

Piper

A Model Genus for
Studies of Phytochemistry,
Ecology, and Evolution



Edited by

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Phylogenetic Patterns, Evolutionary Trends, and the Origin of Ant–Plant Associations in *Piper* section *Macrostachys*: Burger’s Hypotheses Revisited

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9.1. INTRODUCTION

In *Flora Costaricensis*, William Burger of the Field Museum in Chicago considered eight of the *Piper* species that occur in Costa Rica to make up the *P. obliquum* complex, and six additional species to be closely allied to it (Fig. 9.1; Burger 1971). *Piper calcariiformis*, an additional species assignable to the *P. obliquum* complex, was later described by Tebbs (1989). Although Burger did not apply a system of infrageneric classification in *Flora Costaricensis* (1971), these 15 species of the *P. obliquum* complex and its allies represent all of the Costa Rican members of *Piper* sect. *Macrostachys* (Miq.) C.DC. Besides discussing the relationships between species, Burger (1972) also made hypotheses concerning evolutionary trends among the Costa Rican *Piper* species. In addition, he was among the first to note that certain *Piper* species have a tendency to support associations with ants. All known obligate ant-plants among New World *Piper* are found within the *P. obliquum* complex and its allies. In this chapter, we revisit Burger’s hypotheses regarding systematic relationships and evolutionary hypotheses, and explore the evolution of ant–plant associations more fully in the broader context of *Piper* sect. *Macrostachys* using a molecular phylogenetic approach.

Molecular phylogenies are exceptionally useful as independent frameworks to study the evolution of ecological associations and the morphological traits related to them (Armbruster 1992, 1993, 1994, Dodd *et al.* 1999). *Piper* sect. *Macrostachys*, for many

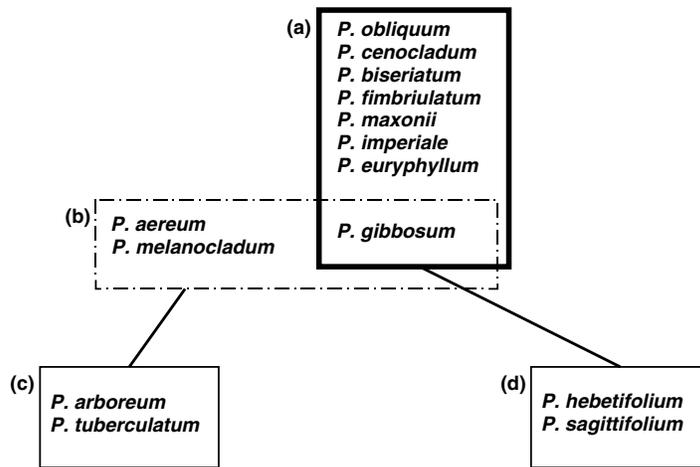


FIGURE 9.1. Systematic relationships among the Costa Rican members of *Piper* subg. *Macrostachys* (i.e., the “*P. obliquum* complex”) as proposed in *Flora Costaricensis* (Burger, 1971). The species in box (a) constitute the “*P. obliquum* complex.” Burger proposed a close relationship between *P. euryphyllum* and *P. gibbosum*, but he also suggested that *P. gibbosum* was closely related to the species in box (b). In addition, he suggested that *P. gibbosum* was a potential link, along with its allies in (b), between the species in (c) and (a), and by itself between (d) and (a). With the exception of *P. calcariformis*, which was described later, the species in this figure represent all of the Costa Rican species of *Piper* subg. *Macrostachys*.

reasons, presents an ideal system for studying the evolution of ant–plant associations using phylogeny. For example, a range of ant associations, from obligate to facultative to none, provides the potential for studying evolutionary patterns and trajectories that may result in obligate mutualisms. The number of origins of obligate associations is of interest since several of the obligate myrmecophytes are morphologically divergent for many traits (e.g., *P. sagittifolium* vs. *P. fimbriatum*), whereas the characters associated with ants are remarkably constant in all five obligate species.

In this chapter, we examine interspecific relationships within the *P. obliquum* complex and allied species *sensu* Burger (1971; i.e., the Costa Rican members of sect. *Macrostachys*), as well as representative species of sect. *Macrostachys* that occur outside of Costa Rica (Table 9.1). We utilize the phylogeny as an independent framework to gain insight into systematic relationships, and evolutionary patterns and trends within the section, including those that led to obligate ant–plant associations. This approach will allow us to address the following questions:

- i) Is the *P. obliquum* species complex *sensu* Burger monophyletic within sect. *Macrostachys*? Do the species of the *P. obliquum* complex, along with allied species, also form a monophyletic group?
- ii) Are Burger’s hypotheses of relationships within the *P. obliquum* complex and its affinities to allied species supported?
- iii) Are the evolutionary trends within the *P. obliquum* complex and allied species supported, as proposed by Burger?
- iv) What are the patterns of morphological change that led to obligate ant–plant interactions in *Piper* sect. *Macrostachys*?

TABLE 9.1
Species Included in This study. Asterisks Denote Species That We Have Observed and Collected in the Field

