. (*Weiblen 1181*), cult. Fairchild Bot. Gard., AY686782, *, AY289262, Cecropieae, *Cecropia peltata* L. (*Treiber 7*), Colombia, KP835220, KP835240, KP835257, Cecropieae, *Cecropia peltata* L. (*Weiblen 1435*), Panama, AY686780, *, AY289263, Cecropieae, *Cecropia putumayonis* Cuatrec. (*Barriga 23*), Ecuador, KP835221, KP835241, KP835258, Cecropieae, *Cecropia sciadophylla* Mart. (*Torres 26*), Colombia, KP835222, KP835242, KP835259, Cecropieae, *Cecropia tacuna* C.C. Berg & P. Franco (*Bevington 64*), Peru, KP835223, KP835243, KP835260, Cecropieae, *Cecropia telealba* Cuatrec. (*Treiber 23*), Colombia, KP835224, KP835244, KP835261, Cecropieae, *Coussapoa latifolia* Aubl. (*Weiblen 1503*), Brazil, AY686769, *, AY289257, Cecropieae, *Coussapoa microcarpa* (Schott) Rizzini (*Weiblen 1188*), Brazil, AY686770, *, AY289260, Cecropieae, *Coussapoa nymphaeifolia* Standl. (*Weiblen 1412*), Costa Rica, AY686771, KP835245, AY289259, Cecropieae, *Coussapoa villosa* Poepp. & Endl. (*Weiblen 1418*), Costa Rica, AY686768, KP835246, AY289261, Cecropieae, *Musanga cecropioides* R. Br. ex Tedlie (*Cabezas 114*), Guinea, KP835227, KP835247, KP835263, Cecropieae, *Musanga cecropioides* R. Br. ex Tedlie (*Jansen 2138*), Liberia, KP835228, KP835248, KP835264, Cecropieae, *Myrianthus arboreus* P. Beauv (*Kami 242*), Republic of the Congo, KP835229, KP835249, KP835265, Cecropieae, *Myrianthus holstii* Engl. (*Mwangoka 3151*), Tanzania, KP835230, *, KP835266, Cecropieae, *Myrianthus serratus* (Trécul) Benth. (*Birnbaum 913*), Mali, KP835231, KP835250, KP835267, Cecropieae, *Myrianthus serratus* (Trécul) Benth. (*Birnbaum 917*), Mali, KP835232, *, KP835268.