Phylogenetics of Cecropieae (Urticaceae) and the evolution of an ant-plant mutualism in *Cecropia*

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Dedication

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Abstract

Mutualisms are common in all ecosystems and can influence ecosystem function in aspects including nutrient cycling and community building. Mutually beneficial interactions between species vary in degrees of specificity from facultative and generalized associations to obligate and specialized associations. Ant-plant mutualisms are especially common in the tropics and are popular for ecological studies of mutualism. Investigating the evolution of tropical ant-plant mutualisms requires a phylogenetic framework. The neotropical ant-plant mutualism involving the large genus *Cecropia* (Urticaceae) and associated ants, including the genus *Azteca*, has been the subject of extensive ecological study but has not been examined from a phylogenetic perspective. Large woody plant genera have often been difficult to treat taxonomically and resolve phylogenetically. It is unknown whether this is due to variation in traditional DNA markers or if other factors are involved. Next generation sequencing techniques can provide more data than direct methods that improve phylogenetic resolution and provide opportunity to infer historical introgression that could also influence phylogenetic resolution. The goal of this dissertation is to investigate evolutionary relationships in *Cecropia* and its closest relatives and to examine the evolutionary history of the mutualism.

Relationships between genera in the tribe Cecropieae (Urticaceae), including *Cecropia*, *Coussapoa*, *Musanga*, *Myrianthus*, and *Pourouma*, were unknown but are necessary to investigate the evolutionary history of the *Cecropia*-ant mutualism. Chapter 1 used molecular phylogenetics to infer relationships between genera in Cecropieae and

investigates the influence of phylogenetic resolution in *Cecropia* on reconstructing the ancestry of myrmecophytism. Bayesian phylogenetic analysis of the NADH dehydrogenase (*ndhF*) chloroplast gene region, the 26S region of nuclear ribosomal DNA, and an exon-primed intron-crossing DNA region supported non-myrmecophytic African *Musanga* within a paraphyletic *Cecropia*. Neotropical *Pourouma* and *Coussapoa* were supported as sister taxa with African *Myrianthus* as their closest relative. Although it was uncertain whether myrmecophytism was the ancestral condition of *Cecropia*, a close relationship between non-myrmecophytic *Cecropia sciadophylla* and *Musanga* suggests that the loss of ant associations did not accompany African colonization by *Musanga*.

In Chapter 2, restriction site associated DNA (RAD) sequencing was used to infer relationships among myrmecophytic and non-myrmecophytic *Cecropia* species. RAD sequence data resolved and supported species level relationships beyond what could be inferred from direct sequencing. The D-statistic to test for introgression among *Cecropia* species was used to examine whether hybridization might account for some of the difficulty associated with diagnosing species in the genus. Most *Cecropia* species sampled were not deeply diverged genetically but a non-myrmecophytic clade included lineages that could be considerably older than most of the ant-associated species. Results of ABBA BABA tests could be interpreted as evidence of recent introgression among closely related myrmecophytic species. However, test results implying geographically implausible introgression between neotropical *C. sciadophylla* and afrotropical *Musanga* suggest that the D-statistic is sensitive to the extent of genetic divergence among clades

and may yield type I error in the case of deeply diverged clades. Evidence from geographically widespread and morophologically hetergenous *C. obtusifolia* and *C. angustifolia* suggests that current synonymy lumps together genetically dissimilar lineages and that future taxonomic revision should consider splitting.

Chapter 3 investigated the origin and evolutionary history of myrmecophytism in *Cecropia* sensu lato (including *Musanga*). The most highly supported phylogeny for *Cecropia* was used in ancestral state reconstructions of ant association and the myrmecophytic traits of domatia (nest cavitiy) and trichilia (food bodies) to investigate the evolutionary history of the mutualism. Although it was unknown whether the common ancestor of *Cecropia* was myremecophytic, the deepest split in the clade revealed ecological differences between the two oldest lineages of *Cecropia* sensu lato. The clade including *C. sciadophylla* and *Musanga* more likely had a non-myrmecophytic ancestor while myrmecophytism was most likely the ancestral state of the more species-rich *Cecropia* sensu stricto. Trichilia were associated with the origin and loss of ant associations where as domatia where not. *Cecropia* is distributed across a broad range of elevation and the absence of ant associations with high montane species was associated with the evolutionary loss of trichilia in two independent cases. A comparative analysis showed that gains and losses of myrmecophytism in *Cecropia* were correlated with the presence or absence of trichillia and domatia. Ant associations were more dependent on the presence of trichilia than on domatia.

A resolved and highly supported phylogeny of over half the genus may be used to inform future ecological and evolutionary studies of the *Cecropia*-ant mutualism. A

phylogenetic framework for *Cecropia* will allow for studies to take relatedness into account when comparing morphologogical traits of species. RADseq provided the data needed to begin to resolve relationships within *Cecropia* and may be what is necessary in other unresolved tropical tree genera. This will also allow for mutualism to be studied in a phylogenetic context where it previously was not possible.

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Preface

Introduction and conclusion sections are included to provide background information for the overall study and an overview of the larger scientific context into which this dissertation fits. Each chapter is meant to stand alone as an individual scientific publication, but these chapters are united in this dissertation by their shared focus on topics related to phylogenetic and mutualism of *Cecropia*. Chapter one has been published in a slightly different form in Systematic Botany and the results presented in chapter two is intended to be published as discrete units after the submission of this dissertation. Any citations to a chapter within this dissertation were altered to include the chapter number (e.g. Treiber et al. 2016, Chapter 1). Each of these publications will have one or more coauthors, so plural pronouns are used throughout, but as first author I am responsible for the content of each chapter.

Chapter 1: Phylogeny of the Cecropieae (Urticaceae) and the Evolution of an Ant-Plant Mutualism

INTRODUCTION

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 1993a; 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 throughout 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 Barthélemy Dumortier (1829), who included *Cecropia* and *Coussapoa* within the family Artocarpideae Dumort. Charles 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. Heinrich Gustav Adolf Engler (1889) placed Conocephaleae Trécul including *Cecropia*, *Coussapoa*, *Pourouma*, *Myrianthus*, and *Poikilospermum* in the subfamily Conocephaloideae (Moraceae). Edred John Henry 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. Sergio Romaniuc-Neto (1999) proposed that all genera of Cecropieaceae 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 1-1, Figure 1-1). 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 FA16180b that 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 and 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 1-1, Appendix 2). 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; 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 data set including all three regions and all taxa listed in Appendix 1, and (5) a concatenated data sets 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 data set 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 for each posterior tree type.

RESULTS

Morphological Phylogeny **–**The Bayesian consensus tree largely supported prior taxonomic groupings (Figure 1-2, Table 1-1). 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 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 base pairs 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 base pairs 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* (Figures 1-3 & 1-4). 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* (Figure 1-5). 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 to 0.99 according to the position of non-myrmecophytic *Cecropia* and *Musanga* in the tree (Figure 1-6). 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 (Figure 1-7). 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 (Figure 1-7).

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

(Figure 1-2) whereas nuclear and chloroplast DNA sequences of *Poikilospermum* are more closely related to members of Urticeae (Figures 1-3 to 1-5). 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* (Figure 1-5). This finding is not surprising given the morphological similarities of *Cecropia* and *Musanga* (Figures 1-1,1-2). 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 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 highly similar vegetative and reproductive characteristics (Table 1-1, Figure 1-8). 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 cauducous bract in place of the spathe (Figure 1-8). 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 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).

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 (Figure 1-6). 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 to 0.95 depending on the position of the other non-myrmecophytic *Cecropia* species (Figure 1-6A, 6B, and 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-mymecophytic *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 to 0.99 (Figure 1-6C, 1-7). 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-myrecophytes 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 (Figure 1-7). 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 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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Table 1-1. 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 globoso 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 cilindrical 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


6 cm
A
1 mm
B
8 cm
E
2 mm
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1.5 mm
D
5 mm
F
1 mm
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H
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2 mm
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5 mm
T
5 cm
N
4 cm
O
1 mm
P
5 cm
Q
1 mm
U
1 mm
V

Figure 1-1. *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 transversal section. G. Staminate flower. H. Pistillate flower. *Coussapa microcarpa*: I. Leafy twig with stipule and pistillate inflorescences. J. Staminate flower. K. Infrutescence Figure 1-1. *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)]

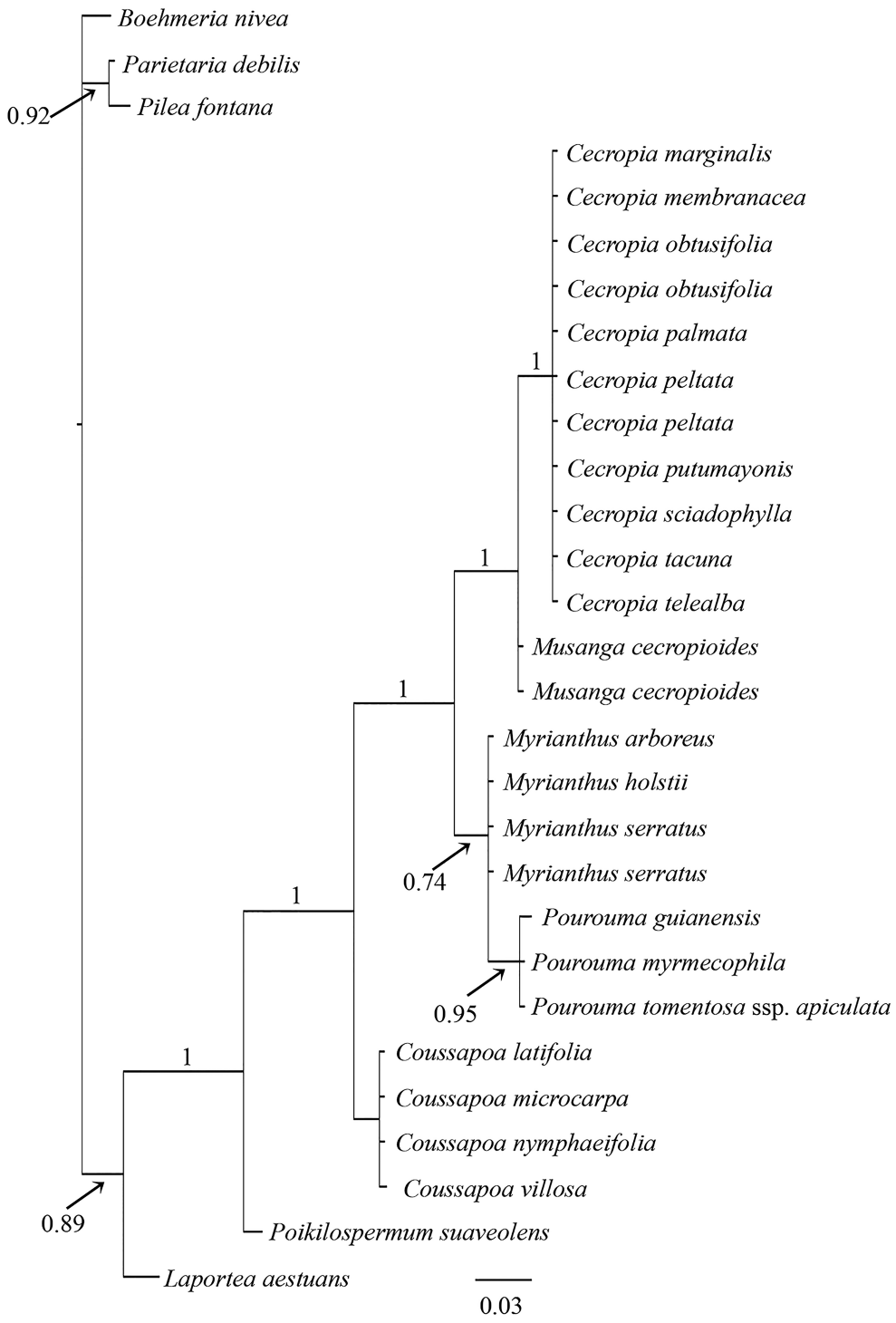

Figure 1-2. Bayesian (>50%) majority consensus rule tree for Cecropieae based on 40 morphological characters. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. Posterior probabilities greater than 0.75 are noted on respective branches.

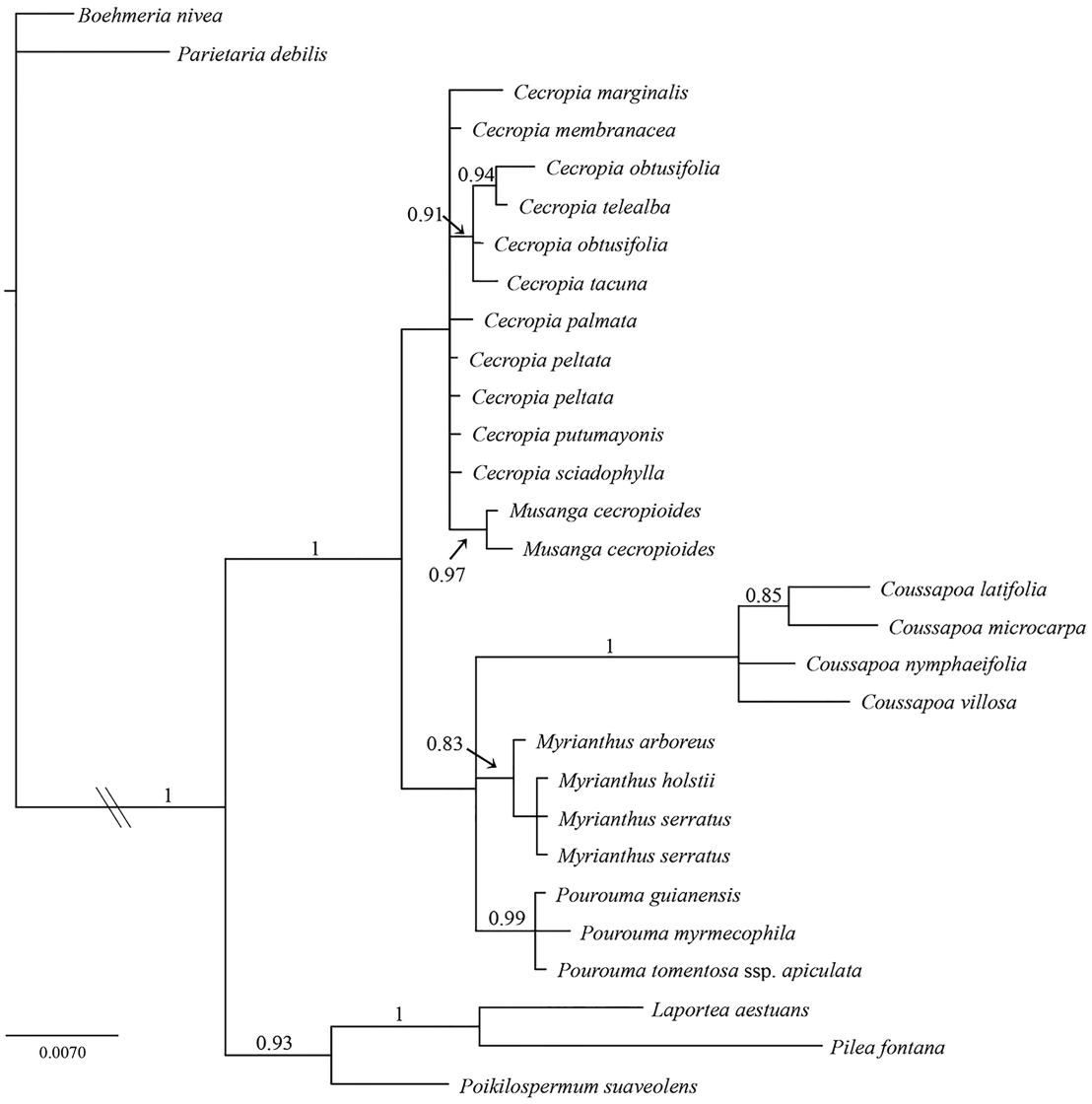

Figure 1-3. Bayesian (>50%) majority consensus rule tree for Cecropieae based on ndhF DNA sequence data. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. The branch bearing double hatch marks indicates that it has been truncated and is not proportional to the rest. The originally length of the truncated branch was approximately 0.04. Posterior probabilities greater than 0.75 are noted on respective branches.

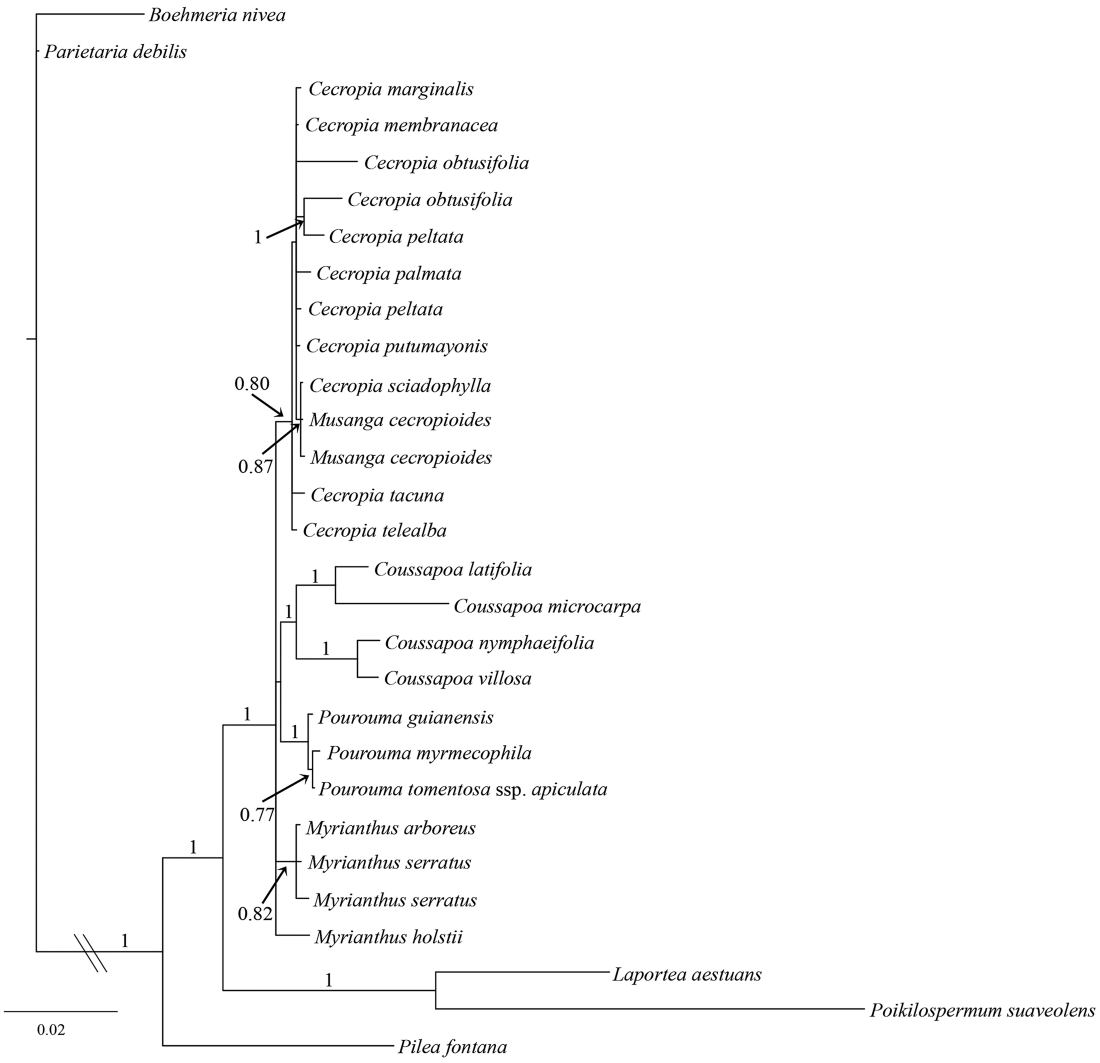

Figure 1-4. Bayesian (>50%) majority consensus rule tree for Cecropieae based on 26S DNA sequence data. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes were used to root the tree. The branch bearing double hatch marks indicates that it has been truncated and is not proportional to the rest. The originally length of the truncated branch was approximately 0.028. Posterior probabilities greater than 0.75 are noted on respective branches.

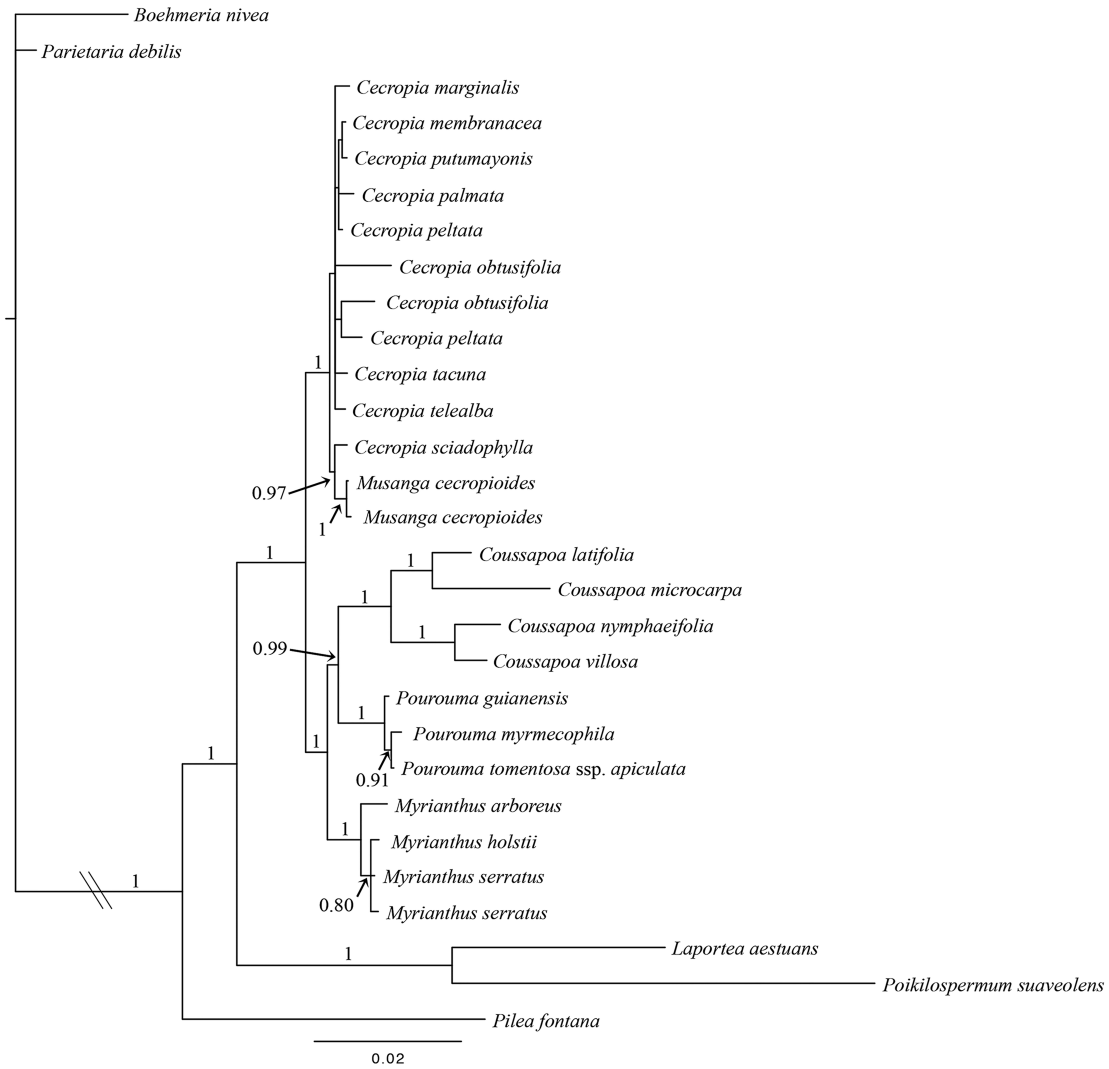

Figure 1-5. Bayesian (>50%) majority consensus rule tree for Cecropieae based on 26S, *ndhF*, and EPIC DNA regions. Five species (*Boehmeria*, *Parietaria*, *Pilea*, *Laportea*, and *Poikilospermum*) from other Urticaceae tribes are used to root the tree. The branches bearing double hatch marks indicates 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.