Species	Collector/Accession #	Source
INGROUP		
<i>P. aereum</i> Trel.	I.A. Chacon 2213 (MO)	Costa Rica
<i>P. arboreum</i> Aublet*	E. Tepe 377 (MU)	Costa Rica
<i>P. archeri</i> T. & Y.	M.A. Jaramillo 87 (DUKE), GenBank AF275178	Colombia
<i>P. begoniicolor</i> T. & Y.	T. Croat 69629 (MO)	Colombia
<i>P. biseriatum</i> C.DC.*	E. Tepe 141 (MU)	Costa Rica
<i>P. calcariformis</i> Tebbs*	A. Estrada 2397 (CR)	Costa Rica
<i>P. cenocladum</i> C.DC.*	E. Tepe 393 (MU)	Costa Rica
<i>P. cernuum</i> Vell.	G. Hatschbach 46665 (MU)	Brazil
<i>P. cogolloi</i> Callejas	R. Callejas 6431 (MO)	Colombia
<i>P. euryphyllum</i> C.DC.*	E. Tepe 410 (MU)	Costa Rica
<i>P. fimbriulatum</i> C.DC.*	E. Tepe 115 (MU)	Costa Rica
<i>P. gibbosum</i> C.DC.*	E. Tepe 411 (MU)	Costa Rica
<i>P. gigantifolium</i> C.DC.	S. Mori 12866 (NY)	Brazil
<i>P. hebetifolium</i> Burger*	E. Tepe 448 (MU)	Costa Rica
<i>P. imperiale-a</i> (Miq.)C.DC.*	E. Tepe 402 (MU)	Costa Rica
<i>P. imperiale-b</i> (Miq.)C.DC.*	E. Tepe 473 (MU)	Costa Rica
<i>P. marsupiatum</i> T. & Y.*	MBG #931716	Cultivated, Missouri Botanical Garden
<i>P. maxonii</i> C.DC.*	E. Tepe 370 (MU)	Costa Rica
<i>P. melanocladum</i> C.DC.*	E. Tepe 134 (MU)	Costa Rica
<i>P. nobile</i> C.DC.	T. Croat 60527 (MO)	Venezuela
<i>P. obliquum</i> R. & P.*	E. Tepe 114 (MU)	Costa Rica
<i>P. obtusilimbum</i> C.DC.*	MBG #930887	Cultivated, Missouri Botanical Garden
<i>P. perareolatum</i> C.DC.	A. Gentry 74657 (MO)	Peru
<i>P. pseudonobile</i> C.DC.	Boyle 3521 (MO)	Ecuador
<i>P. sagittifolium</i> C.DC.*	E. Tepe 116 (MU)	Costa Rica
<i>P. subglabribracteatum</i> C.DC.	A. Cogollo 7784 (MO)	Colombia
<i>P. sp. nov.*</i>	E. Tepe 94 (MU)	Costa Rica
OUTGROUP		
<i>P. auritum</i> Kunth*	M.A. Jaramillo 63 (DUKE), GenBank AF 275175	Colombia
<i>P. friedrischtalii</i> C.DC.*	E. Tepe 131 (MU)	Costa Rica
<i>P. garaganum</i> C.DC.*	M.A. Jaramillo 73 (DUKE), GenBank Af275162	Colombia
<i>P. lacunosum</i> Kunth*	E. Tepe 443 (MU)	Costa Rica
<i>P. multiplinervium</i> C.DC.*	M.A. Jaramillo 139 (DUKE), GanBank AF 275168	Colombia

9.2. TAXONOMIC HISTORY OF *Piper* sect. *Macrostachys* (MIQ.) C.DC.

The taxonomy of *Piper* L. has been problematic since tropical exploration increased in the 18th and 19th Centuries. Despite the attention of many notable botanists (Kunth 1839, Miquel 1843–1844, de Candolle 1869, 1923), the taxonomy of *Piper* remains difficult and enigmatic (Yuncker 1958, Bornstein 1989). Among factors contributing to a

difficult taxonomy are that many species have been described within the past 100 years on the basis of sterile and/or fragmentary material (e.g., Trelease 1929, Trelease and Yuncker 1950), and that several recent authors have applied drastically different species concepts (Trelease 1929, Trelease and Yuncker 1950, Tebbs 1989, 1990, 1993).

More recently, a number of Floras have aided in clarifying the taxonomy and reducing the number of accepted names in *Piper* (Standley and Steyermark 1952, Burger 1971, Yuncker 1972, 1973, 1974, Howard 1988, Steyermark 1984, Callejas 2001), as have a smaller number of revisionary studies (Steyermark 1971, Smith 1975, Callejas 1986, Bornstein 1989). In addition, the circumscription of *Piper* has been debated, with varying numbers of segregate genera recognized by different authors (Kunth 1839, Miquel 1843–1844, de Candolle 1923, Trelease and Yuncker 1950). Great strides have been made in recent years toward a more robust system of infrageneric classification based on cladistic analyses of morphological and molecular evidence (Callejas 1986, Jaramillo and Manos 2001, E. Tepe *et al.*, unpubl. data; Chapter 10). These phylogenetic studies support *Piper* L. in a broad sense as well as many of the traditional sections recognized by earlier authors (i.e., Kunth 1839, Miquel 1843–1844, de Candolle 1869, 1923).

Kunth (1839) was the first author to attempt to provide a system of infrageneric classification for *Piper*, and thus was the first to group all of the known species that would come to constitute *Piper* sect. *Macrostachys* in a section denoted *Species Piperi obliquo . . . propinqua*. Miquel (1843–1844) transferred these species to *Artanthe* Miq. (now synonymous with *Piper*) sect. *Macrostachys* Miq. In the most recent genuswide classification, de Candolle (text: 1869, key: 1923) submerged *Artanthe* into an expanded *Piper* L., but separated the species of *Piper* sect. *Macrostachys* into several different sections on the basis of differing leaf venation patterns and bract characters. However, Callejas (1986) and Tebbs (1989) regrouped these into *Piper* sect. *Macrostachys* (Miq.) C.DC. on the basis of inflorescence, fruit, leaf base, petiole, and bract characters. *Piper* sect. *Macrostachys* is one of seven currently recognized Neotropical sections (Tebbs 1989; however, see Jaramillo and Manos 2001 and Chapter 10 for a different view), and contains approximately 50 species.

9.3. NATURAL HISTORY OF *Piper* sect. *Macrostachys*

Most species of *Piper* sect. *Macrostachys* (Figs. 9.2–9.4; Fig. 9.4 is typical *Macrostachys*) are large-leaved shrubs and small trees with pendulous inflorescences that are typically longer than the leaves. They are characteristic of deep-shade areas in the moist understory of wet tropical forests ranging from southern Mexico to northern Argentina. Several species of sect. *Macrostachys* represent stages along a continuum of ant associations, ranging from casual and periodic to highly specialized and obligatory (Risch *et al.* 1977, E. Tepe, unpubl. data). The obligate ant-plants in sect. *Macrostachys*—reported heretofore only from Costa Rica—are characterized by a number of adaptations (Fig. 9.2). In young plants, ants nest inside petiolar cavities that are tightly folded into a tube (Fig. 9.2; Burger 1972, Risch *et al.* 1977, Letourneau 1998). As the plant increases in size, the stem becomes hollow (Fig. 9.2; Risch *et al.* 1977). A pore between the petiolar and stem cavities allows for movement of ants between the two domatia. The primary source of nutrients for the ants appears to be plant-produced pearl bodies. The pearl bodies are located on the

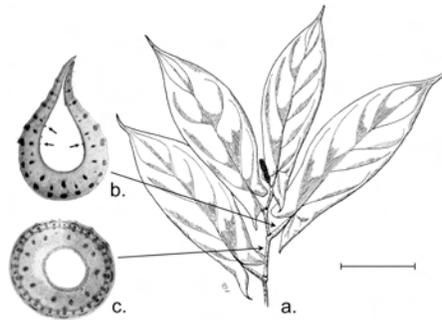


FIGURE 9.2. Obligate myrmecophyte. (a) *Piper sagittifolium* (a morphologically atypical member of *Piper* sect. *Macrostachys*). (b) Petiole cross section of *P. sagittifolium*, arrows indicate pearl bodies. (c) Stem cross section of *P. sagittifolium* from third internode. Scale bar = 5 mm for sections and 10 cm for illustration.