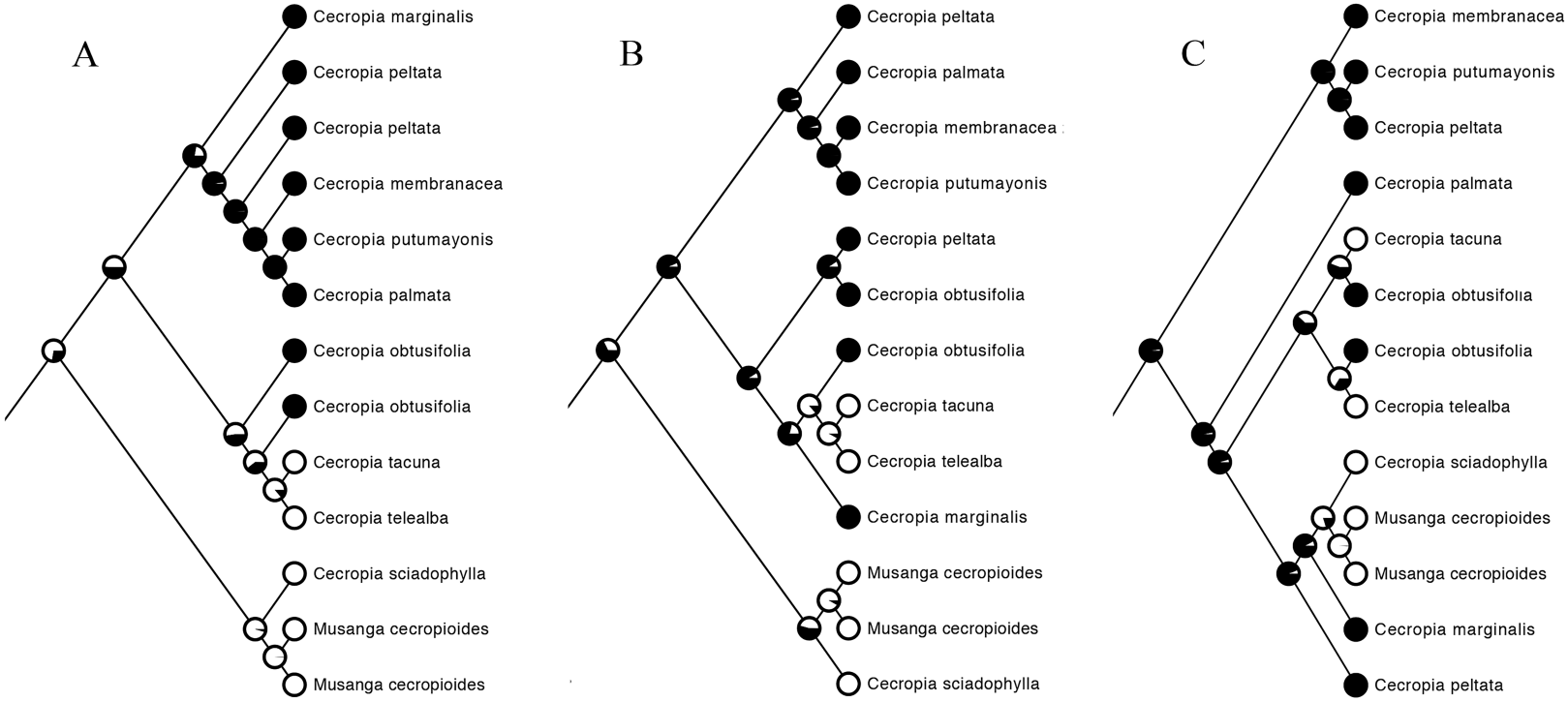

Figure 1-6. Ancestral reconstruction of ant associations on sample of phylogenies from the Bayesian posterior distribution. Circles at tips indicate if a species has ant associations (black) or is not ant associated (white) and circles at node represent probability of ancestor for each state. Ancestral state reconstructions done on (A) tree similar to consensus, where *C. sciadophylla* and *Musanga* are sister to the other *Cecropia* with the other antless *Cecropia* branching off earlier, on (B) tree like A, but with other antless *Cecropia* branching embedded in the *Cecropia* clade, and (C) on tree with *C. sciadophylla* and *Musanga* embedded within the *Cecropia* clade.

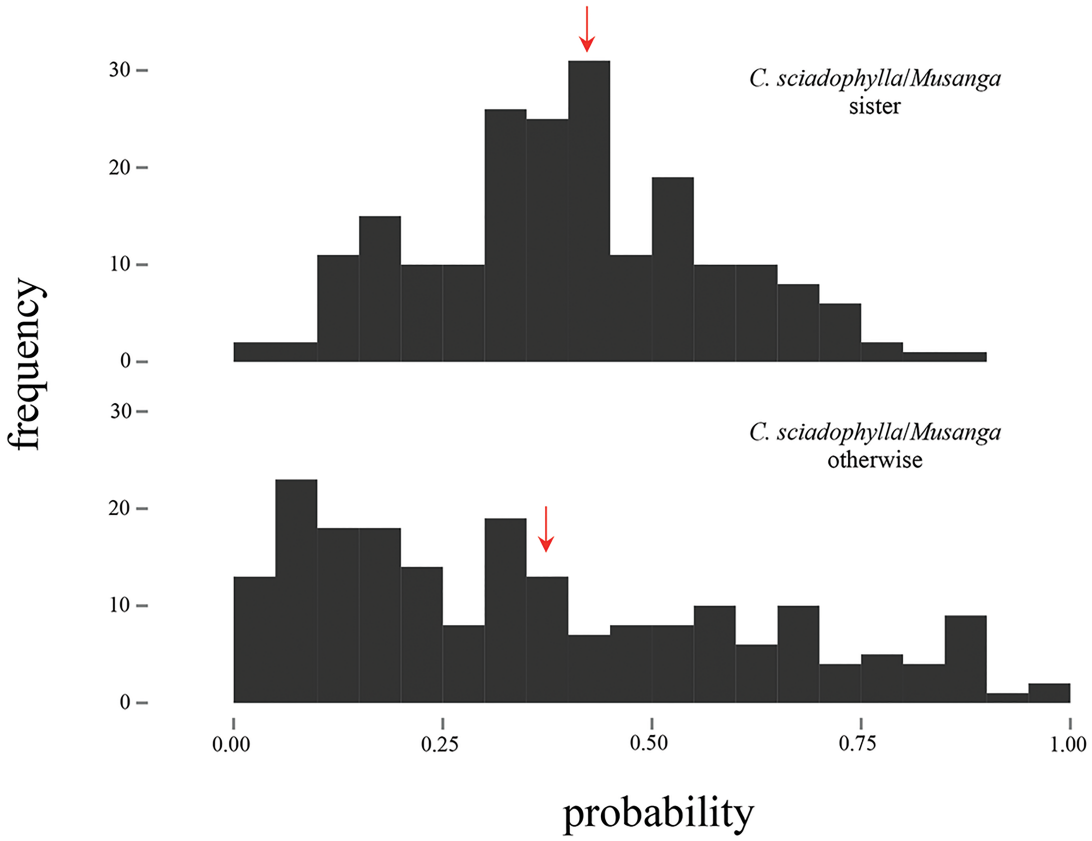

Figure 1-7. Probability of a myrmecophytic ancestor for samples from the posterior of a Bayesian analysis. In the upper panel is the probability distribution for 200 random samples from a subset of the posterior trees that had topologies with *C. sciadophylla* and *Musanga* sister to the remaining *Cecropia* samples. Approximately normal distribution around a mean of 0.40. The lower panel had the probability distribution for 200 random samples 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 red arrows.

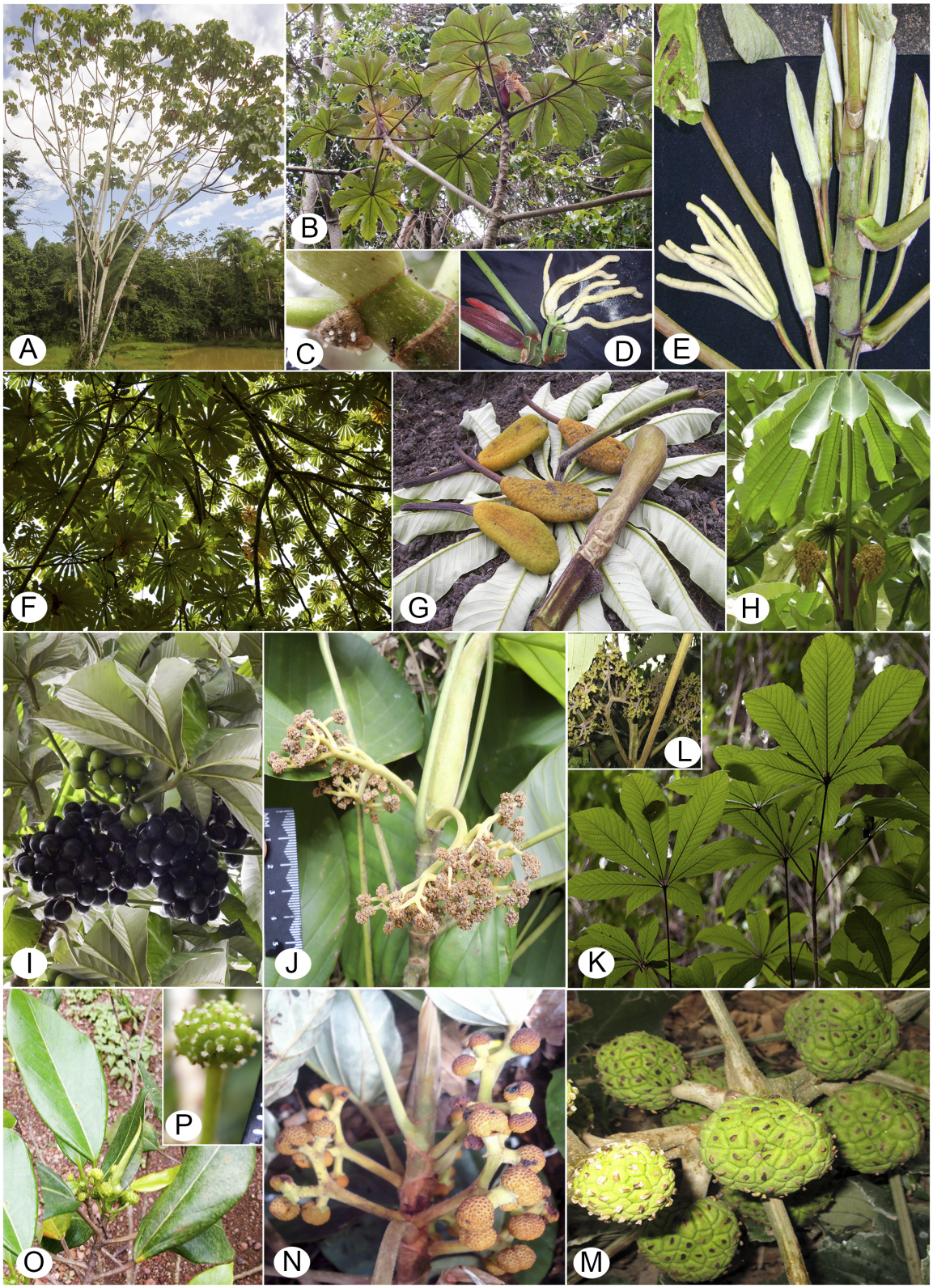
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Figure 1-8. Morphological diversity of tribe Cecropieae: (A) *Cecropia peltata* (tree); (B) *Cecropia glaziovii* (leafy twigs, leaves, and stipule); (C) *Cecropia pachystachya* (trichilia, Müllerian bodies, and ant); (D) *Cecropia glaziovii* (staminate inflorescence); (E) *Cecropia pachystachya* (spathes, and staminate inflorescence); (F) *Musanga cecropioides* (tree, and leaves); (G) *Musanga cecropioides* (leaf, stipule, and pistillate inflorescences); (H) *Musanga cecropioides* (leaves, stipule, and staminate inflorescences); (I) *Pourouma cecropiifolia* (leaves, and infructescences); (J) *Pourouma mollis* (stipule, and staminate inflorescences); (K), (L), (M) *Myrianthus holstii* (leaves [K], staminate inflorescence [L], infructescence [M]); (N) *Coussapoa villosa* (stipule, and staminate inflorescences); (O) *Coussapoa microcarpa* (leaves, and pistillate inflorescences). Photos: F-H from J. Nakos, K-L from E. Kami.

Chapter 2: The phylogenetic utility of RAD Sequencing in Cecropia (Cecropieae:Urticaceae)

INTRODUCTION

Resolving phylogenetic relationships among species in large, woody plant genera is often difficult (Blattner 2001; Davies et al. 2001; Richardson et al. 2001; Rønsted et al. 2007; Triono et al. 2007). It is not known if this is generally due to slow rates of molecular evolution, poor species concepts, hybridization, introgression, or other factors. The problem is especially common in tropical woody plants where species relationships in the largest genera are not well known. Smith and Donoghue (2008) showed that rates of molecular evolution are slower in woody plants than in herbs, but the lack of resolution in species-level phylogenies in trees and shrubs may also be due to insufficient molecular variation in the conventional markers used for phylogenetic studies. As sequencing technologies advance, new techniques promise to provide amount of variation needed to resolve phylogenetic relationships in large tropical tree genera.

For the past two decades, plant systematists have relied on the method of Sanger (or direct) sequencing of individual loci to resolve phylogenetic relationships (Soltis et al. 1992; Soltis 1998). It is both difficult to identify loci with sufficient molecular variation to resolve phylogenetic relationships and challenging to find loci that may be consistently amplified and sequenced across the taxa of interest (Rubin et al. 2012). Limited variation in traditional loci for plant phylogenetics has proved to be a particularly common pattern in species-rich genera. Lack of molecular variation in traditional loci may be blamed for poor phylogenetic resolution in large genera of tropical trees. For instance, published phylogenies for *Inga* (Fabaceae) and *Pouteria* (Sapotaceae), each with approximately 300 species, failed to support any node with >95% bootstrap or posterior probability in *Inga* and only >90% posterior probability support for ~80% of the nodes in a small subset of

Pouteria (Richardson et al. 2001; Triono et al. 2007). *Ficus*, with approximately 800 species worldwide, had only 50% of nodes supported among a sample of two hundred species (Rønsted et al. 2007), while the most recent phylogenies of *Macaranga* had only 10-14 % of its nodes strongly supported (Blattner 2001; Davies et al. 2001). The recurring pattern of low resolution and support in these studies demonstrates the difficulty in resolving relationships with Sanger sequencing whereas new sequencing technologies may offer promise.

Next generation sequencing provides megabases of data in a single procedure (Straub et al. 2012). One type of next generation sequencing, restriction-site-associated DNA (RAD) sequencing, which samples multiple regions throughout the genome, may provide a more cost effective alternative whole genome sequencing for molecular phylogenetic studies (Davey et al. 2011; Rubin et al. 2012). Recent phylogenetic studies have used RAD sequencing (RADseq) to rapidly identify single nucleotide polymorphisms (SNPs) in model species (Baird et al. 2008), and to examine phylogeography in recently diverged mosquitos (Emerson et al. 2010), species boundaries in recently radiated African cichlids (Wagner et al. 2012), and phylogeny in flowering plants (Eaton and Ree 2013; Cavender-Bares et al. 2015; Eaton et al. 2015). The application of RAD sequence data could provide the amount of sequence data required for phylogenetic resolution that has been lacking in many systematic studies of species rich tropical plant groups.

RAD sequencing also provides sufficient data to test hypotheses about introgression among species. Hybridization is common in plants and can be an important factor to consider when inferring phylogenetic relationships. The large number of SNPs

generated can be used in statistical tests for introgression (Green et al. 2010; Eaton and Ree 2013). The D-statistic has been used to examine introgression in Neanderthals and humans (Green et al. 2010), herbaceous plants (Eaton and Ree 2013) and trees (Eaton et al. 2015). The D-statistic looks at the frequency of different incongruent SNP patterns, which are expected to be similar if they are due to stochastic processes. If one pattern is statistically more frequent, it is evidence for hybridization or introgression between species (Eaton and Ree 2013). In this paper, we examine RAD sequence data to detect patterns of introgression and to resolve phylogeny in the tropical tree genus *Cecropia*.

A common Neotropical genus of fast growing pioneer trees, *Cecropia* (Urticaceae), is important in forest regeneration throughout Central and South America (Berg and Franco-Rosselli 2005). *Cecropia* occupies a similar habitat as *Macaranga*, a related genus in the Old World tropics. *Cecropia* species are commonly described as myrmecophytic (having a symbiotic relationship with ants), but the history of this ant-plant mutualism is not well understood despite speculation about the evolution of *Cecropia* and the associated ants (Janzen 1969; 1973a). Lack of a phylogenetic framework for *Cecropia* has hindered our understanding of the origin, maintenance, and dynamics of the mutualism.

Cecropia trees range from 5 to 20 m in height and have few branches with a candelabrum-like branching pattern (Berg and Franco-Rosselli 2005). Stipule scars on the main stem and branches are usually conspicuous. Leaves of adult trees are large and peltate, with incisions between the radiating main veins that can be shallow or extend to the petiole. At the base of adult leaves, there is a concentration of trichomes called trichilia where food bodies, known as Müllerian bodies, are produced (Berg and Franco-

Rosselli 2005). All *Cecropia* species are dioecious, with pistillate and staminate flowers on separate plants. The staminate inflorescences are often pendulous. The pollen is dry and easily released by perturbation, which suggests that *Cecropia* is likely wind pollinated (Epperson and Alvarez-Buylla 1997; Berg and Franco-Rosselli 2005).

Morphological similarity has made taxonomy difficult in close relatives of *Cecropia* and in the genus, itself. Until recently relationships among genera in the tribe Cecropieae were unknown. A recent phylogenetic study including all five genera of Cecropieae discovered the African genus *Musanga* to be embedded in *Cecropia* (Chapter 1) instead of more closely related to the other African genus *Myrianthus*. Another recent study placed *Musanga* sister to *Cecropia* but the relationship was not well supported (Gutierrez-Valencia et al. 2017). Chapter 1 also found the other three genera of Cecropieae (*Coussapoa*, *Myrianthus*, and *Pourouma*) to be members of a clade that is sister to *Cecropia*/*Musanga*.

Morphological variation within *Cecropia* species and similarities among species also complicate the taxonomy of the genus. A recent monograph by Berg and Rosselli (2005) reduced the size of the genus from ~165 to 61 species. Species identification is difficult due to geographic and ecotype variation in traits. For example, *C. angustifolia* varies greatly in indumentum, leaf venation, and inflorescence construction, which appear to correlate with annual precipitation and elevation. Berg and Rosselli (2005) grouped thirteen previously named species under *Cecropia angustifolia*. Similarly, fourteen names were synonyms of *C. obtusifolia*, which exhibits substantial morphological variation and is geographically. In contrast, species such as *C.*

sciadophylla and *C. membranacea* with somewhat smaller geographic ranges do not vary much morphologically.

Molecular data are needed to clarify species boundaries and resolve species relationships, but sequence variability at conventional loci (*rbcL*, *matK*, trnL-trnF, and *ndhF*) appear to be insufficient in *Cecropia* (Gutierrez-Valencia et al. 2017). We expect the amount of data provided by RADseq to better resolve species relationships at recent and deeper nodes. Gutierrez-Valencia et al. (2017) assumed species monophyly and combined sequences drawn from different populations in their estimate of *Cecropia* phylogeny. We tested this assumption by examining species monophyly with RADseq data. If morphological variation is predictive of genetic variation, we expect morphologically homogeneous species to form monophyletic groups and morphologically heterogeneous to not be monophyletic. We also tested for evidence of introgression among *Cecorpia* species as a possible explanation for the difficulty of resolving species relatedness in the genus.

This work is necessary to investigate the evolution of the genus and its mutualism with ants. Most previous studies did not consider the mutualism in a phylogenetic context. For instance, studies looking at differences in herbivory among species (Latteman et al. 2014) and structural variation of parenchyma in septa (Valverde and Hanson 2011) among myrmecophytic and non-myrmecophytic species did not consider phylogeny. A phylogenetic framework allows us to account for evolutionary history when answering ecological questions that are dependent on knowledge of relatedness. In addition, measures of species specificity and the costs and benefits of species interactions require clear and biologically meaningful definitions of species.

MATERIALS AND METHODS

Sanger Sequencing

Sampling – We collected *Cecropia* and outgroup accessions from Central and South America from field surveys or collaborators (S. Madriñán, M.F. Torres, C.L. Sagers, A.L. Gaglioti, P. Barriga, and G.D. Weiblen). We examined 91 samples representing 26 species of *Cecropia*, 1 *Coussapoa* species, and 3 species of *Pourouma* (Table 2-1). Samples were chosen to explore the variation in conventional plant systematic markers and the ability to sequence samples for multiple loci.

DNA extraction and sequencing – Five regions were sequenced including two chloroplast regions, psbK-psbI and trnH-psbA, (Kress et al. 2010) and three nuclear loci: glyceraldehyde 3-phosphate dehydrogenase (Strand et al. 1997) and 2 nuclear exon-primed intron-crossing (EPIC) markers, FA32910 and FA16180b, that were developed for Moraceae (Yao et al. 2013). These regions were targeted because they are more variable than those used in Chapter one to resolve the position of Cecropia within the subfamily Cecropieae (Urticaceae). DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Valencia, California, USA) with 20mg of silica gel preserved leaf fragments. PCR amplification of psbK-psbI and trnH-psbA chloroplast regions followed those outlined in Kress et al. (2010). PCR amplification of the G3pdh nuclear regions followed procedures outlined in Strand et al. (1997) and the two EPIC regions from Yao et al. (2013). PCR products for all regions were cleaned by ethanol precipitation and quantified using a NanoDrop 2000c (Thermo Scientific Inc, Waltham, MA, USA). Sequencing was performed in 10μL reactions using Big Dye sequencing reagents and protocols (Applied Biosystems, Foster City, CA, USA), and data were collected using an ABI Prism 3730xl

DNA Analyzer (Applied Biosystems). 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.

Attempts were made to sequence the internal transcribed spacer region of ribosomal DNA and triose phosphate isomerase; conventional markers used in plant systematics (Baldwin et al. 1995; Strand et al. 1997; Li et al. 2011), but were abandoned due to poor rates of amplification and multiple copies.

Phylogenetic analyses – Phylogenetic analyses were performed using a matrix with no missing data and a sparse matrix that included samples with at least three DNA regions sequenced. Analyses were performed using Mr. Bayes v.3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012) on the CIPRES Science Gateway (Miller et al. 2010) with gamma-distributed rate variation across sites, and a parameter for the proportion of invariable sites. 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".

Restriction Site Associated (RAD) Sequencing

Sampling – We examined 47 samples collected representing 31 species of *Cecropia* and 4 other members of the Cecropieae tribe (*Coussapoa*, *Musanga*, *Myrianthus*, and *Pourouma*). The sampling of Cecropia included 26 species for which Sanger sequence data was also collected and five additional species lacking Sanger data. This sampling design did not permit direct comparison of Sanger and RADseq results from identical

sample sets but rather was aimed at quantifying the relative performance data sets in resolving and supporting phylogeny. Other members of Cecropieae were included to root the tree and *Musanga* was included to confirm its ingroup position, embedded in *Cecropia* as suggested previously (Chapter 1) verus its position sister to *Cecropia* (Gutierrez-Valencia et al. 2017). To test hypotheses concerning morphological and genetic variation within species, we included three to four samples per species for four *Cecropia* species. All four species were widespread and we choose two that were morphologically homogeneous throughout their range (*C. sciadophylla* and *C. membranacea*) and two that were morphologically homogeneous throughout their range (*C. obtusifolia* and *C. angustifolia*) (Table 2-2).

DNA Preparation and Sequencing – Silica dried material collected in the field was used for DNA extractions, except for one sample for which we only had herbarium material. DNA was extracted using a modified CTAB method (Doyle and Doyle 1987) with at 2% CTAB buffer and extended incubations. Each sample was extracted in duplicate to provide more material per sample for sequencing. Each sample for RADseq was required to contain 50uL of high molecular weight DNA with no degradation or contaminating material at a concentration of 20 ng/uL. Samples were sent to Floragenex Inc. (Eugene, OR) for RAD library preparation and sequencing. Libraries were prepared using the *PstI* restriction enzyme following the methods of Baird et al. (2008). The library was created from 95 pooled and barcoded samples sequenced on an Illumina Hi Seq 2000 to generate 100 bp single end reads. Samples were combined for each collection when demultiplexing the library.

Sequence Assembly – Sequences were demultiplexed using *ea-utils* (Aronesty 2001) with default setting which allowed for 1 mismatch in the barcode sequence. The remaining steps of quality filtering and assembly of sequences into *de novo* loci were done using *pyRAD* v. 3.0.63 (Eaton 2014). Bases with a score of <20 were converted into unknown base pairs (Ns) and reads with >5 Ns were discarded. After filtering, reads were clustered within samples at thresholds between 82% and 98%. Average parameter values estimated in *pyRAD* were used when making consensus base calls and clusters with minimum depth of <5 were excluded. Additional analyses were also done with higher minimum depth coverage (<15) to examine the effects of missing data. After removing loci containing more than two alleles as potential paralogs, consensus loci were clustered across samples using the same threshold used in the previous within sample clustering. Assembled loci were exported as a supermatrix with missing data converted to Ns for phylogenetic analysis.

Phylogenetic Analysis – Maximum likelihood analyses were performed on each assembled data set using RAxML version 8.2.4 (Stamatakis 2014) on the CIPRES Science Gateway (Miller et al. 2010). Bootstrap support was estimated from 300 replicate searches from random starting trees run using the GTR+Γ model of nucleotide substitution model.

Tests for Introgression – The D-statistic (Green et al. 2010; Eaton and Ree 2013) was used to test for evidence of introgression in the data. The D-statistic detects introgression between lineages based on the frequency SNPs that are discordant with a phylogenetic hypothesis. Although discordant site patterns often occur due to lineage sorting of ancestral polymorphisms, the different patterns occur at a mostly equal frequency due to

the stochasticity of the process. The D-statistic calculates the asymmetry in the relative occurrence of the two discordant site patterns to test for introgression (Eaton et al. 2015). We first tested the hypothesis that introgression is more common in species that are morphologically heterogeneous than in morphologically homogeneous species. We used *pyRAD* v. 3.0.63 to calculate the D-statistic using 1000 bootstrap replicates. Both morphologically homogeneous species were monophyletic and were tested against close relatives. The location of *C. sciadophylla* in the phylogeny, sister to the rest of *Cecropia*, required the D-statistic test to be performed against all other *Cecropia* samples. The morphologically heterogeneous samples were tested against closely related taxa.

Finally, we used the D-statistic to test for introgression in clades that had lower support or taxa that changed position in different analyses. *C. herthae* was sister to different closely related clades in different analyses. *Cecropia telenitida*, *C. gabrielis*, *C. plicata* were tested due to the consistently low support of the clade they formed.

RESULTS

Direct Sequencing

The complete matrix of 5 gene regions with no missing data included 36 samples representing 15 *Cecropia* species (Tables 2-1). Of the 2,330 aligned nucleotide positions 10% were variable and 3% were phylogenetically informative (Table 2-3). The sparse matrix including samples with at least three gene regions consisted of 91 samples representing 29 *Cecropia* species (Table 2-1). This matrix had 14% variable sites with 8% being phylogenetically informative (Table 2-3).

Bayesian analyses of the complete data matrix and the sparse matrix resulted in poorly resolved phylogenies (Figures 2-2 & 2-3). The complete matrix with fewer samples and species had 13 of 38 nodes with >95% posterior probability and in the case of the sparse matrix there were only 11 of 87 nodes with high support (Table 2-3). Few relationships among species were strongly supported. In both the complete and sparse matrixes, *C. sciadophylla* was highly supported as monophyletic. The complete matrix also supported the monophyly of *C. membranacea* but not the sparse matrix. There was also support for a clade including *C. ficifolia* and *C. tacuna* based on the complete data matrix whereas *C. marginalis* and *C. litoralis* formed a clade in the sparse matrix tree.

RAD Sequencing

Seven samples were excluded from the data analysis due to insufficient coverage and one due to apparent contamination by *Pourouma* DNA, resulting in a total of 39 samples (Table 2-2). Six matrices were assembled using different minimum depth, minimum coverage, and clustering thresholds that varied numbers of loci and phylogenetically informative sites (Table 2-4). The matrices ranged from 71,410 loci with a minimum coverage depth of five and clustering threshold of 0.98 to 24,371 loci with respective parameters of 15 and 0.82 (Table 2-4). Phylogenetically informative characters decreased from 299,910 to 26,561 with increasing minimum depth of coverage from five to 15 and clustering threshold from 0.82 to 0.98 (Table 1-4).

Matrices varying in stringency of clustering and numbers of samples sharing a locus did not affect inferred relationships much at all. The data sets with a higher minimum depth for base calling prior to clustering (15) and a higher number of required samples per locus (15) had fewer informative sites, but yielded higher support values. As

the clustering algorithms became more stringent, from 82% to 98%, the number of informative characters decreased as did support. Lower support values corresponded to reduced numbers of informative sites.

The resulting phylogenies had a consistent topology with only three differences. The most highly supported phylogeny was from the matrix using a minimum depth of 15 and a clustering threshold of 0.82 (Figure 2-4). The main difference in topology was the branching order of *C. marginalis* and *C. goudotiana* in how they were split from the majority of *Cecropia*. Analyses based on a matrix minimum depth of coverage of five and clustering threshold of 90% and 98% resulted in *C. goudotiana* in a deeper position in the tree than *C. marginalis*, while the four other topologies supported *C. goudotiana* diverging from the majority of *Cecropia* after *C. marginalis.* In the trees with clustering thresholds of 90% and 98%, the deeper position of *C. goudotiana* had low support with bootstrap values below 60. In the other four trees, *C. marginalis* diverging before *C. goudotiana* and the rest was well supported (Figure 2-4). Different topologies strongly supported *C. herthae* as sister to the *obtusifolia*/*longipes*/*multisiana* clade (as shown in Fig. 2-4) except for one case where it grouped with the clade including *litoralis*, *sararensis*, *angustifolia*, *reticulate*, *engleriana*, and *metensi*s, but with low support (bootstrap = 60). In addition, the two phylogenies resulting from the clustering threshold of 98% supported *C. gabrielis* sister to a *C. telenitida*/*C. plicata* clade*,* while the remaining phylogenies supported *C. plicata* sister to the *C. gabrielis/C. telenitida* clade (as shown in Fig. 2-4). Bootstrap support for this clade was variable 34 and 76 when *C. gabrielis/C. telenitida* formed a clade and 63 when *C. telenitida*/*C. plicata* formed a clade

. All other relationships in the phylogeny were consistent across all the phylogenetic analyses, so we present only the phylogeny with the highest support (Figure 2-4).

The phylogeny was highly resolved and was highly supported for the majority of species. Nodes that had lower support values corresponded to the differences in topology described above. As expected, *Musanga* was embedded within the *Cecropia* clade and was highly supported as sister to *C. sciadophylla*. The *Musanga*/*Cecropia* clade was also highly supported as sister to and deeply diverged from the remaining *Cecropia* species (Figure 2-4). Morphological homogenous *C. sciadophylla* and *C.* membranacea were monophyletic whereas *C. angustifolia* and *C. obtusifolia* were not.

Tests for Introgression – When *Musanga* and morphologically homogeneous *C. sciadophylla* were tested for evidence of introgression with the core *Cecropia* clade, a fifth of the comparisons involving African *Musanga* and core *Cecropia* were statistically significant but none of the tests involving *C. sciadophylla* and core *Cecropia* were significant (Figure 2-5A).

The D-statistic test results for another morphologically homogeneous species, *C. membranacea,* suggested historical introgression with *C. latiloba* but not with an even closer relative, *C. glaziovii* (Figure 2-5B). Similar results were observed for morphologically heterogeneous species, *C. obtusifolia* and *C. angustifolia*. For example, there was no significant evidence of introgression between *C. angustifolia* (Cundinamarca, CO) and its closest relatives (Figure 2-5B) nor when *C. angustifolia* (Boyaca, CO) was tested with its closest relatives (Figure 2-5E). Statistical significance was instead observed when tests involved species belonging to different clades. For example, half of the 22 tests between the *C. angustifolia*/*C. peltata* clade with the *C.*

obtusa/*C. pupuruscens*/*C. ficifolia* clade suggested historical introgression. Some test results involving *C. angustifolia* (Antioquia, CO) and the two subclades of its sister group were also significant (Figure 2-5C).

Again, in the case of *C. obtusifolia*, no tests involving its closest relative, *C. longipes,* were significant (Figure 2-5E) but some tests involving the *C. obtusifolia*/*C. longipes* clade yielded significant results (Figure 2-5D).

Tests that included *C. herthae* tended to detect significant patterns of introgression with other species. For example, all tests were significant for introgression between *C. herthae* and the *C. longipes*/*C. multisiana*/*C. obtusifolia* clade (Figure 2-5D). When *C. herthae* was tested with the remaining large clade (Figure 2-5D), approximately one third of the tests were significant and were equally distributed between individual members of the clade except for *C. sararensis*.

DISCUSSION

Resolving species relationships with conventional phylogenetic markers has remained difficult in large woody tropical tree genera perhaps due to slower rates of molecular evolution. Species-level phylogeny in *Cecropia* was not supported by a small set of low-copy number loci and results from phylogenetic analyses using RADseq demonstrate that more data can resolve the *Cecropia* phylogeny at the species level with greater confidence. In addition, D-statistic tests suggest that there may have been a history of hybridization among species but the large volume of RADseq data resolves species relationships despite some evidence of introgression.

Analyses based on five DNA regions confirmed the difficulty of resolving species-level relationships in *Cecropia* with conventional plant phylogenetic markers. Resolution was low when no data were missing and decreased substantially as more species were added and the amount of missing data increased. With few phylogenetically informative sites and many taxa, search algorithms were not able to estimate relatedness with any confidence. Given the cost of adding more markers by direct sequencing, the use of RADseq technology offered a more effective approach to resolving species relationships.

The phylogeny based on RAD sequencing had a comparable number of species and amount of missing data as the larger of the two Sanger sequence data sets, but with a much higher percentage of highly supported nodes. The RADseq data set also had about a hundred times more phylogenetically informative sites than either Sanger data set. This suggests that that the lack of variation in conventional markers and an insufficient number of loci is the cause of limited support for *Cecropia* phylogeny based on Sanger sequence data (Gutierrez-Valencia et al. 2017). Slower rates of molecular evolution among woody plants (Smith and Donoghue 2008) along with rapid bursts of species divergence (Madriñán et al. 2013; Vásquez et al. 2016) may make it difficult to resolve phylogeny in groups like *Cecropia*. In their recent study, Gutierrez-Valencia et al. (2017) few nodes in the phylogeny of 33 *Cecropia* species were supported. In addition, these authors pooled genetic data from multiple individuals for a given species, often from different geographic regions (Gutierrez-Valencia et al. 2017). Our examination of the *Cecropia* species concept demonstrates that this approach is problematic because only two of the four species tested were monophyletic. That individuals of *C. angustifolia* and

C. obtusifolia from different geographic locations were not monophyletic suggests that further population genetic analysis and taxonomic assessment in Cecropia is needed. Despite the different taxon samples in our studies, the high support of the RADseq phylogeny suggests that it is necessary to sample more loci than is practical with direct sequencing if alternative phylogenetic hypotheses are to be evaluated (Massatti et al. 2016).

RADseq analyses strongly supporting *Musanga* embedded in the *Cecropia* confirms the result of our molecular analysis of the tribe Cecropieae (Chapter 1). The previous study sampled rather few *Cecropia* species and adding over half of all recognized *Cecropia* also supported the *Musanga*/*C. sciadophylla* clade. This result contradicts a recent molecular study based on seven loci (Gutierrez-Valencia et al. 2017) that found weak supported for *Musanga* as sister to *Cecropia*. As discussed in Chapter 1, our study did not sample the second species of *Musanga* but this taxon is an east African montane endemic and likely derived from the widespread Afrotropical *M. cecropioides*. *Musanga* is similar to *Cecropia* in both habitat and morphology, but lacks structures (i.e. trichilia) that are associated with the ant mutualism. Janzen and Mckey (1977) stated that if *Musanga* occurred within the range of *Cecropia* it would have been placed in the genus. The sister relationship of *C. sciadophylla* and *Musanga* suggests the need for taxonomic revision to either transfer *C. sciadophylla* to *Musanga* or to synonymize *Musanga* with *Cecropia*.

Berg and Franco-Rosselli (2005) acknowledged that few groupings could be recognized within *Cecropia* but they did define a few groups based on similar morphological characteristics and geography. With molecular data for most of the species

they recognized, we can begin to compare how their groupings compare with phylogenetic relatedness. Berg recognized two main groups of species based on morphology, the *peltata*-group and the *telenitida*-group. Our analysis placed four members of the *peltata*-group (*C. sararensis, C. litoralis, C. engleriana*, and *C. metensis*), in a clade that also included (*C. angustifolia* Antioquia, CO and *C. reticulata*). However, *C. peltata*, for which the group was named, was part of an even larger clade including four species not grouped with it by Berg and. Our study also lacked four species of the *peltata*-group so further sampling is needed to assess its taxonomic validity.

We sampled only two out of seven species in the *telenitida*-group (*C. gabrielis* and *C. telenitida*) but they did form a clade. In addition to the *peltata*- and *telenitida*-groups, Berg recognized agroup of seven species based on their presence in the Guayana region and broad leaf segments. The three species from this group that we sampled (*C. ficifolia*, *C. obtusa*, and *C. purpurascens*) formed a clade. The Guayana Shield is known for its distinctive weathered soils and high level of plant endemism. It is not uncommon to find habitat specialists this region with narrow geographic ranges or occassionally in similar habitats in neighboring regions (Berry and Riina 2005). Three species restricted to the Guyana region (*C. granvilleana*, *C. kavanayensis*, and *C. angulata*) have yet to be sequenced. Although a more complete sample is needed, it appears that geography and morphological similarity may predict a modest degree of relatedness in *Cecropia*. Future studies need to consider more intensive sampling of populations within species especially those taxa having multiple synonyms. For instance *C. peltata* has eleven synonyms according to Berg and Franco-Rosselli (2005) but we sampled only a single individual.

This species possesses the same combination of morphological heterogeneity, broad geographical range, and multiple synonyms as *C. angustifolia* and *C. obutusifolia*. Our findings based on sampling within species calls into question the broad species concept of Berg and Roselli (2005). More detailed genetic studies are needed to improve the *Cecropia* species concept and our understanding of phylogeny (Herrera and Shank 2016).

Both of the morphologically homogeneous species that we examined in detail, *C. membranacea* and *C. sciadophylla*, were monophyletic (Figure 2-4). We speculated that heterogeneous species might show more evidence of introgression than homogeneous species but this was not the case. Tests with the D-statistic involving *C. membranacea* and close relatives were significant with *C. latiloba* and each *C. membranacea* individual. No tests involving individuals of morphologically heterogeneous *C. obtusifolia* and their closest relatives detected patterns of introgression whereas tests for two of out three *C. angustifolia* individuals were signigicant.

Test results for *C. sciadophylla*, *Musanga*, and the core *Cecropia* clade call into question the validity of the D-statistic as a test for introgression among divergent lineages. No tests involving *C. sciadophylla* and rest of *Cecropia* were significant whereas one-fifth of the tests with African *Musanga* and neotropical *Cecropia* pointed to a history of introgression. This seems highly unlikely given that *Musanga* is geographically isolated from core *Cecropia* and RADseq branch lengths (Figure 2-3) suggest that might have been in isolation for several million years. The D-statistic might be sensitive to the extent of genetic divergence among clades and could yield type I error in the case of deeply diverged clades. Eaton et al. (2015) demonstrated the difficulty of inferring introgression over deep evolutionary time scales when studying oaks and

missing taxa also make it difficult to infer introgression. These considerations limit the strength of our conclusions about introgression until we have a more complete sampling of the genus and a better understanding of the D-statistic.

Introgression might account for the unstable placement of *C. herthae* in the phylogenies based on different matrices. Significant deviations from ABBA BABA expectations were detected between *C. herthae* and many close relatives (Figure 2-5D) frequently but strong support for its position as sister to the *C. obtusifolia/longipes/multisiana* clade in the majority of the phylogenetic analyses suggests that the large amount of data generated with RADseq might overcome the effect of introgression on phylogenetic inference. It is important to note that as more species are added in analyses of *Cecropia* phylogeny our understanding of introgression and its impact on resolving species-level relationships is likely to change. It will not only be necessary to include a complete sample of species but also multiple samples per species (Eaton et al. 2015).

Our analyses strongly supported most nodes in a phylogeny of 27 *Cecropia* species and confirmed the inclusion of *Musanga* in the *Cecropia* clade. With a phylogenetic framework for almost half the genus, we can now address questions about the evolution of the mutualism with ants. Previous work on Cecropieae suggested that a more complete sample of *Cecropia* might improve our ability to gain insight on the origin of the mutualism (Chapter 1) and it is possible that the addition of 18 species would remove some of the uncertainty about the ancestral state of *Cecropia*, whether myrmecophytic or not. With this new phylogenetic hypothesis, we can also begin to address questions about the evolution of morphological traits that may have been

associated with the origin of the mutualism and our phylogenetic framework will make it possible for future studies to consider an evolutionary perspective.

Our work compared to previous studies demonstrate that direct sequencing is not a time or cost effective way to resolve *Cecropia* phylogeny (Gutierrez-Valencia et al. 2017) but RAD sequencing is a promising alternative. The amount of resolution in the phylogeny with RAD sequencing suggests this approach can provide the information necessary to resolve relationships among the remaining *Cecropia* species. Beyond resolving relationships, it will be important to include multiple samples (especially in morphologically heterogeneous taxa) to accurately define species boundaries in the genus and determine how to deal with the taxonomy of *Musanga*. The level of resolution obtained using RAD sequencing in *Cecropia* is also promising for phylogenetic studies of other large, tropical woody genera. Our study supports the notion that RADseq, by examining a large portion of the genome, is able to overcome the lack of phylogenetically informative characters provided by conventional phylogenetic markers.

Table 2-1. Plant specimens used for Sanger sequencing, locality, and GenBank accession numbers for DNA regions sequenced, where EPIC #1 refers to FA16180b and EPIC #2 refers to FA32910.