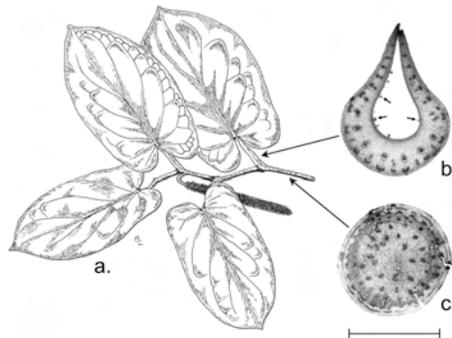


FIGURE 9.3. Obligate myrmecophyte. (a) *Piper calcariformis*. (b) Petiole cross section of *P. calcariformis*, arrows indicate pearl bodies. (c) Stem cross section of *P. sagittifolium* from third internode. Scale bar = 5 mm for sections and 15 cm for illustration.

adaxial side of the petioles (i.e., inside the tube, Fig. 9.2(B), arrows) such that they are only available to organisms inside the petiolar cavity, and are produced in large numbers only in the presence of the ant mutualist *Pheidole bicornis* Forel (Risch and Rickson 1981). Separate studies have produced different estimates of the nutritive composition of the pearl bodies with values ranging from 22 to 48% (dry weight) for lipids, negligible amounts of up to 2.1% for carbohydrates, and 10 to 24% for proteins (Risch *et al.* 1977, Fischer *et al.* 2002). *Piper calcariformis*, an obligate myrmecophyte with closed petioles and pearl bodies (Fig. 9.3), is unique among the obligate ant-plants in that it consistently has solid stems (Fig. 9.3).

The mutualism between *Ph. bicornis* and the myrmecophytic species of *Piper* is appropriately referred to as obligate because the fitness and survival of the plants are compromised when uninhabited by ants (Risch 1982, Letourneau 1983, 1998), and because *Ph. bicornis* appears to be specific to these *Piper* species; further, *Ph. bicornis* seemingly never forages off of its host plants (Risch *et al.* 1977, Letourneau 1998). The plants benefit from the obligate association with ants primarily through reduced damage by stem borers and a lower incidence of fungal infections of the inflorescences and infructescences (Letourneau 1998).

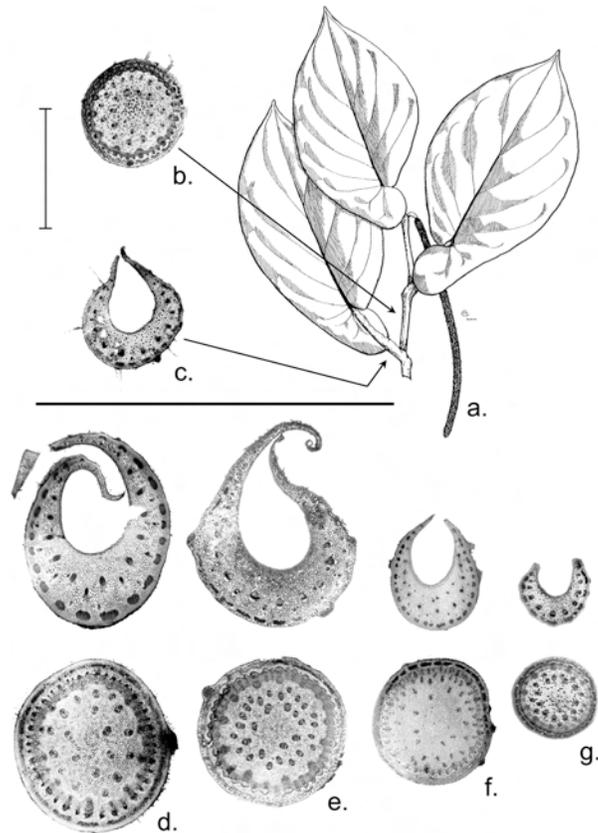


FIGURE 9.4. Facultative and nonmyrmecophytes. (a) *Piper biseriatum* (typical *Macrostachys*). (b) Stem cross section of *P. biseriatum*. (c) Petiole cross section of *P. biseriatum*. (d) Petiole and stem cross sections of *P. imperiale*, individual a facultative myrmecophyte. (e) *P. eurphyllum*, predicted, but not observed facultative myrmecophyte. (f) *P. imperiale*, individual a nonmyrmecophyte, compare with (d) for intraspecific variation in petiole closure. (g) *P. gibbosum*, nonmyrmecophyte. Scale bar = 5 mm for sections and 15 cm for illustration.

Myrmecophytic species of *Piper* have characteristic sheathing petioles that become tightly folded into a tube, whereas nonobligate myrmecophytes have solid stems and petioles that are closed to varying degrees (Fig. 9.4). In some facultative ant-plant species, colonies of ants are occasionally found nesting inside petiolar cavities (E. Tepe, unpubl. data); however, the ant residents are members of a suite of arboreal ant species that nest opportunistically in a variety of available, nonterrestrial cavities (Ward 1991, Byrne 1994, Alonso 1998, Orivel and Dejean 1999). Both the ant and plant species involved in facultative associations are capable of surviving without the other, and it is likely that these ant-plant associations are ephemeral (Alonso 1998). Ants apparently excavate the pith of the obligate myrmecophytes (Risch *et al.* 1977, Letourneau 1998); however, medulary vascular bundles are often scattered throughout the pith in species with stems that do not become hollow (e.g., Fig. 9.3) and may preclude excavation by ants. Tough tissue appears to deter pith excavation in some species of *Macaranga* (Euphorbiaceae; Fiala and Maschwitz 1992a).

Finally, in all remaining species of sect. *Macrostachys* encountered in the field, the petioles are either very small or the margins do not come together to form a tube (Fig. 9.4(F–G)), and no ants have been observed living on or in these plants (E. Tepe, pers. obs.). In summary, five species of *Piper* have obligate associations, two or more species have facultative associations, and numerous species have no associations with ants. All of these species are members of *Piper* sect. *Macrostachys*. It is within this continuum of ant–plant interactions that clues may be found to better understand the evolution of these mutually beneficial interactions.