Species	Collector & Number	Locality	GenBank accessions				
			trnH-psbA	psbK-psbI	G3pdh	EPIC #1	EPIC #2
Cecropia andina Cuatrec	Sagers 5067	Ecuador	KY941744	KY941657	KY941831	KY941510	KY941595
Cecropia andina Cuatrec.	Sagers 5069	Ecuador	KY941745	KY941658	KY941832	KY941511	-
Cecropia angustifolia Trécul	Torres 100	Valle del Cauca, CO	KY941746	KY941659	KY941833	KY941512	-
Cecropia angustifolia Trécul	Torres 102	Valle del Cauca, CO	KY941747	KY941660	KY941834	KY941513	-
Cecropia angustifolia Trécul	Torres 51	Antioquia, CO	KY941748	KY941661	KY941835	KY941514	KY941596
Cecropia angustifolia Trécul	Torres 52	Antioquia, CO	KY941749	KY941662	KY941836	KY941515	KY941597
Cecropia angustifolia Trécul	Torres 66	Antioquia, CO	KY941751	KY941663	KY941838	KY941516	KY941599
Cecropia angustifolia Trécul	Torres 81	Antioquia, CO	KY941752	KY941664	KY941839	KY941517	-
Cecropia angustifolia Trécul	Zalamea 46	Boyaca, CO	KY941753	KY941665	KY941840	KY941518	KY941600
Cecropia angustifolia Trécul	Zalamea 47	Boyaca, CO	KY941754	KY941666	KY941841	KY941519	KY941601
Cecropia angustifolia Trécul	Zalamea 48	Boyaca, CO	KY941755	KY941667	KY941842	KY941520	KY941602
Cecropia angustifolia Trécul	Zalamea 49	Boyaca, CO	KY941756	KY941668	-	KY941521	KY941603
Cecropia bullata	Sagers 5076	Ecuador	KY941757	KY941669	KY941843	KY941522	-
Cecropia engleriana Snethl.	Barriga 2009-009	Ecuador	KY941758	KY941670	KY941844	KY941523	KY941604
Cecropia engleriana Snethl.	Barriga 2009-153	Peru	KY941759	KY941671	KY941845	KY941524	KY941605
Cecropia engleriana Snethl.	Sagers 5082	Ecuador	KY941760	KY941672	KY941846	KY941525	KY941606
Cecropia ficifolia Warb. & Snethl.	Barriga 2009-006	Ecuador	KY941761	KY941673	-	KY941526	KY941607
Cecropia ficifolia Warb. & Snethl.	Barriga 2009-168	Peru	KY941762	KY941674	KY941847	KY941527	KY941608

Cecropia ficifolia Warb. & Snethl.	Sagers 5083	Ecuador	KY941763	KY941675	KY941848	KY941528	KY941609
Cecropia ficifolia Warb. & Snethl.	Torres 22	Amazonas, CO	KY941764	KY941676	-	KY941529	-
Cecropia gabrielis Cuatrec.	Sagers 5078	Ecuador	KY941765	KY941677	KY941849	KY941530	KY941610
Cecropia gabrielis Cuatrec.	Torres 79	Antioquia, CO	KY941767	KY941679	-	-	-
Cecropia gabrielis Cuatrec.	Treiber 26	Tolima, CO	KY941766	KY941678	-	KY941531	-
Cecropia goudotiana Trécul	Treiber 10	Tolima, CO	-	KY941680	-	KY941532	KY941611
Cecropia herthae Diels	Barriga 2009-021	Ecuador	KY941768	KY941681	KY941850	KY941533	KY941612
Cecropia herthae Diels	Barriga 2009-091	Peru	KY941769	KY941682	KY941851	KY941534	KY941613
Cecropia herthae Diels	Sagers 5084	Ecuador	KY941770	KY941683	KY941852	KY941535	KY941614
Cecropia insignis Liebm.	Zalamea 70	Tolima, CO	KY941771	KY941684	-	KY941536	KY941615
Cecropia latiloba Miq.	Barriga 2009-052	Ecuador	KY941772	KY941685	-	KY941537	KY941616
Cecropia litoralis Snethl.	Barriga 2009-039	Ecuador	KY941773	KY941686	KY941853	KY941538	KY941617
Cecropia litoralis Snethl.	Sagers 5070	Ecuador	KY941774	KY941687	-	KY941539	KY941618
Cecropia litoralis Snethl.	Sagers 5085	Ecuador	KY941775	KY941688	KY941854	KY941540	-
Cecropia longipes Pittier	Torres 87	Antioquia, CO	KY941776	-	-	KY941541	-
Cecropia marginalis Cuatrec.	Barriga 2009-004	Ecuador	KY941777	KY941689	KY941855	KY941542	KY941619
Cecropia marginalis Cuatrec.	Barriga 2009-008	Ecuador	KY941778	KY941690	-	KY941543	KY941620
Cecropia marginalis Cuatrec.	Sagers 5086	Ecuador	KY941779	KY941691	KY941856	KY941544	KY941621
Cecropia membranacea Trécul	Barriga 2009-001	Ecuador	KY941780	KY941692	KY941857	KY941545	KY941622
Cecropia membranacea Trécul	Barriga 2009-014	Ecuador	KY941781	KY941693	KY941858	KY941546	KY941623
Cecropia membranacea Trécul	Barriga 2009-015	Ecuador	KY941782	KY941694	KY941859	KY941547	KY941624

Cecropia membranacea Trécul	Barriga 2009-016	Ecuador	KY941783	KY941695	KY941860	KY941548	KY941625
Cecropia membranacea Trécul	Barriga 2009-156	Peru	KY941784	KY941696	KY941861	KY941549	-
Cecropia membranacea Trécul	Barriga 2009-159	Peru	KY941785	KY941697	KY941862	KY941550	KY941626
Cecropia membranacea Trécul	Torres 24	Amazonas, CO	KY941786	KY941698	KY941863	KY941551	KY941627
Cecropia membranacea Trécul	Torres 92	Valle del Cauca, CO	KY941787	KY941699	KY941864	KY941552	KY941628
Cecropia membranacea Trécul	Treiber 3	Cundinamarca, CO	KY941789	KY941700	KY941865	KY941553	-
Cecropia membranacea Trécul	Zalamea 54	Casanare, CO	KY941788	KY941701	KY941866	KY941554	-
Cecropia metensis Cuatrec.	Zalamea 52	Boyaca, CO	KY941790	KY941702	KY941867	-	KY941629
Cecropia montana Snethl.	Sagers 5072	Ecuador	KY941791	KY941703	-	KY941555	-
Cecropia multisecta P. Franco & C.	Torres 89	Antioquia, CO	KY941792	KY941704	KY941868	KY941556	KY941630
Cecropia multisiana Mildbr.	Zalamea 73	Cundinamarca, CO	KY941793	KY941705	-	KY941557	-
Cecropia obtusifolia Bertol.	Torres 106	Valle del Cauca, CO	KY941794	KY941706	KY941869	KY941558	KY941631
Cecropia obtusifolia Bertol.	Torres 50	Antioquia, CO	KY941795	KY941707	KY941870	KY941559	
Cecropia obtusifolia Bertol.	Torres 56	Antioquia, CO	KY941796	KY941708	-	-	
Cecropia obtusifolia Bertol.	Torres 83	Antioquia, CO	KY941797	KY941709	KY941871	KY941560	KY941632
Cecropia obtusifolia Bertol.	Torres 85	Antioquia, CO	KY941798	KY941710	KY941872	KY941561	KY941633
Cecropia obtusifolia Bertol.	Torres 88	Antioquia, CO	KY941799	KY941711	KY941873	KY941562	KY941634
Cecropia obtusifolia Bertol.	Torres 94	Valle del Cauca, CO	KY941800	KY941712	KY941874	KY941563	-
Cecropia obtusifolia Bertol.	Treiber 4	Cundinamara, CO	KY941801	KY941713	KY941875	KY941564	KY941635
Cecropia obtusifolia Bertol	Treiber 29	Tolima, CO	KY941802	KY941714	KY941876	KY941565	-
Cecropia obtusifolia Bertol.	Weiblen 2798	Panama	KY941803	KY941715	KY941877	KY941566	KY941636
Cecropia obtusifolia Bertol.	Weiblen 2800	Hawaii	KY941804	KY941716	KY941878	KY941567	KY941637
Cecropia pastasana Diels	Sagers 5071	Ecuador	KY941805	KY941717	KY941879	KY941568	KY941638
Cecropia pastasana Diels	Sagers 5074	Ecuador	KY941806	KY941718	KY941880	KY941569	KY941639
Cecropia peltata L	Treiber 7	Cundinamarca, CO	KY941807	KY941719	KY941881	KY941570	KY941640

Cecropia peltata L	Weiblen 1435	Panama	KY941808	KY941720	KY941882	KY941571	KY941641
Cecropia peltata L.	Zalamea 72	Tolima, CO	KY941810	KY941721	-	KY941572	-
Cecropia peltata L.	Torres 108	Cauca, CO	KY941809	KY941722	-	KY941573	-
Cecropia plicata Cuatrec.	Torres 104	Valle del Cauca, CO	KY941811	KY941723	-	KY941574	-
Cecropia plicata Cuatrec.	Torres 58	Antioquia, CO	KY941812	KY941724	-	KY941575	-
Cecropia putumayonis Cuatrec.	Barriga 2009-010	Ecuador	KY941813	KY941725	KY941883	KY941576	-
Cecropia putumayonis Cuatrec.	Barriga 2009-023	Ecuador	KY941814	KY941726	KY941884	KY941577	KY941642
Cecropia putumayonis Cuatrec.	Sagers 5089	Ecuador	KY941815	KY941727	KY941885	KY941578	-
Cecropia sararensis Cuatrec.	Torres 37	Vichada, CO	KY941816	KY941728	KY941886	KY941579	KY941643
Cecropia sciadophylla Mart	Torres 26	Amazonas, CO	KY941818	KY941729	KY941887	KY941580	KY941644
Cecropia sciadophylla Mart	Torres 30	Amazonas, CO	KY941819	KY941730	KY941888	KY941581	KY941645
Cecropia sciadophylla Mart	Torres 41	Vichada, CO	KY941820	KY941731	KY941889	KY941582	KY941646
Cecropia sciadophylla Mart	Zalamea 57	Meta, CO	-	KY941732	KY941890	KY941583	KY941647
Cecropia sciadophylla Mart.	Sagers 5090	Ecuador	KY941817	KY941733	-	KY941584	KY941648
Cecropia tacuna C.C.Berg &	Bevington 64	Peru	KY941821	KY941734	KY941891	KY941585	KY941649
Cecropia tacuna C.C.Berg &	Bevington 69	Peru	KY941822	KY941735	KY941892	KY941586	KY941650
Cecropia telealba Cuatrec.	Torres 101	Valle del Cauca, CO	KY941825	KY941736	-	KY941587	-
Cecropia telealba Cuatrec.	Torres 21	Quindío, CO	KY941824	KY941737	KY941894	KY941588	-
Cecropia telealba Cuatrec.	Treiber 23	Quindío, CO	KY941823	KY941738	KY941893	KY941588	KY941651
Cecropia telenitida Cuatrec.	Sagers 5075	Ecuador	KY941826	KY941739	KY941895	KY941590	KY941652
Coussapoa villosa	Sagers 5097	Ecuador	KY941827	KY941740	-	KY941591	KY941653
Pourouma bicolor	Sagers 5091	Ecuador	KY941828	KY941741	-	KY941592	KY941654
Pouruoma cecropiifolia	Zalamea 63	Meta, CO	KY941829	KY941742	-	KY941593	KY941655
Pourouma guianensis	Sagers 5092	Ecuador	KY941830	KY941743	KY941896	KY941594	KY941656

Table 2-2. Summary of RAD sequencing data for specimens included in phylogenetic analyses including: species, collection information and loci per sample for the largest data set (RADseq5) and the smallest (RADseq15) used for analyses.

Species	Collector & Number	Locality	RADseq5 loci per sample	RADseq15 loci per sample
Cecropia angustifolia Trécul	Torres 81	Antioquia, Colombia	37544	21342
Cecropia angustifolia Trécul	Treiber 01	Cundinamarca, Colombia	40237	27795
Cecropia angustifolia Trécul	Zalamea 48	Boyaca, Colombia	43298	32258
Cecropia engleriana Snethl.	Barriga & Alvia 2009-009	Ecuador	42982	32628
Cecropia ficifolia Warb. & Snethl.	Barriga & Bellota 2009-168	Peru	31185	15220
Cecropia gabrielis Cuatrec.	Treiber 26	Colombia	32078	15360
Cecropia glaziovii Snethl.	Gaglioti 156	Brazil	41835	33857
Cecropia goudotiana Trécul	Treiber 10	Colombia	41585	33136
Cecropia herthae Diels	Barriga 2009-091	Ecuador	45504	34634
Cecropia hispidissima Cuatrec.	Treiber 49	Colombia	37843	24837
Cecropia insignis Liebm.	Zalamea 70	Colombia	37880	23503
Cecropia latiloba Miq.	Barriga & Alvia 2009-052	Ecuador	41914	32670
Cecropia litoralis Snethl.	Barriga 2009039	Ecuador	41408	31403
Cecropia longipes Pittier	Torres 87	Colombia	22458	8282
Cecropia marginalis Cuatrec.	Barriga 2009-004	Ecuador	43015	32993
Cecropia membranacea Trécul	Barriga 2009-001	Ecuador	42802	33683
Cecropia membranacea Trécul	Torres 23	Amazonas, Colombia	38314	25225
Cecropia membranacea Trécul	Zalamea 54	Casanare, Colombia	41791	33076
Cecropia metensis Cuatrec.	Zalamea 52	Colombia	39019	26330
Cecropia mutisiana Mildbr. ex Cuatrec.	Zalamea 73	Colombia	43820	34298
Cecropia obtusa Trécul	Gaglioti 159	Brazil	39435	29640
Cecropia obtusifolia Bertol.	Barriga 2010-010	Costa Rica	33754	16605
Cecropia obtusifolia Bertol.	Treiber 02	Colombia	42079	30869

Cecropia obtusifolia Bertol.	Weiblen 2798	Panama	46780	34821
Cecropia peltata L.	Treiber 07	Colombia	38756	25076
Cecropia plicata Cuatrec.	Torres 104	Colombia	12857	1666
Cecropia purpurascens C.C.Berg	Gaglioti 174	Brazil	25829	9674
Cecropia putumayonis Cuatrec.	Barriga & Alvia 2009-010	Ecuador	42249	33083
Cecropia reticulata Cuatrec.	Torres 78	Colombia	37334	20260
Cecropia sararensis Cuatrec.	Torres 37	Colombia	17584	5315
Cecropia sciadophylla Mart.	Barriga & Alvia 2009-090	Ecuador	37446	25472
Cecropia sciadophylla Mart.	Gaglioti 124	Brazil	39467	27043
Cecropia sciadophylla Mart.	Torres 30	Amazonas, Colombia	39031	28311
Cecropia sciadophylla Mart.	Zalamea 57	Meta, Colombia	37438	23956
Cecropia tacuna C.C.Berg & P.Franco	Bevington 64	Peru	41549	33572
Cecropia telenitida Cuatrec.	Torres 69	Colombia	39686	26951
Coussapoa floccosa Akkermans & C.C.Berg	Gaglioti 104	Brazil	37446	8901
Musanga cecropioides R. Br. ex Tedlie	Cabezas 114	Guinea	39435	31477
Myrianthus arboreus P. Beauv	Kami 242	Republic of the Congo	21739	16227
Pourouma tomentosa Mart. ex Miq.	Gaglioti 139	Brazil	18124	6704

Table 2-3. The percentage of missing data and nodes with high support, number of species, samples, total loci, variable sites, and phylogenetically informative sites (pis) for matrices compiled using Sanger and RAD sequencing data. RADseq5 was the matrix assembled with an 82% clustering threshold. All loci were shared by at least 5 samples before and after clustering and RADseq15 had the same clustering threshold with all loci shared by at least 15 samples.

data set	% missing data	# species (ingroup)	# samples	total loci	variable sites	pis (w/ outgroup)	% nodes with high support (>0.95 pp and 95 bootstrap)
Sanger - complete	0	15	36	5	242	73	35
Sanger - sparse	33	29	91	5	326	185	9
RADseq5	40	27	37	61,022	817,026	299,910	89
RADseq15	34	27	37	36,789	469,717	161,718	94

Table 2-4. Numbers of loci in matrices, variable sites, and parsimony informative sites for runs with differing minimum depth for base calling (min. depth), the number of samples that must share a locus to be included (min. coverage), and the clustering threshold for matrix assembly for RADseq data.

run	min. depth	min. coverage	clustering threshold	# final loci	variable sites	phylogentically informative sites
1	5	5	0.82	61,022	817,026	299,910
2	5	5	0.90	62,876	705,144	254,199
3	5	5	0.98	71,410	185,696	53,117
4	15	15	0.82	36,789	469,717	161,718
5	15	15	0.90	36,863	420,430	146,160
6	15	15	0.98	24,371	95,409	26,561

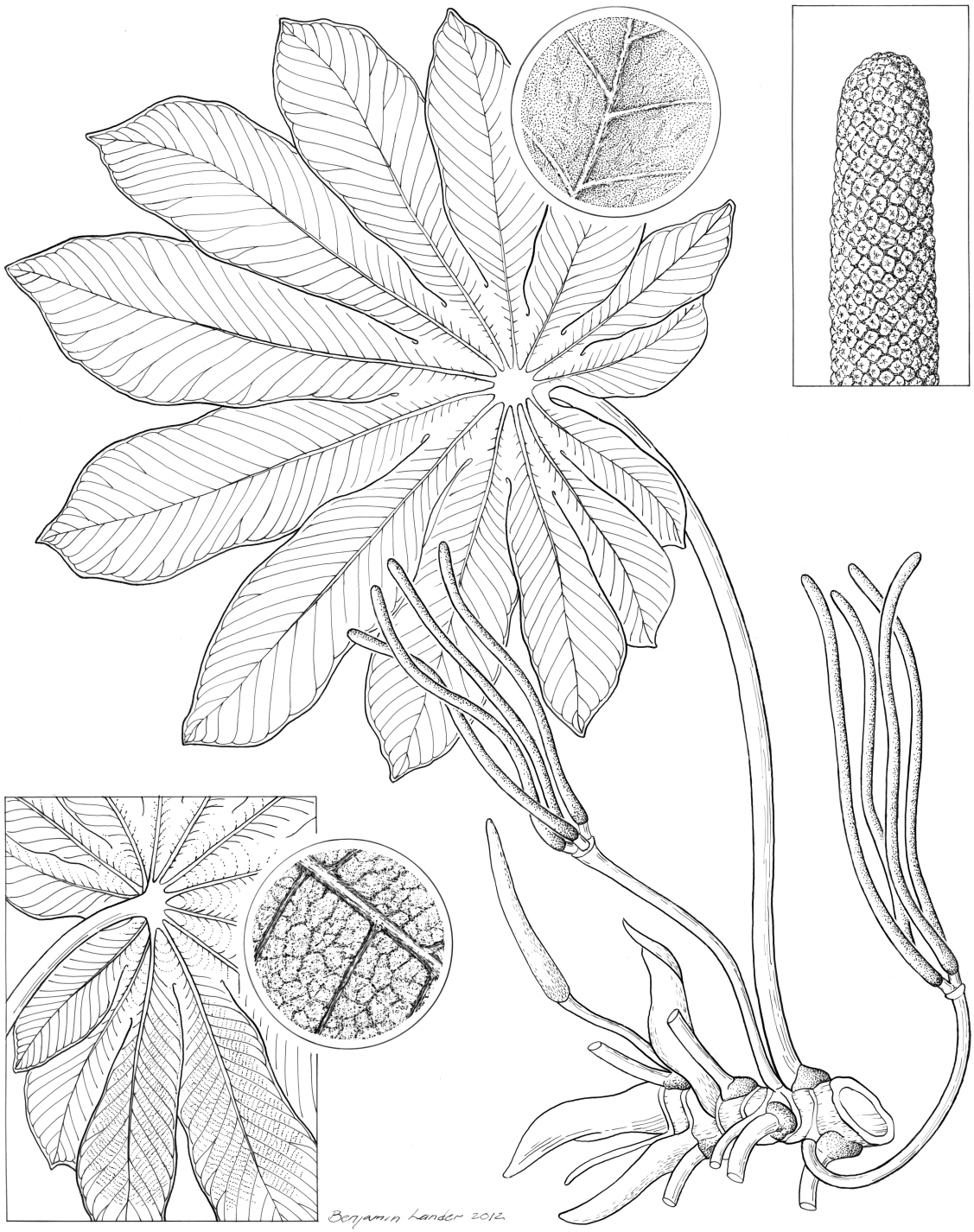

Figure 2-1. *Cecropia obtusifolia*: A. Leafy twig with stipule, pistillate inflorescences (0.25X) and close up of adaxial surface of leaf (40X). B. Pistillate inflorescence without spathe (4X). C. Detail of petiole attachment (0.5X) and close up of abaxial leaf surface (40X). [from Weiblen 2800, MIN #903982]

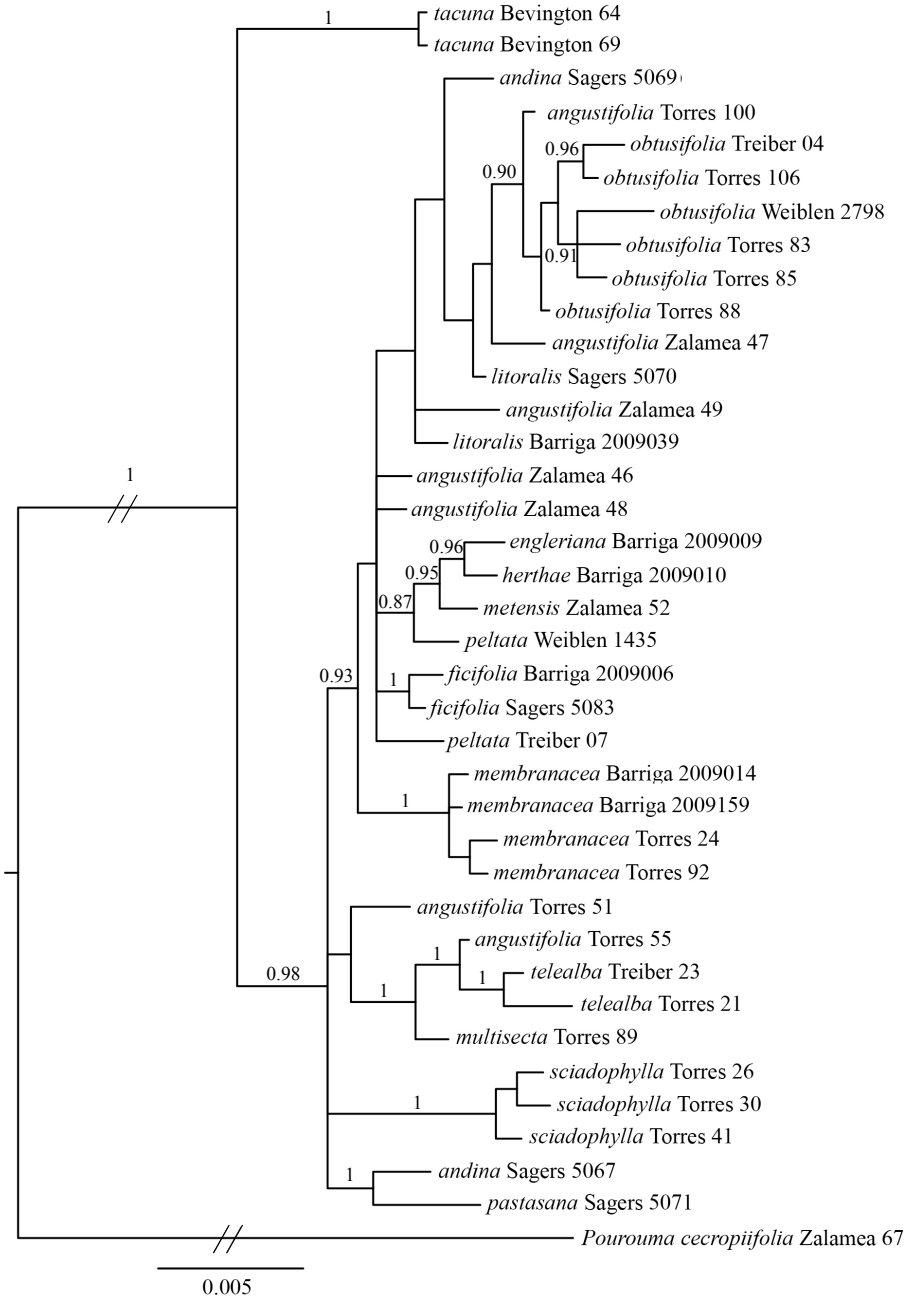

Figure 2-2. Bayesian (>50%) majority consensus rule tree for *Cecropia* based on the complete data set with no missing data. Close relative *Pourouma cecropiifolia* was used to root the tree. Posterior probabilities greater than 0.75 are noted on respective branches. The branches bearing double hatch marks are truncated and are not proportional to the rest.