9.4. PHYLOGENETIC RELATIONSHIPS IN *Piper* sect. *Macrostachys*

Our phylogenetic analyses of nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA for 32 taxa (27 ingroup and 5 outgroup species) converged upon a single Maximum Likelihood (ML) tree (Fig. 9.5) and an identical Bayesian tree. Trees with similar topologies were also recovered in four equally most parsimonious trees (MPT) in an analysis of ITS in which gaps were treated as missing data, and 27 MPTs when gaps were coded as separate presence/absence characters following Simmons and Ochoterena (2000). The topology recovered in the ML and Bayesian analyses is not in conflict with any of the MPTs with or without gap coding (Fig. 9.6), and none of the topologies of the MPTs contradict the hypotheses discussed here. The only differences among MPT and ML/Bayesian topologies is the level of support for clades C and D when gaps are treated as missing data, and support for clade C when gaps are coded as presence/absence characters. (For methods see Appendix 9.1)

Our results are concordant with several recent studies that support the monophyly of *Piper* sect. *Macrostachys* (Miq.) C.DC. (Callejas 1986, Jaramillo and Manos 2001, Tepe *et al.*, unpubl. data). Its monophyly is supported in studies with broad taxonomic sampling (Jaramillo and Manos 2001; Chapter 10) and in this study with the selection of closely related sister species. Section *Macrostachys s.l.* (Fig. 9.5, clade A), including sect. *Hemipodium* (Miq.) C.DC., represented here by *P. arboreum* (Tebbs 1989, Jaramillo and Manos 2001; Chapter 10), is strongly supported as monophyletic, with a Bayesian posterior probability (PP) of 1.0 and a bootstrap (BS) value of 94%. Section *Hemipodium* is sister to the remainder of sect. *Macrostachys* (Fig. 9.5, clade B), which is likewise strongly supported (1.0 PP, 76% BS). Posterior probabilities and bootstrap values have been considered the upper and lower limits of support, respectively (Douady *et al.* 2003), with bootstrap representing an overly conservative estimate of support in many cases (Wilcox *et al.* 2002). Short branch lengths and unresolved clades corroborate the results of Jaramillo (Chapter 10) that sect. *Macrostachys* appears to be recently derived through rapid diversification.

The sect. *Macrostachys* clade (Fig. 9.5, clade A) is somewhat unresolved, particularly in the parsimony analysis, with one major clade weakly supported within it (clade C). Within clade C, two subclades (D and E) are resolved, but weakly supported. Within the unresolved region of the *Macrostachys* clade, a small subclade that includes *P. melanocladum* and *P. aereum* represents a subgroup of *Macrostachys* species characterized by relatively small leaves lacking basal lobes, and with distributions restricted to

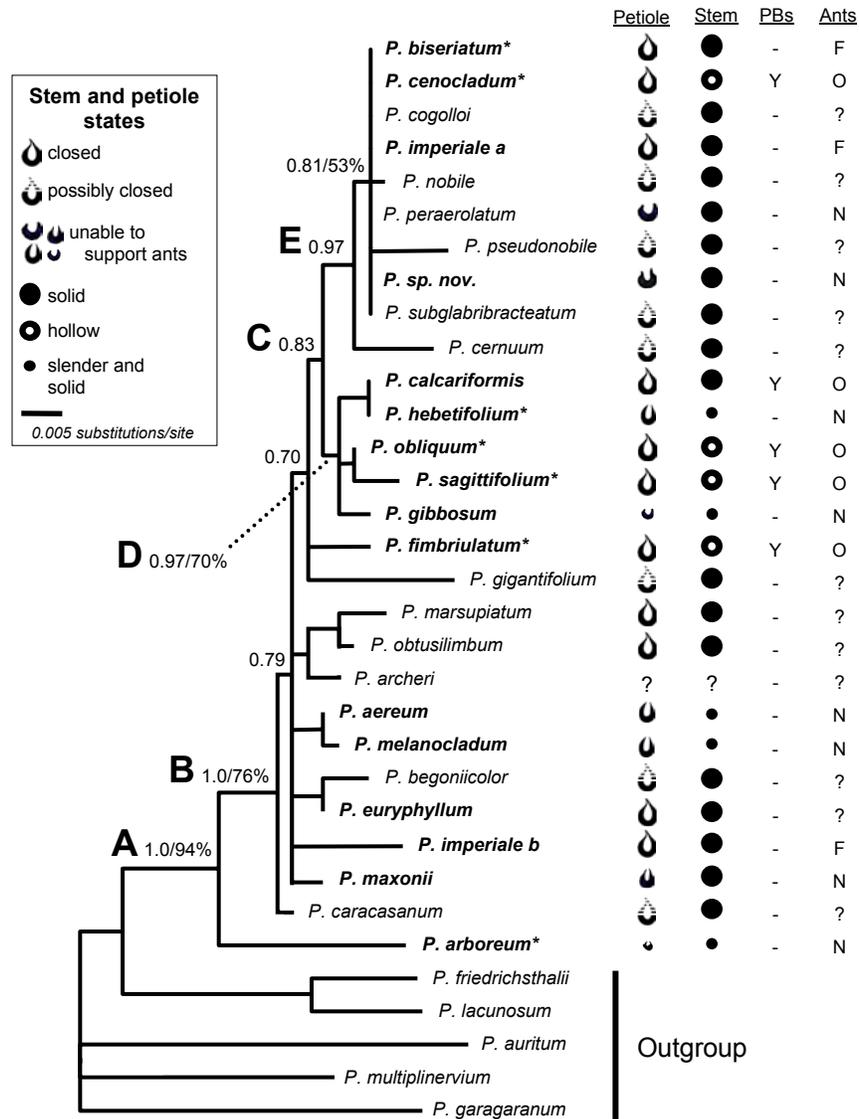


FIGURE 9.5. The single tree for *Piper* sect. *Macrostachys* from ML analysis of ITS sequence data (CI=0.82, RI=0.78). Numbers indicate Posterior Probabilities/Bootstrap values above 50%. Support for branches not indicated on tree are included in text. Species names in bold are found in Costa Rica, and asterisks (*) denote species in which more than one collection from different locations were included in the analysis and were resolved in the same position on the tree. Stem and petiole columns represent cross sections. PBs are pears bodies found inside the petiole chambers. The ant column indicates whether obligate "O", facultative "F", or no "N" associations with ants have been observed. Species that have not been observed, but that are predicted to have facultative associations with ants are indicated by "?" See Appendix 9.1 for methods.

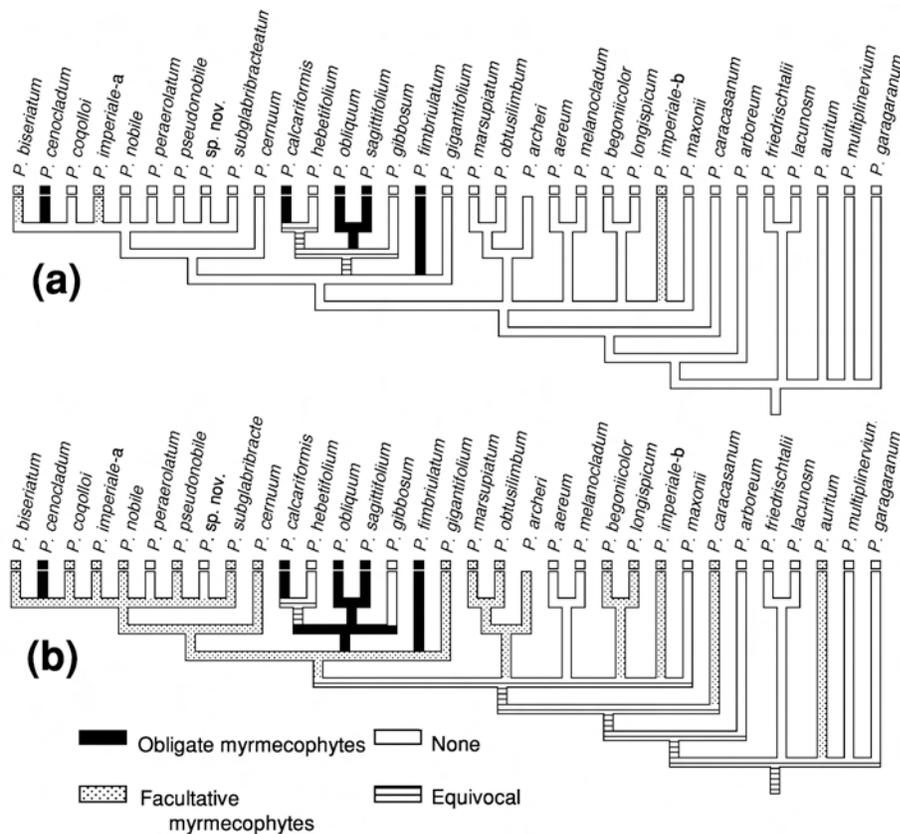


FIGURE 9.6. Reconstruction of the evolutionary history of myrmecophytism in *Piper* subg. *Macrostachys* based on (a) observed associations and (b) predicted associations.

southern Central America and adjacent northwestern South America (1.0 PP, 88% BS). Another clade includes *P. euryphyllum* (= *P. longispicum* C.DC.) and *P. begoniicolor* (1.0 PP, 98% BS). These two species, along with several others (including at least one species that occurs at high altitudes in Costa Rica), have been informally recognized as an Andean lineage within sect. *Macrostachys* by R. Callejas (pers. comm.). This Andean subclade is morphologically distinct and is resolved by our data; thus it appears, pending the inclusion of additional species in future analyses, that Callejas' informal rank is supported. However, the small clade that includes *P. archeri*, *P. obtusilimbium*, and *P. marsupiatum*, which are all South American species, is not strongly supported (0.74 PP, 20% BS).

The major clade within sect. *Macrostachys* (Fig. 9.5, clade C), although not present in all MP trees, is present in both ML and Bayesian analyses. Within clade C, two sister subclades (D and E) are present. The unresolved placements of *P. fimbriulatum* and *P. gigantifolium* are sister to clade C. In several of the MP trees, *P. fimbriulatum* and *P. gigantifolium* are sister to each other, but are placed in a large, unresolved portion of the tree. Subclade D includes rare species (except *P. obliquum*, which is widespread) that

have distributions restricted to Costa Rica and adjacent Panama. Subclade E is internally unresolved by the ITS, with the exception of *P. cernuum*, which is resolved as basal to the remainder of sect. *Macrostachys s.s.* It includes species of Costa Rican endemics, as well as South American species.

Many of the species included in our analyses were represented by more than one individual from more than one location (asterisks, Fig. 9.5). In all cases except for *P. imperiale*, all replicates of a given species are resolved as closest relatives and were therefore excluded in final analyses. In contrast, different collections of *P. imperiale* are placed distant to each other: in clade E, and at the base of the tree as unresolved (Fig. 9.5). Taxonomic determinations for some collections from Costa Rica are difficult because they tend to have traits that are characteristic of both *P. imperiale* and *P. biseriatum*. Furthermore, these ambiguous specimens are unequivocally resolved among individuals of *P. biseriatum* and *P. imperiale* in our ITS trees. It is possible that these problematic specimens represent hybrids, or species not yet identified. Although the collections included in this study were unambiguously identified, the placement of *P. imperiale* collections in two distant parts of the tree may indicate possible gene flow between *P. biseriatum* and *P. imperiale*. If hybridization is in fact implicated, this would represent the first report of hybridization within *Piper* in the wild. Thus far, the only report of hybridization in *Piper* is of artificial crosses between the cultivated pepper, *P. nigrum*, and several of its close relatives (Sasikumar *et al.* 1999).

9.5. BURGER'S HYPOTHESES REVISITED

9.5.1. *Systematic Relationships*

The *P. obliquum* complex (*sensu* Burger 1971) comprises eight Costa Rican species that Burger recognized as being closely related (Fig. 9.1). In addition to this complex, he posited that six other Costa Rican species were more closely allied to this complex than to any other *Piper* species that occur in Costa Rica (Fig. 9.1). The *P. obliquum* complex along with the allied species constitute all of the Costa Rican members of sect. *Macrostachys*, and, although these 14 species are each other's closest relatives among Costa Rican *Piper* species (the geographic extent of Burger's study), based on the ITS phylogeny, neither the *P. obliquum* complex nor the allied species constitute a monophyletic group when analyzed with additional species of sect. *Macrostachys* that occur outside of Costa Rica (Figs. 9.5 and 9.6). To further test the nonmonophyly, we constrained our ML analysis to force monophyly of the *P. obliquum* complex. The resulting likelihood scores of the trees were significantly higher than that of the most likely tree ($P = 0.001$, Shimodaira–Hasegawa test; Shimodaira and Hasegawa 1999). This result is consistent with all analyses of the ITS. In addition, it is clear that Burger did not believe the *P. obliquum* complex to be monophyletic because he proposed several close relationships between species in the *P. obliquum* complex with members of sect. *Macrostachys* not present in Costa Rica.