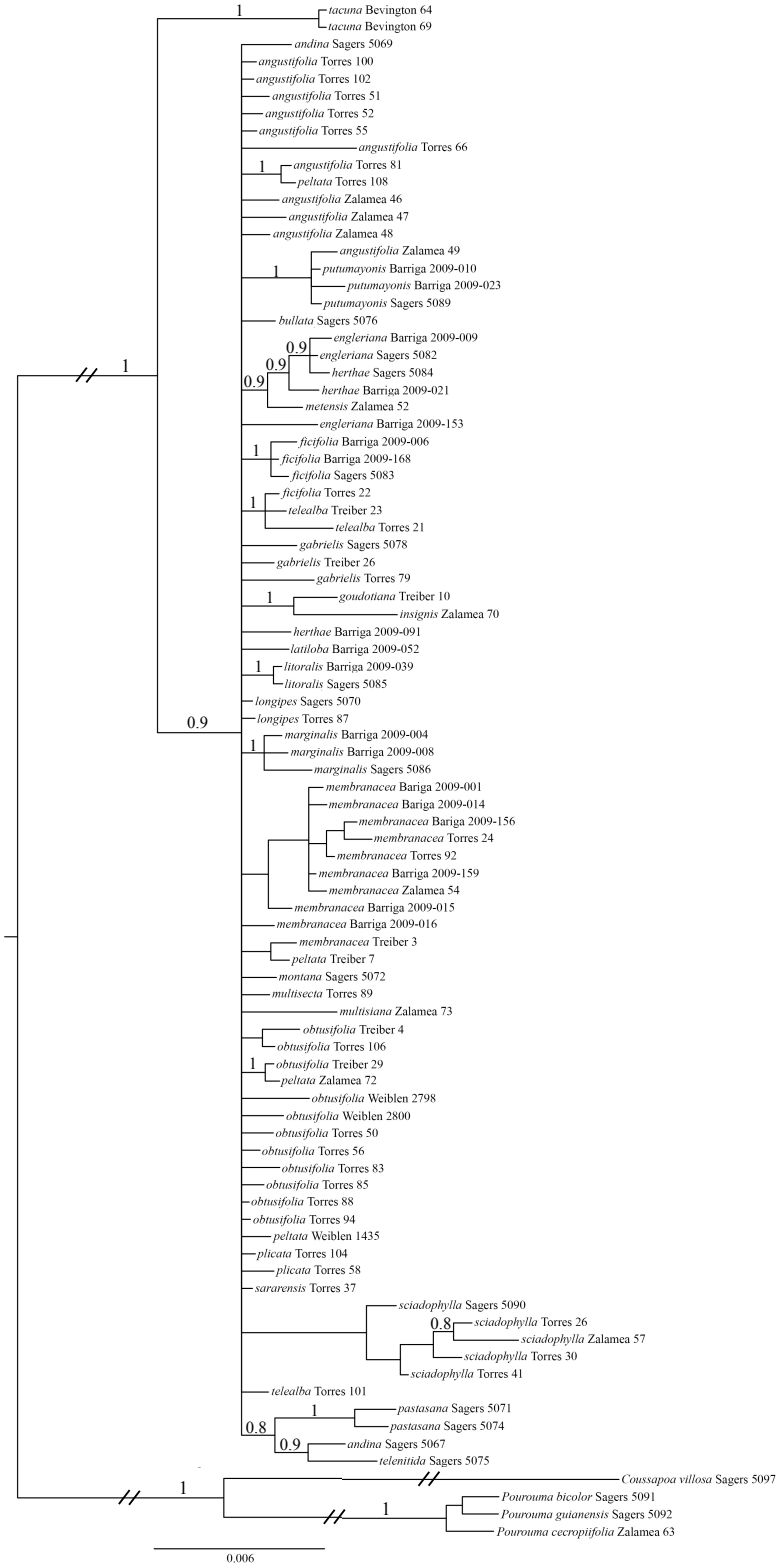
tacuna Bevington 64
tacuna Bevington 69
andina Sagers 5069
angustifolia Torres 100
angustifolia Torres 102
angustifolia Torres 51
angustifolia Torres 52
angustifolia Torres 55
angustifolia Torres 66
angustifolia Torres 81
peltata Torres 108
angustifolia Zalamea 46
angustifolia Zalamea 47
angustifolia Zalamea 48
angustifolia Zalamea 49
putumayonis Barriga 2009-010
putumayonis Barriga 2009-023
putumayonis Sagers 5089
bullata Sagers 5076
engleriana Barriga 2009-009
engleriana Sagers 5082
herthae Sagers 5084
herthae Barriga 2009-021
metensis Zalamea 52
engleriana Barriga 2009-153
ficifolia Barriga 2009-006
ficifolia Barriga 2009-168
ficifolia Sagers 5083
ficifolia Torres 22
telealba Treiber 23
telealba Torres 21
gabrielis Sagers 5078
gabrielis Treiber 26
gabrielis Torres 79
goudotiana Treiber 10
insignis Zalamea 70
herthae Barriga 2009-091
latiloba Barriga 2009-052
litoralis Barriga 2009-039
litoralis Sagers 5085
longipes Sagers 5070
longipes Torres 87
marginalis Barriga 2009-004
marginalis Barriga 2009-008
marginalis Sagers 5086
membranacea Bariga 2009-001
membranacea Bariga 2009-014
membranacea Bariga 2009-156
membranacea Torres 24
membranacea Torres 92
membranacea Barriga 2009-159
membranacea Zalamea 54
membranacea Barriga 2009-015
membranacea Barriga 2009-016
membranacea Treiber 3
peltata Treiber 7
montana Sagers 5072
multisecta Torres 89
multisiana Zalamea 73
obtusifolia Treiber 4
obtusifolia Torres 106
obtusifolia Treiber 29
peltata Zalamea 72
obtusifolia Weiblen 2798
obtusifolia Weiblen 2800
obtusifolia Torres 50
obtusifolia Torres 56
obtusifolia Torres 83
obtusifolia Torres 85
obtusifolia Torres 88
obtusifolia Torres 94
peltata Weiblen 1435
plicata Torres 104
plicata Torres 58
sararensis Torres 37
sciadophylla Sagers 5090
sciadophylla Torres 26
sciadophylla Zalamea 57
sciadophylla Torres 30
sciadophylla Torres 41
telealba Torres 101
pastasana Sagers 5071
pastasana Sagers 5074
andina Sagers 5067
telenitida Sagers 5075
Coussapoa villosa Sagers 5097
Pourouma bicolor Sagers 5091
Pourouma guianensis Sagers 5092
Pourouma cecropiifolia Zalamea 63
0.006

Figure 2-3. Bayesian (>50%) majority consensus rule tree for *Cecropia* based on the sparse Sanger sequence data set. Species of close relatives *Coussapoa* and *Pourouma* were used to root the tree. Posterior probabilities greater than 0.75 are noted on respective branches. The branches bearing double hatch marks are truncated and are not proportional to the rest.

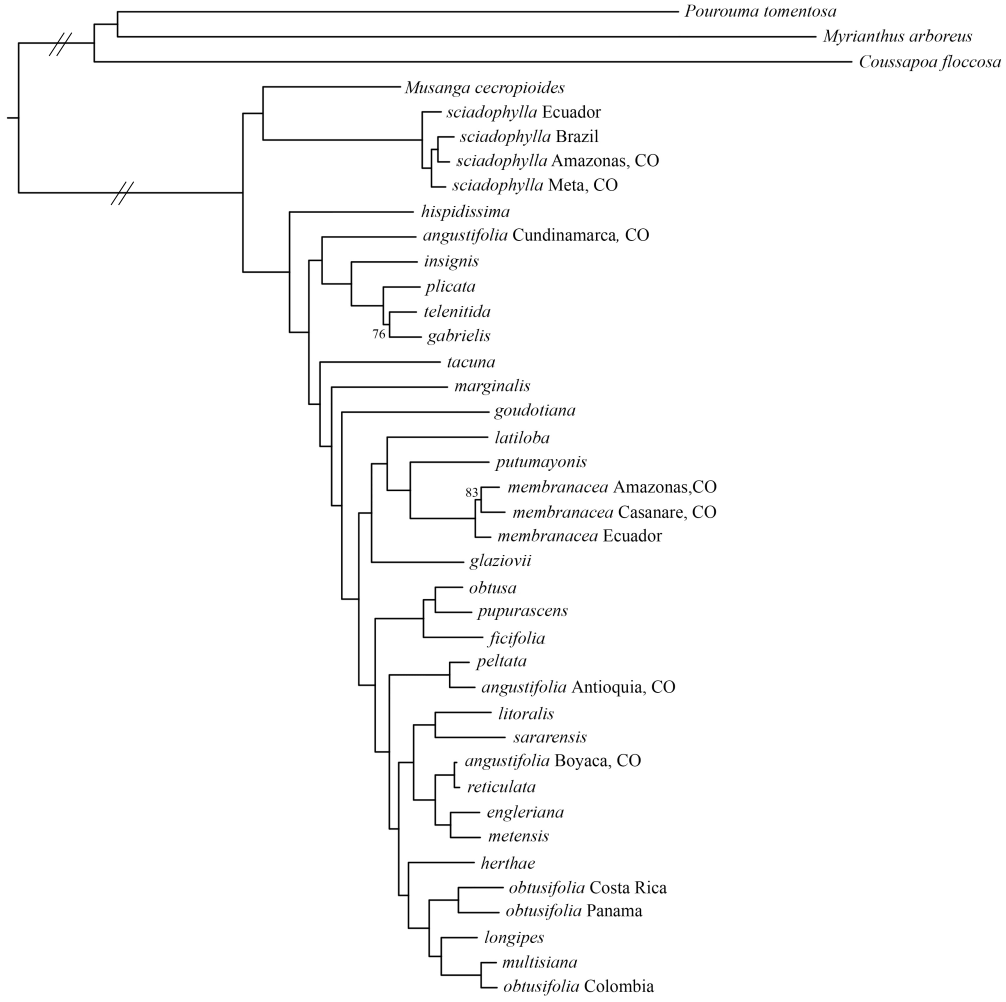
Pourouma tomentosa
Myrianthus arboreus
Coussapoa floccosa
Musanga cecropioides
sciadophylla Ecuador
sciadophylla Brazil
sciadophylla Amazonas, CO
sciadophylla Meta, CO
hispidissima
angustifolia Cundinamarca, CO
insignis
plicata
telenitida
76
gabrielis
tacuna
marginalis
goudotiana
latiloba
putumayonis
83
membranacea Amazonas,CO
membranacea Casanare, CO
membranacea Ecuador
glaziovii
obtusa
pupurascens
ficifolia
peltata
angustifolia Antioquia, CO
litoralis
sararensis
angustifolia Boyaca, CO
reticulata
engleriana
metensis
herthae
obtusifolia Costa Rica
obtusifolia Panama
longipes
multisiana
obtusifolia Colombia

Figure 2-4. Maximum likelihood phylogeny inferred from the concatenated RADseq data set with the highest minimum depth (15) and lowest clustering threshold (0.82). The phylogeny was rooted using other members of the Cecropieae tribe (*Coussapoa*, *Myrianthus*, and *Pourouma*). Bootstrap support was 100 except where indicated. The branches bearing double hatch marks have been truncated and are not proportional to the rest.

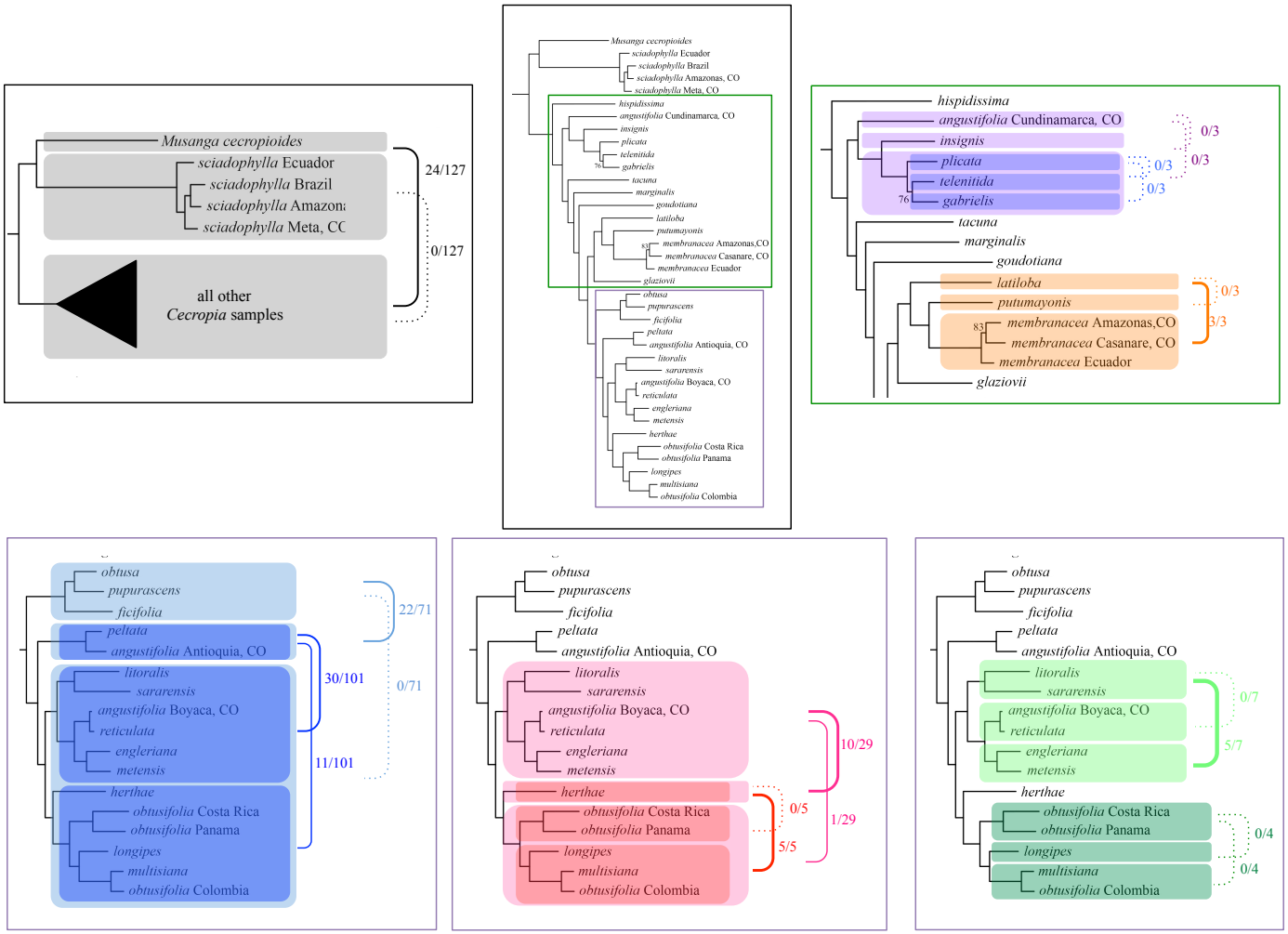
Musanga cecropioides
sciadophylla Ecuador
sciadophylla Brazil
sciadophylla Amazoni
sciadophylla Meta, CC
all other
Cecropia samples
24/127
0/127
Musanga cecropioides
sciadophylla Ecuador
sciadophylla Brazil
sciadophylla Amazonas, CO
sciadophylla Meta, CO
hispidissima
angustifolia Cundinamarca, CO
insignis
plicata
telenitida
gabrielis
tacuna
marginalis
goudotiana
latiloba
putumayonis
membranacea Amazonas,CO
membranacea Casanare, CO
membranacea Ecuador
glaziovii
obtusa
pupurascens
ficifolia
peltata
angustifolia Antioquia, CO
litoralis
sararensis
angustifolia Boyaca, CO
reticulata
engleriana
metensis
herthae
obtusifolia Costa Rica
obtusifolia Panama
longipes
multisiana
obtusifolia Colombia
hispidissima
angustifolia Cundinamarca, CO
insignis
plicata
telenitida
gabrielis
tacuna
marginalis
goudotiana
latiloba
putumayonis
membranacea Amazonas,CO
membranacea Casanare, CO
membranacea Ecuador
glaziovii
0/3
0/3
0/3
0/3
0/3
3/3
obtusa
pupurascens
ficifolia
peltata
angustifolia Antioquia, CO
litoralis
sararensis
angustifolia Boyaca, CO
reticulata
engleriana
metensis
herthae
obtusifolia Costa Rica
obtusifolia Panama
longipes
multisiana
obtusifolia Colombia
22/71
30/101
11/101
0/71
obtusa
pupurascens
ficifolia
peltata
angustifolia Antioquia, CO
litoralis
sararensis
angustifolia Boyaca, CO
reticulata
engleriana
metensis
herthae
obtusifolia Costa Rica
obtusifolia Panama
longipes
multisiana
obtusifolia Colombia
10/29
0/5
5/5
1/29
obtusa
pupurascens
ficifolia
peltata
angustifolia Antioquia, CO
litoralis
sararensis
angustifolia Boyaca, CO
reticulata
engleriana
metensis
herthae
obtusifolia Costa Rica
obtusifolia Panama
longipes
multisiana
obtusifolia Colombia
0/7
5/7
0/4
0/4

Figure 2-5. Results of D-statistic tests using RADseq SNP data for *Cecropia*. Shaded regions indicate clades in which species tested and colors represent different groups of P1, P2 and P3 for the different tests. Brackets indicate tests between P3 and P1 or P2 with solid lines for comparisions with significant results and dashed lines for comparisions with no significance. Brackets connecting P3 with P1 or P2 show the number of significant tests (after Bonferroni correction) out of the total for each group.

Chapter 3: Evolution of Myrmecophytism in *Cecropia* (Urticaceae)

INTRODUCTION

Mutualisms are ubiquitous; in fact, every species on earth is thought to be involved, indirectly or directly, in one or more partnerships where both participants benefit (Bronstein et al. 2004). Because mutualisms are prevalent in all systems, they can have a strong influence on the ecosystems where they occur. For example, plant associations with mycorrhizal fungi are important for nutrient cycling and maintaining species diversity (van der Heijden et al. 1998) and the function of some ocean systems depends on the association between coral and their photosynthetic dinoflagellate symbionts (Hay et al. 2004). Mutualisms can be extremely specialized, as with endosymbionts and their hosts, or more generalized, as with plants and their pollinators. Facultative interactions can range from general or transient interactions to long-term, specialized interactions. One example of a generalized association involves ants that visit plant extrafloral nectaries (EFN), structures secreting nectar outside of flowers. The multiple ant species and other insects that visit EFN (Bronstein et al. 2006) help protect plants from herbivores (Marazzi et al. 2013). In somewhat more specialized interactions, plants provide food rewards and/or hollow stems (domatia) for ant partners in exchange for protection from herbivory (Beattie 1985; Bronstein et al. 2006). The specificity of a mutualism is one factor that may affect the strength of the selective pressure on each partner, which in turn can affect the course of evolution in either partner. Characterizing how dynamic mutualisms have been in the past will allow for better predictions of how changes in the environment may affect mutualistic interactions in the future.

Ant-plant mutualisms are especially amenable study systems for investigating the dynamics of mutualism because the degree of specificity varies among species (Bronstein

1998). For example, ant-plant mutualisms have been used to demonstrate the reciprocal benefits of mutualism, how benefits vary with interaction strength, and what are the costs to participants (Bronstein 1998). It has been suggested that the extent of host specificity and the strength of interactions are likely to shape the evolution of ant-plant mutualisms (Davidson and McKey 1993a). Different levels of specialization, ranging from facultative to obligate, and the abundance of ant-plant mutualisms make them ideal systems for investigating evolutionary ecology questions.

Phylogenetic information has been used to examine ecological questions in several ant-plant mutualisms (Chomicki and Renner 2015). For example, ancestral state reconstruction was used to infer if stem texture and domatia in *Macaranga,* a genus that occupies similar habitats as *Cecropia* from Africa to Malesia, were associated with the origin of mutualistic ant associations (Quek et al. 2004). Another study found that myrmecophytism evolved multiple times in *Macaranga* and each time with a group of different morphological characteristics (Davies et al. 2001). Heil *et al.* (2009) used phylogeny to ask how investment-payoff regimes and exploiters may affect diversity in myrmecophytic species of *Acacia*. Phylogeny has also been used to estimate the time of the origin of the yucca-yucca moth mutualism (Pellmyr and Leebens-Mack 1999). These studies, and along with others, demonstrate that phylogenetic approaches are valuable for answering questions about the evolution of mutualism.

Cecropia, a genus of fast growing pioneer trees, is important in forest regeneration throughout Central and South America (Figure 3-1). *Cecropia* species are commonly described as myrmecophytic (having a symbiotic relationship with ants), but the history of this ant-plant mutualism is not well understood despite much speculation

about the evolution of *Cecropia* and associated ants including the genus *Azteca*. Ecological studies have examined many aspects of the mutualism including the effects of geographic factors on host preferences of *Azteca* ants (Vieira et al. 2010), the benefit of ants for *Cecropia* trees (Vasconcelos and Casimiro 1997; Agrawal and Dubin-Thaler 1999; Fáveri and Vasconcelos 2004), the importance on *Cecropia* food rewards for ants (Sagers et al. 2000), and the effect of ant protection of *Cecropia* (Longino 1989; Longino 1991a). These and other ecological studies have improved our understanding of how *Cecropia* trees and associated ants interact, yet they lack the phylogenetic information needed to gain insight into how the mutualism originated and how dynamic it has been over time.

Some traits of *Cecropia* and associated ants appear to be adaptations for housing mutualistic symbionts (Bailey 1922; Wheeler and Bequaert 1929). For instance, *Cecropia* produce hollow stems with partitioned cavities, known as domatia, which provide housing for nesting ants. *Cecropia* that do not associate with ants may lack domatia or have domatia filled with mucilage. *Cecropia* species have distinct weakened areas that appear as spots, or prostomata, in the wall of the internodes where colonizing queen ants first enter the domatia (Berg and Franco-Rosselli 2005) and in trees occupied by ants these appear as small holes along the stems. *Cecropia* trees produce two types of food bodies: Müllerian bodies are produced at the base of the petiole in a cluster of dense hairs (trichilia) and are consumed by inhabiting ants, while smaller pearl bodies, on abaxial leaf surfaces, provide some nutrition to mutualistic ants but mainly herbivores (Berg and Franco-Rosselli 2005; Dejean et al. 2012). For these food rewards, aggressive ants actively protect the trees from herbivores, encroaching vegetation, and deposit nitrogen-

rich debris (Janzen 1969; Longino 1991a; Vasconcelos and Casimiro 1997; Sagers et al. 2000; Bronstein et al. 2006) providing benefits back to the trees.

The association between *Cecropia* and nesting ants has been shown to be neither one-to-one nor obligate (Janzen 1973a; Fáveri and Vasconcelos 2004) and the associations may vary inter- and/or intra-specifically. For example, different individuals of a single *Cecropia* species may associate with different ant genera (Berg and Franco-Rosselli 2005). Some *Cecropia* lack ants because their elevational distribution extends above that in which ants are found (Brown 1973; Janzen 1973a). *Cecropia* associate most frequently with the genus *Azteca* (Berg and Franco-Rosselli 2005). Only twelve of more than 100 species of *Azteca* form associations with *Cecropia* (Bolton et al. 2007). *Azteca*, which are exclusively Neotropical, also commonly associate with other plants (Dejean et al. 2009; Pringle et al. 2012); however, ants of this genus often live in freestanding carton nests, which are formed by masticated wood mixed with honeydew (Berg and Franco-Rosselli 2005; Pringle et al. 2012). In addition to *Azteca*, *Cecropia* are known to associate with at least seven other species in four genera: *Pachycondyla*, *Camponotus*, *Crematogaster*, and *Pheidole* (Davidson and Fisher 1991; Davidson and McKey 1993b; Berg and Franco-Rosselli 2005).

The absence of phylogenetic information for *Cecropia* has hindered our ability to investigate the origin and evolutionary dynamics of the mutualism. A phylogenetic framework can be used to test evolutionary hypotheses on the origin of the mutualism and which plant characteristics may have been important in causing some ants to shift from the free-nesting habit to nesting in *Cecropia* stems. *Cecropia* also presents the opportunity to investigate whether the appearance of food rewards or nesting cavities in

Cecropia was associated with ancient colonization of *Cecropia* by ants. A recent study investigated these questions in *Cecropia* but the phylogenetic analysis combined sequence data from multiple individuals per species and included a small set of commonly used gene regions (Gutierrez-Valencia et al. 2017). We showed in Chapter 2 that is a questionable approach given problems with the *Cecropia* species concept and the insufficient variation of conventional plant phylogenetic markers in *Cecropia*.

In this chapter we examine the evolution of mutualism in *Cecropia* with a highly supported phylogeny based on a much larger sequence dataset than that of Gutierrez-Valencia et al. (2017). The RAD-seq phylogeny presented in Chapter 2, including nearly half of the genus, was used to examine the origin and loss of myrmecophytism in *Cecropia*. First, we aimed to infer whether myrmecophytism had a single origin in *Cecropia* followed by multiple losses or if there were multiple independent origins. We also inferred the ancestral states of ant traits including trichilia and domatia to see how their evolutionary history is correlated with that of myrmecophytism. In addition, we compared the elevational distributions of myrmecophytic and non-myrmecophytic species to examine whether inferred evolutionary losses of ant associations are related to high montane environments.

MATERIALS AND METHODS

Taxonomy – Recent molecular phylogenetic studies support the genus *Musanga* embedded in the *Cecropia* clade (Chapter 1; Chapter 2). *Musanga* is sister to *C. sciadophylla* and there is a deep split between this small clade and the remaining *Cecropia* species we sampled (Chapter 2). More complete taxon sampling will be

necessary to determine if some species currently in *Cecropia* need to move to the genus *Musanga* or if *Musanga* should be merged into *Cecropia*. In this chapter, however, we will refer to *Cecropia* including *Musanga* as *Cecropia* s.l. and the clade with the remaining species as *Cecropia* s.s. (Chapter 1).

Character matrix – We examined ant association and plant characters that are likely associated with this mutualism to create a matrix of discrete characters. Ant associations were scored as absent (0) or present (1). In addition, the matrix included characters for trichilia and domatia. Due to the variable nature of the trichilia among species and how discrete characters are coded, we included two different coding sets for this character. In the first, if a species had trichilia present even some of the time it was coded as present (1) rather than absent (0). For the second set, if a species had trichilia lacking in some individualss it was coded as absent rather than present. Because of the variability of trichilia within species, characters were not ordered. Domatia were coded as absent (0) or present (1). Character states designations were mainly based on Berg and Franco-Rosselli (2005) and data from specimen collections (Chapter 2). Additional sources were used to confirm ant associations, trichilia, and domatia (Longino 1989; Berg et al. 1990; Agrawal 1998; Dejean et al. 2012; Latteman et al. 2014).

To investigate ancestral history of elevation, which could play a role in ant associations, we recorded elevation information for each sample from the phylogeny in a character matrix. To get a sense of how our collection elevation compared to a sample of *Cecropia* collections, we generate frequency distributions from data for collections downloaded from the Global Biodiversity Index Facility (GBIF) (gbif.com) for four

widespread species (*C. angustifolia*, *C. membranacea*, *C. obtusifolia*, and *C. sciadophylla*).

Ultrametric tree - We used the phylogeny from Chapter 2 for all analyses. To convert the tree to ultrametric for reconstructions, we used three different smoothing algorithms from the *APE* package in R Studio v1.0.136 (RStudio Team 2016). There were multiple samples of some species in the phylogeny, so we removed duplicate samples from the phylogeny before analyses. Because all samples of *C. sciadophylla* and *C. membranacea* were in the same clade we removed all but one sample of each. However, *C. obtusifolia* and *C. angustifolia* samples were in different parts of the tree, so only samples that clustered together were removed. We also randomly removed two of the outgroup samples. Using the *chronos* function, all analyses were calibrated using pollen data with a minimum root age of 65 MYA (Burnham and Johnson 2004) and the node for *Coussapoa* with a minimum age of 8 MYA and a maximum age of 65 MYA (Burnham and Graham 1999). The three smoothing algorithms used were a strict clock, a relaxed clock, and penalized likelihood. The lambda value was also varied from each model from 0-1.5 to determine the best fit. The ultrametric tree with the highest likelihood was used for subsequent analyses.

Ancestral reconstruction –Three discrete traits were reconstructed to investigate ancestral states of ant association and ant related traits (domatia and trichilia). The maximum likelihood reconstructions were done using the *GEIGER* (Harmon et al. 2008) and *PHYTOOLS* v0.4-56 (Revell 2012) packages in RStudio. Reconstructions for each trait (and variation) were performed using three models of trait evolution: equal rates (ER), all rates different (ARD), and symmetrical (SYM). Likelihood ratio tests were used to

compare models. Ancestral reconstructions of the continuous variable elevation were done using Maximum likelihood in *PHYTOOLS*.

Correlated trait evolution – We performed correlation analyses between traits in Mesquite v3.2 (Maddison and Maddison 2011; 2017). The program uses the method of Pagel (1994), which estimates the rates of change of discrete characters based on branch lengths, but is not dependent of the reconstruction of the characters. Two models are tested: one with characters evolving independently and one where character evolution is correlated. A likelihood ratio tests statistic is used to compare the models and a p-value is obtained (Pagel 1994). We tested whether evolutionary gains and losses of ant association were correlated with gains and losses of domatia or trichilia. We examined whether evolutionary shifts in plant traits were dependent on changes in ant association or vice versa. In the case of trichilia, two alternative codings of character states were compared to explore the sensitivity of results to different assumptions about the distribution of the character in polymorphic species.

RESULTS

Ancestral Reconstructions – Ancestral state reconstructions are reported for models assuming equal, symmetrical, and variable rates (Table 3-1). We were unable to reject the simplest model assuming equal rates of change for the three characters. Reconstructing ant associations (Figure 3-2) had an equal probability of the ancestor being either myrmecophytic or non-myrmecophytic but the ancestor of the *C. sciadophylla*/*Musanga* clade had higher probability of being non-myrmecophytic. In the sister group to *C.*

sciadophylla/*Musanga*, hereafter "core *Cecropia*", the probability of a myrmecophytic ancestor was 0.72 (Figure 3-2). The probability of ancestral myrmecophytism was generally high in core *Cecropia* ancestor except near where non-myrmecophytic species were located in the phylogeny. The sister group to *C. tacuna*, including many recently diverged species, most probably had a myrmecophytic ancestor. An alternative coding of *C. sciadophylla* as myrmecophytic resulted in a similar pattern, but with slightly lower probabilities of myrmecophytism in the common ancestor of core *Cecropia*.

The two different codings of states for trichilia resulted in similar patterns, but as expected, with lower probabilities of trichilia in the most recent common ancestors of the polymorphic species *C. tacuna* and *C. gabrielis* (Figure 3-3). *Cecropia s.l.* (including *Musanga*) had equal probability of ancestral trichilia presence or absence. When trichilia were coded as present in polymorphic species, the probability of the *C. sciadophylla*/*Musanga* ancestor having trichilia was 0.44 and decreased to 0.15 when coded as absent (Figure 3-3). The ancestor of core *Cecropia* most probably had trichilia (0.60) and probability increased to 0.91 when coded as present in polymorphic species. The probability of ancestral nodes having trichilia in core *Cecropia* was even higher and reached absolute probability in the large sister group to *C. tacuna* (Figure 3-3).

Ancestral state reconstruction of domatia was the only trait with unequal probability of presence or absence in the common ancestor of *Cecropia* s.l. (Figure 3-4). The probability of domatia present at the root of the clade was 0.67 while for the common ancestor of the *C. sciadophylla/Musanga* clade it was 0.57 (Figure 3-4). Domatia followed a similar pattern in core *Cecropia* as the other characters with the

probability of ancestral presence being absolute or nearly so at most nodes, with C. gabrielis being the only member of the core clade that lacks domatia (Figure 3-4).

Ancestral state reconstruction of elevation based on specimen localities suggests that the ancestor of *Cecropia* s.l. may have occupied middle elevations (Figure 3-5). Within core *Cecropia* there appeared to be a clade of higher elevation specialists including *C. telenitida*, *C. gabrielis* and relatives. The other high elevation specialist, *C. tacuna*, was sister to a large clade of mostly lowland species (Figure 3-5). A similar pattern was observed when maximum elevation from species counts was used instead of specimen location (Figure 3-6). Altitudinal distributions based on herbarium records of *C. membranacea*, *C. obtusifolia*, and *C. sciadophylla* show that these species occur primarly in the lowlands between 0 and 500 meters (Figure 3-7) whereas *C. angustifolia* showed a bimodal distribution with a large number of collections between 1150 and 2000 meters (Figure 3-7).

Correlated trait evolution – Evolutionary gains or losses of ant association were positively correlated with myrmecophytic traits, trichilia and domatia (Table 3-2) and alternative coding of trichilia in polymorphic species yielded the same result. Gains or losses of ant association were dependent on the state of trichilia while trichilia were independent of ant associations. In the case of domatia, changes in the plant trait were dependent on gains or losses of ants while ant associations were independent of this trait (Table 3-2).

DISCUSSION

Ancestral states for myrmecophytism and associated plant traits in the common ancestor of *Cecropia* are uncertain due to the deep divergence of the *C. sciadophylla*/*Musanga* clade from *Cecropia* s.s. Character state reconstructions favored the absence of ant associations and trichilia in the ancestor of *C. sciadophylla*/*Musanga* and the origin of myrmecophytism, trichilia, and domatia in the common ancestor of *Cecropia* s.s. It is not surprising the ancestor of the *C. sciadophylla/* clade was most probably non-myrmecophytic since ants and trichilia are absent in both species while nearly all of *Cecropia* s.s. have ants and trichilia. Domatia were found to be most probably present in the common ancestor of *Cecropia* s.l., a finding that can be attributed to the presence of the trait in *C. sciadophylla*. However, it remains unclear from our analysis where exactly myrmecophytism originated. There was either a single origin in the common ancestor of *Cecropia* s.l. and at least three losses of myrmecophytism or it originated in *Cecropia* s.s. and was lost at least twice in this lineage. In any event, evolutionary changes in ant associations, trichilia, and domatia were significantly correlated owing to repeated losses of myrmecophytism.

Coding of myrmecophytism as a binary character is challenging because ant associations can vary intraspecifically as well as interspecifically. For example, species such as *C. angustifolia*, have ranges extending to 2000-2400 meters, above the habitable zone for ants, and may thereofer lack ant associations in part of their alititudinal range (Janzen 1973a; Janzen 1973b). Ant associates also vary within species such as *C. hispidissima* that usually but not exclusively host *Pachycondala* rather than *Azteca*. It is also commonly observed that species associated with *Azteca* may be inhabited by other ant genera (Wheeler 1942, Treiber, personal observation). Understanding this variability

and its causes requires intensive sampling throughout the geographic range of many species and experimental work that was beyond the scope of our comparative study. Our simple coding of ant associations as either present or absent therefore provides limited insight on the evolution of myrmecophytism.

Domatia were inferred as most probably present in the common ancestor of *Cecropia* s.l. because of the phylogenetic position of *C. sciadophylla*, which lacks ant associations and trichilia but has domatia. The only other species lacking domatia in our sample, *C. gabrielis*, was embedded in *Cecropia* s.s. There are seven species of *Cecropia* s.l. that have either entirely or partially filled stem pith (11% of the genus) and our study included a slightly lower percentage of these taxa that lack domatia (7%) so it is possible that we overestimated the frequency of ancestral domatia (Brown 1973; Janzen 1973a; Berg and Franco-Rosselli 2005).

Ancestral reconstruction of maximum elevation supports the idea that the genus was historically limited to middle altitudes and that high elevation specialists have evolved repeatedly. Upper montane specialists correspond to clades of *Cecropia* s.s. that include non-myrmecophytic species such as *C. gabrielis* and *C. tacuna*. Ccomparing the altitudinal distributions of *C. angustifolia, C. membranacea*, *C. obtusifolia*, and *C. sciadophylla* based on herbarium records suggests that three of these four species are most abundant at low elevations below 500 meters whereas *C. angustifolia* was abundant either below 500 meters or above 1,200 meters. Assuming equal collecting effort with respect to altitude, the disjunct distribution of *C. angustifolia* along with its lack of monophyly according to RAD seq (Chapter 2) supports the need for a revaluation of the *Cecropia* species concept.

Sampling approximately half of the recognized species in the genus, we closely approximated the genus-wide ratio of character states for ant association and trichilia (~16%). However, we recognize that inferrences of ancestral states could change depending on how the species we did not sample are related to those included in our analysis. For instance, if even a few myrmecophytic species joined the *Musanga*/*C. sciadophylla* clade, we would most probably infer a myrmecophytic ancestor for the entire clade. At present, it appears that myrmecophytism evolved once and was lost several times. Based on morphology we predict that at least some antless species, such as *C. holeoluca*, which lack trichilia and is the only *Cecropia* s.l. other than *Musanga* to lack a spathe enclosing the emerging inflorescence (Chapter 1;Wheeler 1942; Berg and Franco-Rosselli 2005), belong to the group with *C. sciadophylla*/*Musanga*. Gutierrez-Valencia et al. (2017) suggested that *C. sciadophylla* and *C. hololeuca* are closely related but the relationship was not highly supported. If *C. hololeuca* is indeed a member of the *C. sciadophylla*/*Musanga* clade, it would strengthen the case for myrmecophytism to have evolved in the sister group, *Cecropia* s.s. Although further study of *Cecropia* is needed, at least we have established a major ancient split in the group with intriguing patterns of ant trait varation between the two clades.

Correlations suggest that ant associations might depend on the evolution of trichilia and this result was consistent with patterns of inferred ancestral states in some parts of the phylogeny but not always. For example, the common ancestor of *Cecropia* s.s. had a higher probably of trichilia than of ant association, suggesting that trichilia might have evolved before ants were acquired. On the otherhand, when trichilia were

coded as absent in the variable species, *C. telenitida* and *C. gabrielis*, the loss of trichilia was more highly probable in their common ancestor than was the loss of ant association.

All of the species that we sampled are either entirely or occassionally myrmecophytic and have trichilia. There are also few reports of ant associations in *Cecropia* that invariably lack trichilia. *Cecropia telenitida* ranges from complete absence of trichilia in some populations to having fully formed trichilia in others and its altitudinal distribution (1400-2600 m) also extends beyond the maximum elevation of the associated ants, suggesting that the trait might be plastic enough to respond to the presence or absence of ants (Janzen 1973a; Berg and Franco-Rosselli 2005). Davies et al. (2001) found in *Macaranga* that myrmecophytism and the aggregation of food bodies coincided in all lineages of the genus, although evolutionary correlations were not tested explicitly. *Cecropia* and its close relatives (*Coussapoa*, *Myrianthus*, and *Pourouma*) all produce food bodies on the foilage (pearl bodies), and so shifting to denser aggregations in the form of trichilia is might be relatively simple (Berg and Franco-Rosselli 2005). It has been suggested that the aggregation of food bodies into trichillia along with changes in chemical composition might reduce visitation by nonspecific ants and thereby favor myrmecophytism (Davidson and McKey 1993b; Davies et al. 2001).

Cecropia peltata, a widespread species, is known to have trichilia in environments where ants are present but is known to lack them on islands where mutualistic *Azteca* are absent (Janzen 1973a; Rickson 1977; Putz 1988). Such observations lend weight to the idea that trichilia could be induced or maintained in the presence of mutualists and rapidly lost in colonists of environments lacking ants such as the upper montate or remote islands.

Correlation analysis suggested that the evolution of domatia depended on the changes in ant association and this result was consistent with patterns of inferred gains of ancestral states but not losses. Ant associations were lost in lineages that retained domatia. The correlation does not take account the ancestral reconstruction, but if the presence of domatia is dependent on the presence of ant association this would suggest that the ancestor of *Cecropia* was myrmecophytic. It could be that domatia were developed early in the evolution of *Cecropia* and if they are low in cost for the plant they have been maintained even with the loss of ant associations in some lineages. *C. tacuna*, a non-myrmecophytic species, has domatia but they are often flooded with mucilage and uninhabitable. In contrast, another study found that domatia evolved several times in *Cecropia* (Chomicki and Renner 2015). This study only included nine species of *Cecropia* and was based on few genes so the phylogeny was not well resolved and *Cecropia* and *Musanga* were not monophyletic (Chomicki and Renner 2015). The under-sampling of *Cecropia* may be the cause for the discrepancy. Our reconstructionsAlthough there have been few studies focused on the evolution of domatia, the same study supported the evolution of domatia in response to the presence of ants. In the study, they found a three-fold higher number of domatia gains than plant-ant origins which implied that the recruitment of myrmecophytic lineages to non-myrmecophytic lineages is followed the evolution of domatia in response (Chomicki and Renner 2015).

To better understand the evolution of myrmecophytism in *Cecropia*, population genetic and phylogenetic studies of the associated ants are needed. *Cecropia* mainly associate with the genus *Azteca*, which has not received broad molecular phylogenetic study and morphological species are thought to be problematic because of conflicting and

homoplasious character states (Ayala et al. 1996). The only phylogenetic analysis of *Azteca* included only eight myrmecophytic lineages and was based on one gene region. These preliminary finding suggested that *Cecropia*-inhabiting *Azteca* were not a monophyletic group (Ayala et al. 1996). *Azteca* species build nests differently in *Cecropia*. For example, some species build spindle-shaped carton nests around the trunks of the trees that deform the trunk at this location are numerous exit holes are observed from domatia in the vicinity of the nest (Longino 1991a; Longino 1991b; Ayala et al. 1996). Other *Azteca* create a cylindrical carton nest that does not deform the trunk and exit holes from domatia are located at at distance from the carton (Longino 1991a; Longino 1991b; Ayala et al. 1996). Gaining better insight into the evolutionary history of myrmecophytism in *Cecropia* requres a deeper investigation of the ecology and systematics of the associated ants.