Although Burger (1971) proposed few systematic relationships within the *P. obliquum* complex itself, he did suggest that several groups of species were closely related to it (Fig. 9.1). He had doubts regarding the affinities of *P. gibbosum*. In some instances, he included it in the *P. obliquum* complex, most closely related to *P. euryphyllum*,

but later in the text he included it in a group with *P. aereum* and *P. melanocladum* (Fig. 9.1). Indeed, *P. gibbosum* has characters in common with both groups. It shares leaf shape, habitat, and the presence of styles (rather than sessile stigmas) with *P. euryphyllum*, and smaller leaf size, leaf texture, and venation patterns with *P. aereum* and *P. melanocladum*. Our analyses do not support a close relationship between *P. gibbosum* and either one of these two species groups but, instead, place *P. gibbosum* in the clade containing *P. obliquum* and several other species (Fig. 9.5, clade D). Although the positions of *P. euryphyllum* and *P. aereum/P. melanocladum* are not fully resolved, they do not appear to be closely related to *P. gibbosum*.

Secondly, Burger (1971) was the first to suggest that *P. arboreum* and *P. tuberculatum* were closely related to species of sect. *Macrostachys* (Fig. 9.1) on the basis of anther form and the lack of an apically developed prophyll. This conclusion has been strongly supported by morphological (Callejas 1986) and molecular data (Jaramillo and Manos 2001; Chapter 10). Again, Burger believed that *P. aereum* and *P. melanocladum* are closely related to each other (Fig. 9.1), and that these two species, along with *P. gibbosum*, form a link between *P. arboreum* and *P. tuberculatum* and the remainder of the *P. obliquum* complex. This conclusion is logical since these five species (i.e., Fig. 9.1(B–C)) are relatively slender and small-leaved plants when compared to the rest of sect. *Macrostachys*. Indeed, *P. arboreum* does represent a group of species in our analyses that includes *P. tuberculatum* and possibly *P. cordulatum* (a Panama endemic, not sampled) that is placed outside of the core *Macrostachys s.s.* clade (i.e., Fig. 9.5, clade B), and *P. aereum* and *P. melanocladum* are supported as closely related, as Burger suggested. However, the affinities of *P. aereum* and *P. melanocladum* to the remainder of the *Macrostachys* species cannot be determined since the placement of this clade is unresolved in the ITS trees. *Piper caracasenum* is placed between *P. arboreum* and the rest of *Macrostachys* (clade B), but its position is not well supported. Increased sampling and the addition of more variable markers may resolve these nodes, and *P. aereum* and *P. melanocladum* may indeed turn out to represent the link that Burger suggested (Fig. 9.1). Again, *P. gibbosum* is resolved in clade D (Fig. 9.5) and thus is apparently not closely related to these species.

Lastly, Burger suggested that, although *P. sagittifolium* is a morphologically unusual species, unlike any other in Costa Rica (Fig. 9.2), it is allied to the *P. obliquum* complex through *P. hebetifolium* and *P. gibbosum* (Fig. 9.1). Our data strongly support a close relationship for these three species, with the addition of *P. obliquum*, and the more recently described *P. calcariformis* (Fig. 9.5, clade D). However, rather than being distantly related to the *P. obliquum* complex, these species form a clade that is nested fully within the core of sect. *Macrostachys*. The placement of *P. sagittifolium* relative to the remainder of the section is the most apparent inconsistency between our analyses and Burger's hypotheses. Burger believed *P. sagittifolium* to be isolated and primitive among the Costa Rican *Macrostachys* species. However, a number of morphological characters appear to support clade D (Fig. 9.5), including flowers with styles (most members of sect. *Macrostachys* outside of clade D have sessile stigmas), long and recurved stigma lobes, apiculate anthers, and a number of vegetative traits that support obligate associations with ants. In fact, with the exceptions of *P. hebetifolium* and *P. gibbosum*, all members of this clade are involved in obligate associations with *Pheidole bicornis*, the obligate plant ant. The most recent circumscription of *Piper obliquum* R&P (Tebbs 1989) suggests that it is an extremely

variable and widespread species. However, this broad circumscription includes a number of well-defined taxa that warrant specific status (Callejas 2001). The form of *P. obliquum* represented in this clade (Fig. 9.6, Clade D) appears to be a unique form of the species that is restricted to southern Central America. Of the species included in our analysis, *P. nobile*, *P. pseudonobile*, *P. archeri*, and *P. caracasenum* have all been considered synonymous with *P. obliquum* at one time or another. This topological distribution does not support the broad concept of *P. obliquum* adopted by some authors.

9.5.2. Evolutionary Trends

In addition to proposing systematic relationships among species of Costa Rican *Piper*, Burger (1972) postulated a number of evolutionary trends. This discussion, however, is limited to those trends related to *Macrostachys* specifically. Burger considered the flowers of *P. sagittifolium* to represent the primitive condition in *Piper*. This conclusion was based on the idea that Piperaceae is derived from an ancestor similar to *Saururus* Mill. (Saururaceae; Burger 1972), the well-supported sister group to the Piperaceae (Tucker *et al.* 1993, Savolainen *et al.* 2000). It follows then, that the flowers of primitive *Piper* species should be plesiomorphic and resemble those of *Saururus*. Indeed, similarities in the flowers of *P. sagittifolium* and *Saururus* include relatively large parts, long styles with long, divergent stigmas, and unusually large anthers (~1 mm!) on long filaments (to ~2 mm). Furthermore, the inflorescences of *P. sagittifolium* are not as tightly packed as is typical of many *Piper* species but, instead, are more loosely associated, similar to those of *Saururus*. Our analyses strongly support the placement of *P. sagittifolium* within sect. *Macrostachys*. If our phylogeny is accurate, parsimony then suggests that any resemblance between the flowers of *P. sagittifolium* and *Saururus* are homoplasies rather than plesiomorphies.

Anthers of the majority of *Piper* have latrorse dehiscence through longitudinal slits. A tendency toward upward dehiscence of anthers is found in several groups of Neotropical *Piper* species, including sect. *Macrostachys*. This shift from lateral to upward dehiscence is presumably due to the tight, cylindrical packing of flowers in several species groups (Burger 1972, Jaramillo and Manos 2001). Upward dehiscence among Neotropical *Piper* species is achieved in two distinct ways. Anthers with parallel thecae and apical dehiscence are found exclusively in a small group of scandent and climbing species (e.g., *P. xanthostachyum* C.DC., sect. *Churumayu*; Burger 1972). In contrast, upward dehiscence has also been attained through broadening of the lower part of the anther connective in species of sections *Radula* and *Macrostachys* (Burger 1972). This results, in the most extreme cases, in the anther thecae being oriented end to end, 180° to each other. Dehiscence is technically lateral, but because of the expanded connective and the altered orientation of the thecae, pollen is effectively released apically. Sections *Radula* (e.g., *P. aduncum* and *P. hispidum*) and *Macrostachys* are exceedingly dissimilar morphologically; thus it is not surprising that Burger (1972) proposed that upward dehiscence via expanded connective evolved independently in these two lines. Molecular evidence, however, strongly supports a sister group relationship between *Radula* and *Macrostachys* (Jaramillo and Manos 2001; Chapter 10) suggesting that a tendency toward upward anther dehiscence through expanded connectives may be a synapomorphy that unites these two sections.