Phylogeny based on RADseq data together with ancestral reconstruction of ant mutualism raises the question of whether an increased rate of species diversification was associated with the origin of myrmecophytism in *Cecropia*. A long branch and low number species in the *C. sciadophylla*/*Musanga* clade compared to short branch lengths and many species in *Cecropia* s.s. suggest that myrmecophytism may be associated with an increased species diversification rate in this clade. In a large-scale comparative study, Weber and Agrawal (2014) found evidence that ant defensive mutualisms were associated with enhanced plant diversification. However, diversification hypothesis can be difficult to test (Weiblen and Treiber 2015) and current methods (i.e. BiSSE) require large sample sets (>50) and even transitions to alternate character states that can be

limiting in small groups (Davis et al. 2013). Even with a larger and more complete sampling of *Cecropia* there may be insufficient statistical power to test this hypothesis.

This study presents a prelimary examination on the origin and loss of myrmecophytism in *Cecropia* in an evolutionary context based on the first highly supported phylogenetic hypothesis in the genus. Our analysis supported a deep split between *C. sciadophylla*/*Musanga* and *Cecropia* s.s. (Chapter 1) and suggested a potential deep split between myrmecophytic and non- myrmecophytic lineages. This possibility reiterates the need for more molecular ecological and taxonomic work on this group. Although we were unable to determine the state of myrmecophytism or in the ancestor of *Cecropia*, our results suggest a non-myrmecophytic ancestor for the *C. sciadophylla*/*Musanga* clade and a myrmecophytic ancestor of *Cecropia* s.s. We also found that trichilia and domatia are correlated with the evolution of the mutualism but potentially in different ways. Correlation analyses suggest that the evolution of ant associations was dependent on the presence of trichilia, and conversely although rather weakly, that the presence of domatia was dependent on the presence of ant associations but these findings are not entirely consistent with patterns of ancestral state reconstuction. In any event, our work illustrates how evolutionary perspectives can inform ecological interpretations and comparisions of ant plant mutualisms.

Table 3-1. Likelihoods for models of evolutionary change in *Cecropia* ant associations, trichilia, and domatia. Models assumed either equal rates (ER), unequal rates (UR), or symmetrical rates (SYM). Trichilia was coded in two ways, either assuming absence (AP0) or presence (AP1) in polymorphic speces. Likelihood ratio tests comparing models were not significant.

character	model	likelihood
ant association	ER	-9.296191
	UR	-9.096795
	SYM	-9.206191
trichilia AP0	ER	-5.472954
	UR	-5.299712
	SYM	-5.472954
trichilia AP1	ER	-8.928169
	UR	-8.924125
	SYM	-8.928169
domatia	ER	-7.908495
	UR	-7.790011
	SYM	-7.908495

Table 3-2. Evolutionary correlations (Pagel 1994) between ant association, trichilia and domatia. In addition to omnibus tests for overall correlation, we tested whether changes in one trait were dependent on the state of another. Non-sigificance is indicated as *n.s.*

X	Y	direction	difference in log likelihood	p-value
ant association	trichilia AP0	omnibus	7.791	<0.001
		X depends on Y	2.639	<0.01
		Y depends on X	2.028	*n.s.*
ant association	trichilia AP1	omnibus	5.909	<0.001
		X depends on Y	3.053	<0.01
		Y depends on X	1.536	*n.s.*
ant association	domatia	omnibus	4.683	<0.01
		X depends on Y	0.935	*n.s.*
		Y depends on X	2.134	<0.05

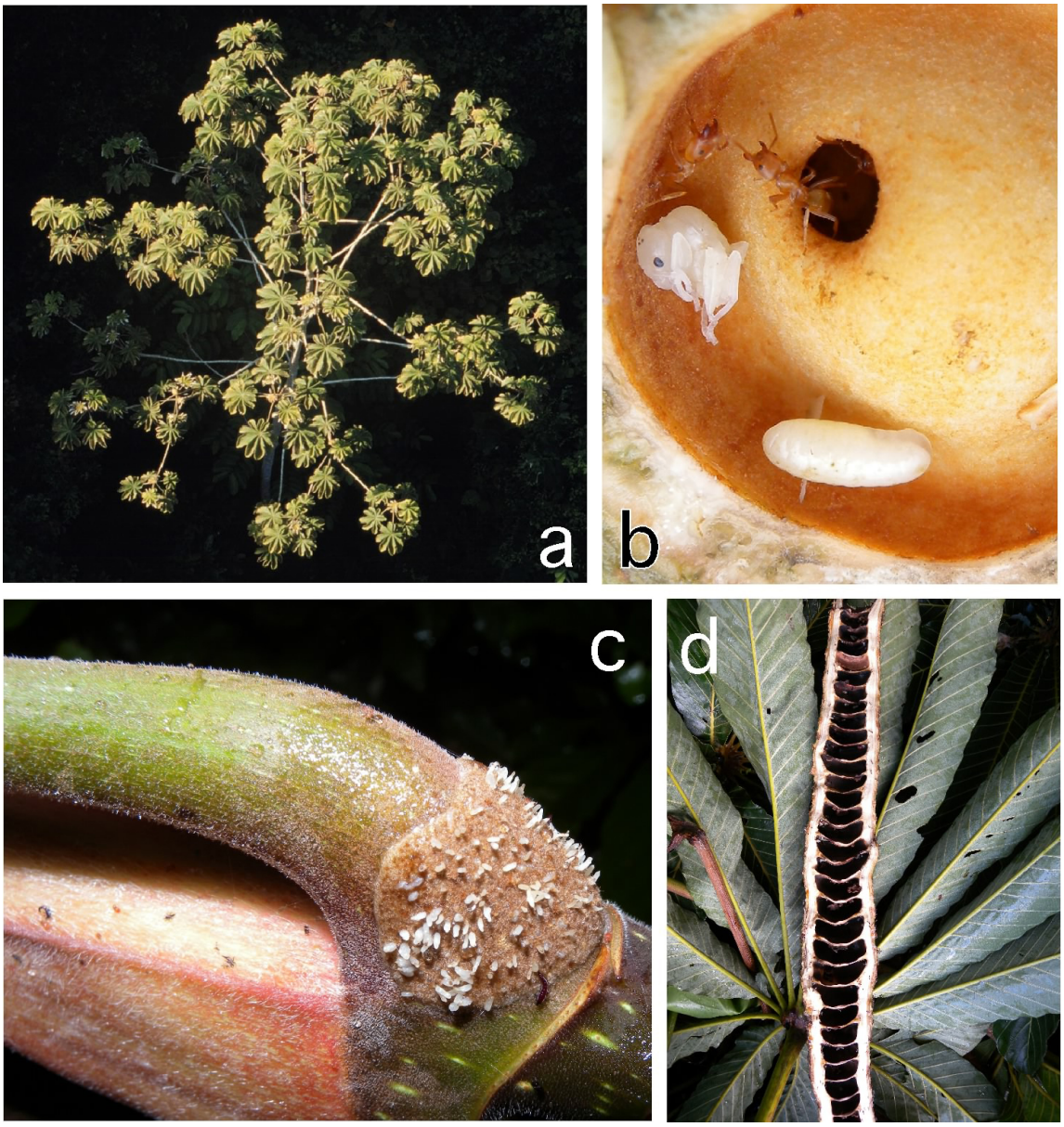

Figure 3-1. The *Cecropia*-ant mutualism. (a) Canopy architecture of *Cecropia peltata* (b) Müllerian bodies produced from glands at the base of a *Cecropia* petiole provide food for ants. (c) Cross-section of a *Cecropia* stem with domatia with *Azteca* larva, pupa and adults. (d) *Cecropia sciadophylla* leaf and stem in longitudinal section. Photos: G. D. Weiblen

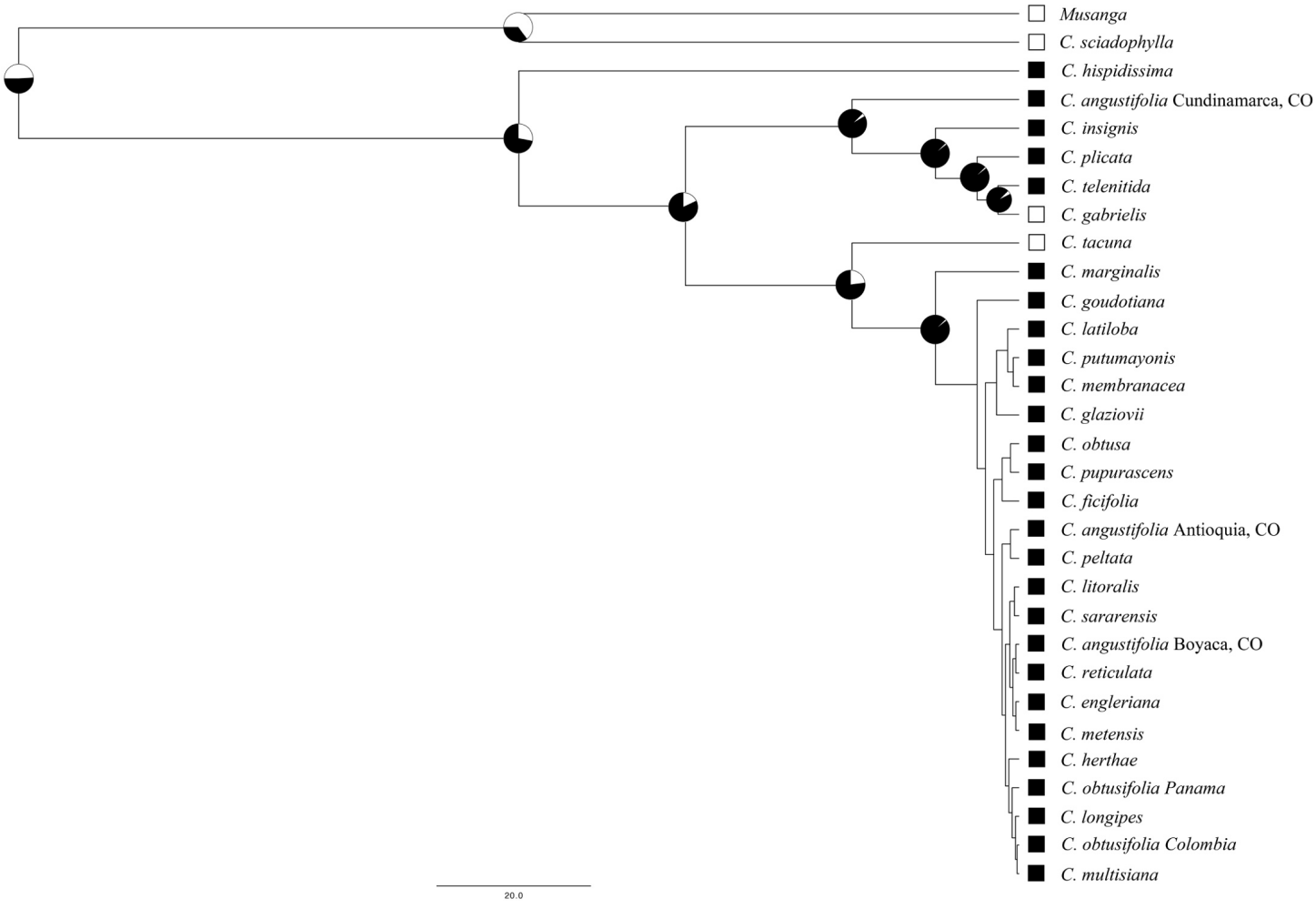
Musanga
C. sciadophylla
C. hispidissima
C. angustifolia Cundinamarca, CO
C. insignis
C. plicata
C. telenitida
C. gabrielis
C. tacuna
C. marginalis
C. goudotiana
C. latiloba
C. putumayonis
C. membranacea
C. glaziovii
C. obtusa
C. pupurascens
C. ficifolia
C. angustifolia Antioquia, CO
C. peltata
C. litoralis
C. sararensis
C. angustifolia Boyaca, CO
C. reticulata
C. engleriana
C. metensis
C. herthae
C. obtusifolia Panama
C. longipes
C. obtusifolia Colombia
C. multisiana
20.0

Figure 3-2. Maximum likelihood ancestral state reconstruction of ant associations in *Cecropia* based on an equal rates model of evolutionary transitions. Pie charts at nodes represent the probability of that ancestor lacking ant associations (white) or with ant associations present (black). Nodes without circles had absolute probability of myrmecophytism. The squares at the tips of the branches indicate the state coded for species in the same format as above. Non- myrmecophytic *Coussapoa* (not shown) was the outgroup.

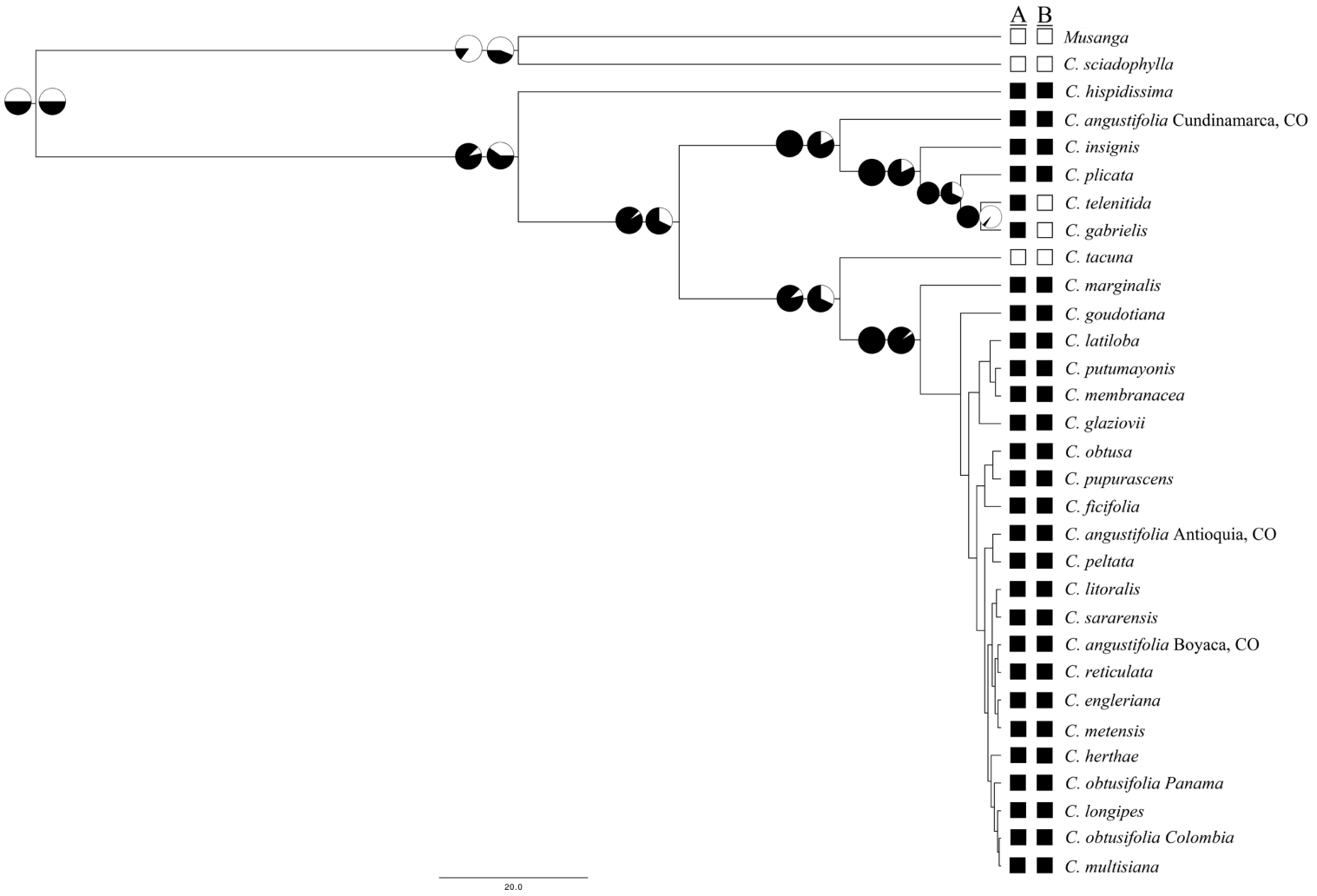
A
B
Musanga
C. sciadophylla
C. hispidissima
C. angustifolia Cundinamarca, CO
C. insignis
C. plicata
C. telenitida
C. gabrielis
C. tacuna
C. marginalis
C. goudotiana
C. latiloba
C. putumayonis
C. membranacea
C. glaziovii
C. obtusa
C. pupurascens
C. ficifolia
C. angustifolia Antioquia, CO
C. peltata
C. litoralis
C. sararensis
C. angustifolia Boyaca, CO
C. reticulata
C. engleriana
C. metensis
C. herthae
C. obtusifolia Panama
C. longipes
C. obtusifolia Colombia
C. multisiana
20.0

Figure 3-3. Maximum likelihood ancestral state reconstruction for trichilia in *Cecropia* using an equal rates model of evolutionary transitions. Pie charts at nodes represent the likelihood of the ancestor lacking trichilia (white) or having trichilia present (black). The circle on the right is the likelihood when states were coded as in column A and the circles on the right when states coded as in column B. Column A represents coding of trichilia presence in polymorphic species and column B represents coding favoring absence. *Coussapoa* (not shown) lacks trichillia and was the outgroup.

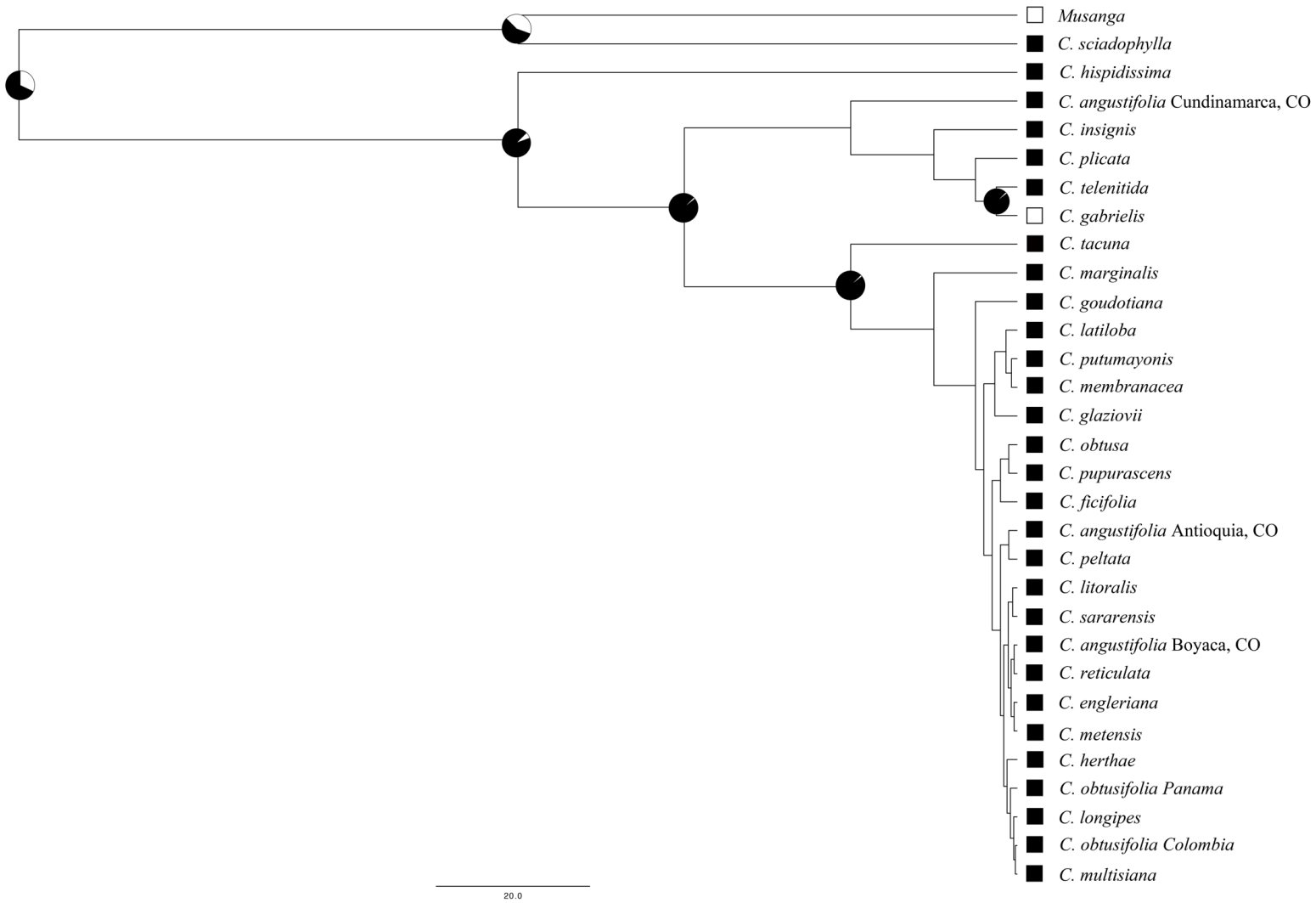
Musanga
C. sciadophylla
C. hispidissima
C. angustifolia Cundinamarca, CO
C. insignis
C. plicata
C. telenitida
C. gabrielis
C. tacuna
C. marginalis
C. goudotiana
C. latiloba
C. putumayonis
C. membranacea
C. glaziovii
C. obtusa
C. pupurascens
C. ficifolia
C. angustifolia Antioquia, CO
C. peltata
C. litoralis
C. sararensis
C. angustifolia Boyaca, CO
C. reticulata
C. engleriana
C. metensis
C. herthae
C. obtusifolia Panama
C. longipes
C. obtusifolia Colombia
C. multisiana
20.0

Figure 3-4. Maximum likelihood ancestral state reconstruction of domatia (open nesting space) in *Cecropia* using an equal rates model of evolutionary transitions. Pie charts at nodes represent the likelihood of the ancestor lacking domatia (white) or having domatia present (black). The squares at the tips of the branches indicate the state coded for species in the same format as above. *Coussapoa* (not shown) was the outgroup.

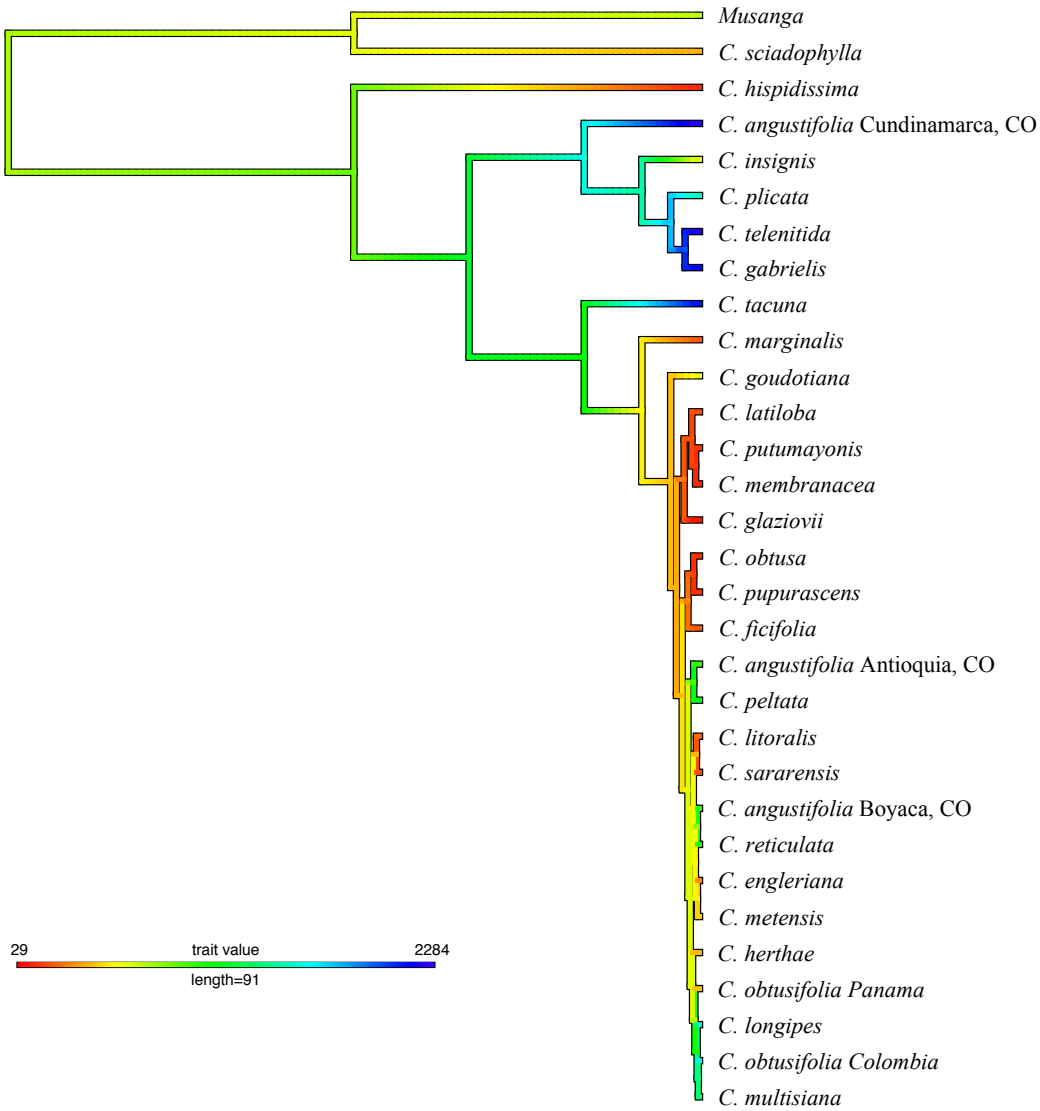

Figure 3-5. Maximum likelihood reconstruction of elevation (meters above sea level) based on sample localities. *Coussapoa* (not shown) was the outgroup.

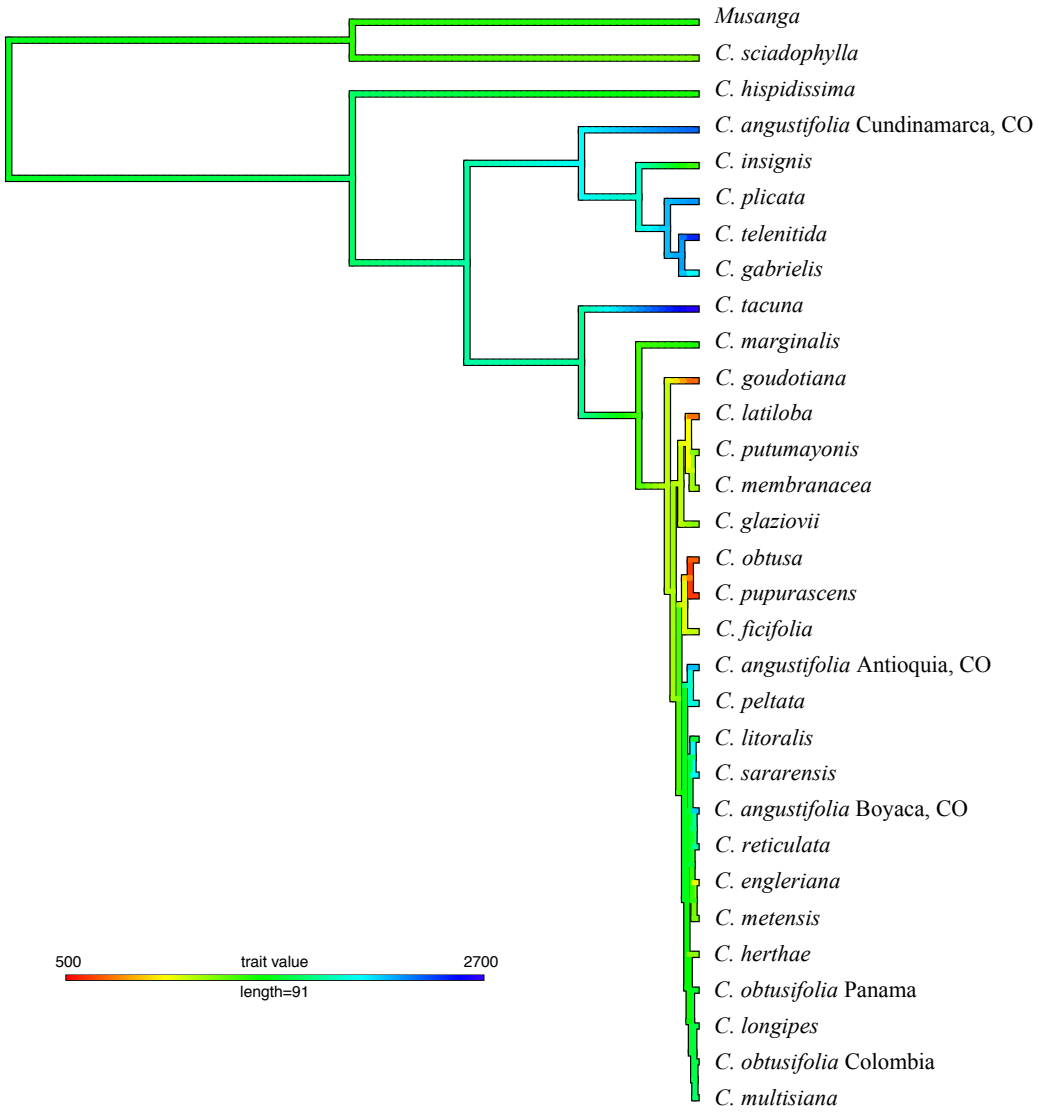

Figure 3-6. Maximum likelihood reconstruction of elevation (meteres above sea level) using maximum elevation from published accounts of each species. *Coussapoa* (not shown) was the outgroup.

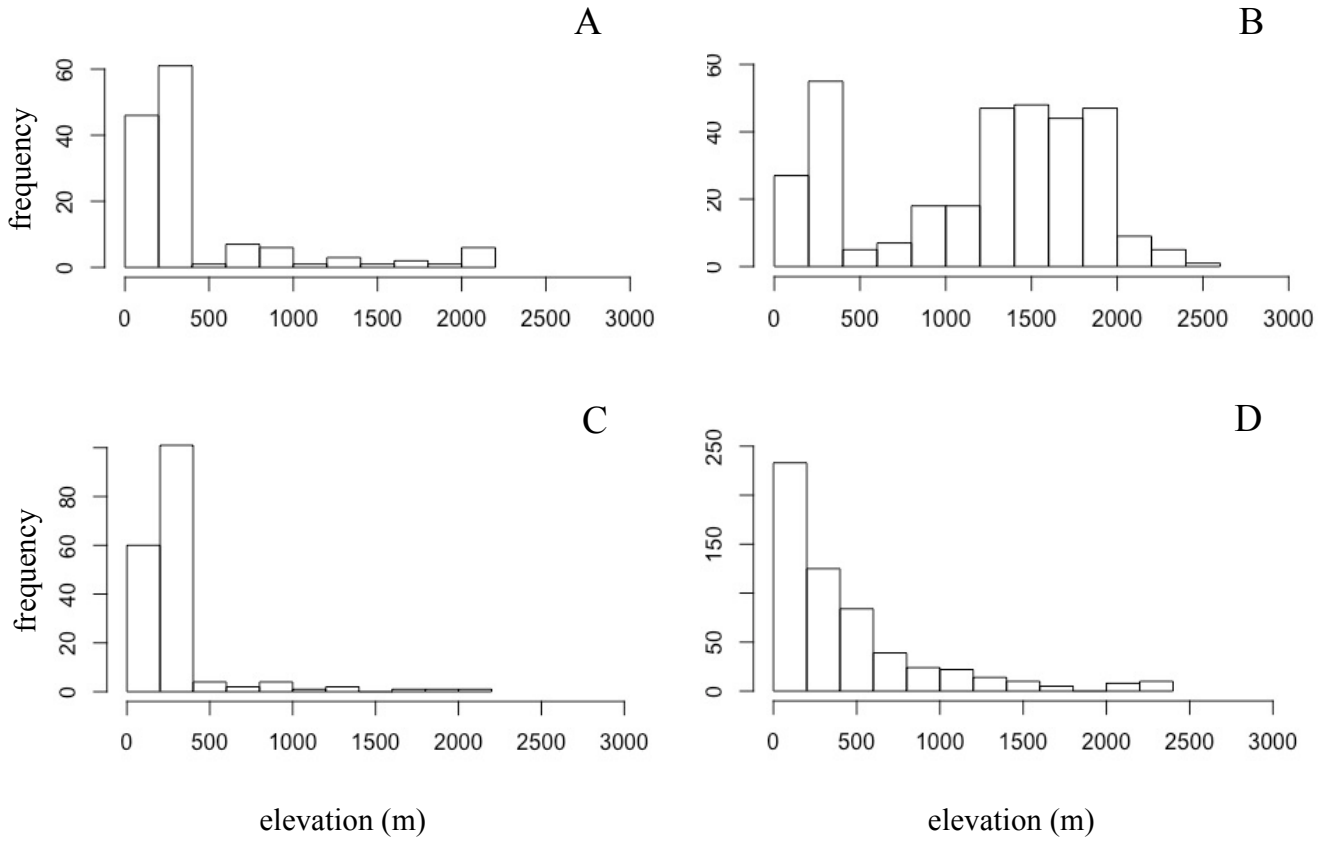

Figure 3-7. Altitudinal distributions of four widespread species based on herbarium specimen records of the Global Biodiversity Information Facility. Species include: (A) *C. membranacea* (n=135), (B) *C. angustifolia* (n=331), (C) *C. sciadophylla* (n=177), and (D) *C. obtusifolia* (n=574).

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Appendix 1. Species and specimens examined with collector numbers, localities, and GenBank accession numbers for 26S, EPIC, and ndhF, analy respecitively. 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.

Appendix 2. Morphological characters and states scored for species in Appendix 1.

1. Growth habit: (0) hemiepiphyte, (1) herb, (2) tree. Herbs are plants without a woody stem and trees are perennial woody plants with secondary thickening, with a clear main stem. We classified *Boehmeria nivea* as an herb, because it has no pronounced secondary growth. We classified *Poikilospermum* hemiepiphytic (epiphytic for one stage of its life cycle but rooted in the soil during another stage). Berg (1978) cited the hemiepiphyte scramblers habit for *Poikilospermum*, while Chew (1963) used the term woody scramblers. *Coussapoa* species we analyzed have a hemiepiphyte habit Berg et al. (1990). Ruiter (1976) and Berg and Franco-Rosselli (2005).

2. Exudate: (0) clear, (1) milky. Romaniuc-Neto (1999) describes the latex as white (milky) in *Coussapoa*. During field sampling white latex was mainly observed in young individuals and vegetative branches of *Coussapoa*, so we classified the exudate as milky for this genus. The exudate in the genera of Cecropieae is often clear becoming black in exposure to air. *Boehmeria*, *Pilea* and *Laportea* have mucilaginous cells and/or secretory ducts that secrete clear exudate. Guérin (1923) and Berg and Franco-Rosselli (2005).

3. Heartwood color: (0) brown to yellowish, (1) whitish to grey. Welle et al. (1992) and Clement (2008).

4. Distinction between heartwood and sapwood: (0) not defined, (1) defined. Because we could not quantitatively measure the difference between "weakly defined" and "strongly defined," we combined these descriptions and interpreted them as "defined." Herbarium and field observation.

5. Wood grain: (0) interlocked, (1) straight. Bonsen (1990) and Welle et al. (1992).

6. Growth ring boundaries: (0) absent, (1) faint or absent. Bonsen and Welle (1983) and Welle et al. (1992).

7. Banded apotracheal parenchyma: (0) absent, (1) present. Parenchyma forming continuous strands or "bands" not associated with the vessels. Bonsen and Welle (1983) and Clement (2008).

8. Paratracheal parenchyma: (0) unlignified, (1) lignified. Axial parenchyma associated with the vessels or vascular tracheids. Bonsen and Welle (1983) and Welle et al. (1992).

9. Druse in parenchyma: (0) absent, (1) present. A globular cluster of crystals, sometimes with an organic core, either attached to the wall by a peg or lying free in the cell (Radford et al. 1974). This character is found in *Poikilospermum* and species of Urticaceae other than Cecropieae, which have rhombic crystals only. Bonsen and Welle (1983).

10. Procumbent uniseriate ray cell: (0) absent, (1) present. Uniseriate rays are wood rays only one cell wide and variable in length. With the exception of *Poikilospermum* (Urticaceae), all Urticales are reported to have uniseriate rays. Bonsen and Welle (1983), Clement (2008) and Clement and Weiblen (2009).

11. Average number of vessels per square mm: (0) 1–3, (1) 1–6, (2) 5–9. This character was used by Bonsen and Welle (1983) to suggest the morphological proximity between *Cecropia – Musanga* group and the *Coussapoa – Myrianthus* group, with *Pourouma* overlapping both groups.

12. Fiber pit location: (0) radial and tangential, (1) radial. Bonsen and Welle (1983) and Humphries and Blackmore (1989).

13. Pore number: (0) two, (1) three, (2) four, (3) greater than 4. Sorsa and Huttunen (1975), Barth (1976), Ruiter (1976) and Barth (1984).

14. Stipules: (0) amplexicaul, (1) not amplexicaul. Cecropieae show amplexicaul stipules, which extend to the side of the stem opposite the main blade. Chew (1963), Ruiter (1976), and Berg (1978).

15. Stipular scars: (0) inconspicuous, (1) horizontal, (2) ascending. This character was used by Berg et al. (1990) in the identification key to genera (*Cecropia*, *Coussapoa* and *Pourouma*).

16. Phyllotaxis: (0) distichous, (1) spiral, (2) decussate. Berg (1978) and Chen et al. (2003).

17. Venation: (0) pinnate, (1) actinodromous (2) palmate. Urticaceae presents pinnate, actinodromous or palmate venation. Based on Hickey (1973) and Radford et al. (1974), we use the term pinnate for leaves entire with a single primary vein (midvein) serving as the origin for the higher order venation; actinodromous for leaves entire with two or more primary veins diverging radially from a single point; and palmate for leaves lobed with three or more primary veins arising from a common point. Berg (1978) points to *Cecropia* and *Musanga* having palmately veined leaves, *Coussapoa* having pinnately to subtriplinerved to palmately veined leaves, *Poikilospermum* having mostly pinnately veined leaves, *Myrianthus* having pinnately to palmately veined leaves and *Pourouma* having pinnately to palmately veined leaves. Chen et al. (2003) use the term pinnately or 3–5-veined to describe venation in *Boehmeria*, *Laportea*, *Parietaria* and *Pilea.*

18. Lamina: (0) simple leaves, (1) with some lobed leaves. The species of Cecropieae often have lobed leaves that are simple when juvenile, or in *Pourouma*, for example,

display a gradient in lamina shape from simple or slightly lobed to lobed. The character state "simple leaves" implies all leaves on the plant are entire, while having "lobed leaves" implies that at least some of the leaves of the plant were lobed.

19. Lamina insertion: (0) not peltate, (1) peltate. Peltate leaves have the petiole attached to the blade not by the margin. This character was used by Berg (1978) in the identification key to distinguish *Cecropia* and *Musanga* from other genera.

20. Uncinate hairs: (0) absent, (1) present. Hooked hairs on the surface of the leaf. Gangadhera and Inamdar (1977) and Metcalfe et al. (1950).

21. Arachnoid hairs: (0) absent, (1) present. The arachnoid or cobwebby indumentum is compound of unicellular hairs which are very thin and interwoven, usually white but sometimes brownish. Bonsen and Welle (1983), Berg et al. (1990), and Berg and Franco-Rosselli (2005).

22. Cystoliths: (0) absent, (1) present. A cystolith is usually an ellipsoidal or globular, calcified body with a silicified stalk. Members of the Cecropieae accumulate silicon in idioblasts of leaves, but do not have cystoliths. Kachroo and Bhat (1981) and Setochi et al. (1993).

23. Hydathodes: (0) absent, (1) present. Hydathodes are water-glands, organs extruding water or fluid. Bonsen and Welle (1983) and Metcalfe and Chalk (1950).

24. Breeding system: (0) monoecious, (1) dioecious. Berg (1978) and Chen et al. (2003).

25. Spathe: (0) absent, (1) present. The inflorescences of *Cecropia* and *Musanga* are completely enclosed by a spathe except for *C. hololeuca*. At anthesis, the spathe opens and drops. Berg and Franco-Rosselli (2005). The other genera of Urticaceae do not present spathe but rather bracts that are usually reduced and caducous.

26. Staminate inflorescence architecture: (0) globose, (1) cyme, (2) spike, (3) fasciculate, (4) paniculate. The staminate inflorescences of *Boehmeria*, *Coussapoa*, *Musanga, Poikilospermum and Pourouma* are often cymosely branched or repeatedly branched with the flowers gathered in capitate, capitulate, glomerate, globose heads, or globose clusters (Chew 1963; Ruiter 1976; Berg 1978; Berg et al. 1990; Chen et al. 2003). We use the term glomerule (a dense cluster of sub-sessile or of small capitula) to standardize the different classifications. The term spike (sessile flowers arranged along the sides of an axis) is used for *Cecropia* by Berg and Franco-Rosselli (2005). The term fascicle refers to a highly contracted cyme, although less than the glomerule (Font Quer 1985).

27. Pistillate inflorescence architecture: (0) globose, (1) cymose, (2) spicate, (3) fasciculate, (4) paniculate. The term spike (sessile flowers arranged along the sides of an axis) is used by Berg and Franco-Rosselli (2005) for *Cecropia* and Ruiter (1976) for *Musanga*.

28. Inflorescence arrangement: (0) solitary, (1) paired, (2) clustered.

29. Interfloral bracts: (0) absent, (1) present. The interfloral bracts are present in some species of *Coussapoa* and were used by Berg et al. (1990) in a key for species identification.

30. Staminate perianth merosity: (0) absent, (1) 2-merous, (2) 3-merous, (3) 4-merous, (4) merosity variable 2–5-merous. There is a wide range of perianth number throughout the Urticaceae, and many species are polymorphic. For example, perianth number in *Poikilospermum* and *Myrianthus* is extremely variable within a species and ranges from 2–5-merous. State 4 separated those species that were polymorphic for perianth number

from those that had a more consistent number of perianth parts. For species in which only generic descriptions were available, each state present was coded.

31. Staminate perianth connation: (0) absent, (1) free, (2) partially connate, (3) completely connate. Fusion among perianth parts within a flower varies from none, to partially connate (less than half the length of the perianth is fused, often the perianth is apically or basally fused), to completely connate (more than half of the length of the perianth is fused).

32. Pistillate perianth connation: (0) absent, (1) free, (2) partially connate, (3) completely connate.

33. Number of stamens per flower: (0) one, (1) two, (2) three, (3) four, (4) five, (5) six.

34. Filaments: (0) straight in bud, (1) inflexed in bud. The species of Cecropieae show only straight filaments in bud, while the other tribes of Urticaceae present inflexed filaments that often explosively straighten at anthesis and throw pollen away from the flower. In *Poikilospermum*, the subgenus *Poikilospermum* shows straight filaments in bud and the subgenus *Ligulistigma* exhibit inflexed filaments in bud. Berg (1978) and Chew (1963).

35. Placentation: (0) basal, (1) lateral. The basal placentation is a synapomorphy of the Urticaceae, only in *Pourouma* the placentation is subbasal to lateral. We consider *Pourouma* with lateral placentation as described by Gaudichaud (1830) when he proposed the "Pouroumées" group (Pouroumeae tribe).

36. Endocarp: (0) chartaceous, (1) crustaceous, (2) woody. Berg (1978) and Chen et al. (2003).

37. Seeds size: (0) small (less than 5 mm long), (1) large (greater than 10 mm long). The seeds size was utilized by Chew (1963) to suggest the transfer of the microspermous genera (*Cecropia*, *Coussapoa*, *Musanga*, and *Poikilospermum*) to the Urticaceae and to leave the megaspermous genera *Pourouma* and *Myrianthus* in the Moraceae. Berg (1978) and Chen et al. (2003).

39. Cotyledons: (0) unequal, (1) equal. Berg (1978) and Chen et al. (2003).

40. Adventitious roots: (0) absent, (1) present. Adventitious roots are lateral roots coming from organs other than main root system, such as the stem. These roots are often present in Cecropieae and function in supporting, fixing and the absorption of water and inorganic nutrients.

41. Myrmecophytism: (0) absent, (1) present. *Cecropia* provide both housing and food for ants. The principal food rewards, Müllerian bodies (MB), are ovoid structures rich in glycogen and containing a small amount of protein (Rickson 1976). MB are produced on hairy platforms termed trichilia, which appear at the bases of leaf petioles at species-specific stages in the development of *Cecropia* seedlings and saplings (Davidson and Fisher 1991). *Pourouma myrmecophilla* presents domatia at the base of the petioles, which are used as shelter for ants. In both cases, the ants provide protection against herbivory. Berg et al. (1990) and O'Dowd (1982) comment that three species of *Coussapoa* have ants (*C. asperifolia*, *C. microcarpa* and *C. villosa*).