9.6. ANT-PLANT ASSOCIATIONS IN *Piper* sect. *Macrostachys*

Several recent studies have used phylogenetic analyses to address the evolution of ant-plant mutualisms (Michelangeli 2000, Blattner *et al.* 2001, Brouat *et al.* 2001, Davies *et al.* 2001). Interestingly, each study has revealed a unique pattern of evolution of ant-plant associations including numbers of origins and losses of ant associations, trends and correlations in associated plant morphologies, and whether or not plants with intermediate morphologies and facultative associations with ants represent plant species that are ancestrally intermediate between plant species with obligate associations and those with none. Our use of the term *intermediate* throughout this paper is not with regard to a stage in the evolutionary process, but rather to describe species that are morphologically intermediate and that have facultative associations with ants, i.e., the associations are neither obligate nor random and ephemeral.

Numerous origins of myrmecophytes have been reported in the Melastomataceae, with representatives in nine genera from several tribes (Gleason 1931, Whiffin 1972, Vasconcelos 1991, Morawetz *et al.* 1992, Michelangeli 2000). A cladistic analysis of morphology for *Tococa* revealed at least two origins of associations with ants with a minimum of one loss (Michelangeli 2000). Furthermore, the presence, location, and morphology of the domatia appear to be rather plastic at the species level and above (Michelangeli 2000). In *Leonardoxa africana* (Fabaceae), a single origin of ant interactions among four subspecies is supported, each representing a different degree of association with ants (McKey 1984, 1991, 2000, Chenuil and McKey 1996, Brouat *et al.* 2001). Brouat *et al.* (2001) sequenced several chloroplast markers for the four subspecies of *L. africana*. The subspecies are not resolved as monophyletic groups, but rather are intermixed. Although evolutionary hypotheses could not be determined for *L. africana*, the study elegantly illustrates how apparent gene flow or insufficient molecular divergence between taxa can obscure evolutionary interpretations (Brouat *et al.* 2001). In the Asian genus *Macaranga* (Euphorbiaceae), up to four independent origins of ant-plant associations and numerous reversals are supported through a molecular phylogenetic analysis (Blattner *et al.* 2001, Davies *et al.* 2001). In addition, several species of *Macaranga* exhibit traits previously thought to be intermediate in the evolution of ant mutualisms (Fiala and Maschwitz 1992a). However, these species appear to represent a distinct clade, separate from other myrmecophytes, with an independent origin of myrmecophytism rather than evolutionary intermediates (Blattner *et al.* 2001, Davies *et al.* 2001). Phylogenetic studies of ants have also been used to study ant-plant associations with similarly diverse results (Ward 1991, 1999, Ayala *et al.* 1996, Chenuil and McKey 1996).

9.6.1. *Origins and Evolutionary Trends*

The somewhat unresolved nature of portions of the ITS tree (Figs. 9.5 and 9.6), and, to some degree, the absence of natural history information for a number of South American species do not permit us to make unambiguous hypotheses regarding the number of origins of obligate and facultative myrmecophytism in species of *Piper* sect. *Macrostachys*. Mapping known ant-plant associations onto the ITS trees, however, suggests that obligate associations evolved independently at least twice, but not more than four times (Fig. 9.6). Again, although

the ML (= Bayesian) tree is presented in this paper (Fig. 9.6), none of the MP trees contradict the hypotheses presented here (see Fig. 9.6). It is possible to detect reliably the presence of obligate ant-plant associations from herbarium specimens owing to the presence of persistent, conspicuous pearl bodies in the petiole chambers, and, in most specimens, the remains of numerous ants or ant parts. Examination of herbarium specimens does not suggest the presence of any obligately myrmecophytic species in sect. *Macrostachys* additional to those included in this study.

The obligate myrmecophyte *P. cenocladum*, placed in clade E (Fig. 9.5) is isolated from the other four species of obligate myrmecophytes and, thus, presumably represents an independent origin. Three of the remaining myrmecophytes are placed in clade D. Two equally parsimonious reconstructions of obligate myrmecophytism are possible in this clade (Fig. 9.6). One reconstruction has a single gain of obligate associations with two independent and complete losses. Alternately, obligate associations could have evolved twice independently within this clade: once in the *P. obliquum/P. sagittifolium* clade (0.96 PP, 96% BS) and again in *P. calcariformis*.

The position of the obligate myrmecophyte, *P. fimbriulatum*, is unresolved. Thus it is unclear whether this species represents an additional origin of obligate myrmecophytism. *Piper fimbriulatum* resembles the rest of the species in clade D (Fig. 9.5) in having long, recurved stigma lobes, but differs markedly in leaf texture and absence of a style (i.e., sessile stigmas). Additional species and more informative markers are required to further evaluate the hypotheses proposed here, and to resolve the ambiguities in our analyses.

Facultative associations are more ephemeral and inconspicuous than obligate associations. Often only one to several petioles on a given plant are occupied by ants (E. Tepe, pers. obs.). On the basis of species that we have actually observed in the field with resident ants, our analyses suggest that facultative associations evolved independently two to three times (Fig. 9.6). However, large sheathing petioles with persistent margins are typical of a majority of *Macrostachys* species, and it is likely that the petioles of many more species than we have observed in the field form closed shelters and are therefore possibly inhabited by ants. Detection of facultative myrmecophytes in herbarium specimens is more ambiguous than it is for obligate associations. The degree of petiole closure is not preserved in dried specimens, but petiole morphology can suggest that facultative associations are likely to occur in a given species. Examination of herbarium specimens has led us to predict that the phenomenon of facultative associations is probably much more common and widespread than we have observed in the field. When predicted facultative associations are mapped onto the tree, a single origin is supported within the *Macrostachys* clade (Fig. 9.6). Consequently, the hypothesis of the number of origins of facultative associations based only on plants observed *in situ* with resident ants is the most conservative, and it is undoubtedly an underestimation.

9.6.2. Evolution of the Mutualism

9.6.2a. Obligate associations and hollow stems

All obligate myrmecophytes in sect. *Macrostachys* are characterized by tightly closed petioles with pearl body production localized on the inner surface of the petiolar

tube. Accordingly, the distribution of these two characters parallels obligate myrmecophytism (Fig. 9.6). Similarly, hollow stems are restricted to the plants with obligate associations. But because the stems never become hollow in *P. calcariformis*, a minimum of two and a maximum of three independent origins are required to explain the distribution of hollow stems, or stems that the ants are capable of or inclined to excavate, as the case may be. None of the facultative ant-plants studied thus far have hollow stems; however nonhomologous cavities are occasionally formed by stem-boring insects (E. Tepe, pers. obs.).

According to our current phylogenetic hypothesis based on the ITS data, the most parsimonious reconstructions of the evolution of obligate associations with ants is three to four independent gains, with up to two losses (Fig. 9.6). The number of gains depends on the placement of *P. fimbriulatum* and the resolution of the clade containing *P. obliquum*; the number of losses depends entirely upon the resolution of the latter. It seems almost certain, however, that the origin of myrmecophytism in *P. cenocladum* is independent of that for the *P. obliquum* clade (Fig. 9.5, clade E).

The hypothesis that obligate associations evolved twice in Clade D (Fig. 9.5), once for *P. obliquum*/*P. sagittifolium*, and independently in *P. calcariformis*, is intriguing because *P. calcariformis* is unique among the obligate ant-plants in that it consistently has solid stems (Fig. 9.3). The stem anatomy of *P. calcariformis* is different from the other four obligate myrmecophytes in that the medulary vascular bundles are scattered throughout the pith (which is the most common arrangement among species of sect. *Macrostachys*). In contrast, the medulary bundles of the obligate species with hollow stems are arranged in a second ring just interior to the primary ring (Fig. 9.2(C)). In these plants, the pith is reportedly excavated by the resident ants (Risch *et al.* 1977, Letourneau 1998, Dyer and Letourneau 1999). It is unknown whether bundles throughout the pith of *P. calcariformis* and the rest of the large-stemmed species of *Macrostachys* (Fig. 9.5) would preclude excavation by ants, but the bundles of the other four obligate myrmecophyte species with hollow stems do not extend into the area of pith that becomes hollow (Fig. 9.2). Alternatively, if the obligate myrmecophytes in the *P. obliquum* clade (Fig. 9.5, clade D) are the result of a single origin, the loss of the associations in *P. gibbosum* and *P. hebetifolium* is not surprising, since neither of these species is morphologically suited to support ant residents; both of these species have small petioles that do not close tightly, and stems that are more slender than any of its close relatives (Fig. 9.5). Furthermore, the petiole margins of *P. gibbosum* are caducous, leaving behind a broadly U-shaped petiole (Fig. 9.4(G)). It appears that the slender stem morphology is derived in these two species, perhaps in response to their mid- to high elevational habitats. It is possible that the mutualism between *Piper* and *Pheidole bicornis* is not as stable at higher elevations as at lower elevations. Myrmecophytic species of *Cecropia* are more frequently found with ant inhabitants at lower elevations, and increasingly less so as altitude increases (Wheeler 1942, Janzen 1973). It is possible that the same phenomenon is responsible for the lack or loss of associations in *P. gibbosum* and *P. hebetifolium*.

Multiple origins and losses of obligate myrmecophytism appear to be common in the ant-plant associations that have been studied in other genera thus far. For example, two to four origins, with numerous losses, are supported in *Macaranga* (Euphorbiaceae; Blattner *et al.* 2001, Davies *et al.* 2001), and a minimum of two gains and one loss are supported

in *Tococa* (Melastomataceae; Michelangeli 2000, also see Davidson and McKey 1993). Davies *et al.* (2001) proposed that multiple origins of myrmecophytism in *Macaranga* may be the result of the combination of certain morphological traits and a specific ecological and biogeographical setting. For example, the ancestors of the Malesian species of *Macaranga* that have given rise to myrmecophytes appear to have had large-diameter stems with soft pith and food bodies, and they occurred in a climate that allowed uninterrupted food body production (Davies *et al.* 2001). In other words, it appears that myrmecophytism has most likely evolved in plants that had a morphological predisposition for supporting such associations, and that were located in constant, tropical environments. According to this hypothesis, the combination of such characters as large, sheathing petioles, large-diameter stems, possibly widespread pearl body production, and a climatologically constant habitat that also includes *Pheidole bicornis* have contributed to the development of ant-plant associations in *Piper*.

A number of studies have demonstrated that ant partners of obligate associations are rarely species-specific, and that no ant-plant association studied thus far is the result of parallel cladogenesis (Mitter and Brooks 1983, Ward 1991, Ayala *et al.* 1996, Chenuil and McKey 1996). The fact that a single ant species, *Ph. bicornis*, is associated with all five obligate species of sect. *Macrostachys*, excluding the possibility of cryptic species of ants, precludes the possibility that species-for-species coevolution between ant and plant species has taken place. In fact, that one ant species is obligately associated with several plant species is not surprising. Frequent host switching of an ant species among host plant species appears to be common among many groups of plant ants, namely *Azteca* (Ayala *et al.* 1996), *Crematogaster* (Blattner *et al.* 2001), and *Pseudomyrmex* (Ward 1991). Thus, it appears that multiple origins of obligate ant-plants, and host switching by obligate plant ants, is common and that *Ph. bicornis* is capable of switching between the different obligate myrmecophyte species in *Piper* as well.

The mutualism with ants in obligate myrmecophytes is not maintained by a single plant character, but rather a suite of characters that is implicated in the associations. As the number of concurrent characters increases among species (i.e., tightly closed petiole sheaths, obligate-type pearl bodies, restricted areas of pearl body production, hollow stems in four of the five species), it becomes increasingly unlikely that these characters evolve in parallel or through convergence. In order to test the possibility that this suite of characters, and thus obligate myrmecophytism, evolved only once, we constrained our analysis to force the monophyly of the obligate myrmecophytes. The MP analysis produced the shortest trees, which were 11 steps longer than the shortest tree in our unconstrained analysis (125 vs. 114 steps). The ML analysis resulted in trees with likelihood values significantly higher than the most likely tree's from the unconstrained analysis when they were compared with the Shimodaira-Hasegawa test ($P = 0.001$). When the ML analysis was constrained to force the obligates into a monophyletic clade with the facultative myrmecophytes as a paraphyletic clade basal to them, so as to suggest that the facultatives are evolutionary transitions between species without associations and with obligate associations, the resulting trees were also longer (MP: 129 vs. 114 steps, ML: $P = 0.002$). Thus, according to our current analyses, neither the obligate nor the facultative myrmecophytes in *Macrostachys* can be explained by a single evolutionary origin, and the facultative myrmecophytes are not transitional between obligate myrmecophytes and species that lack associations with ants.

9.6.2b. *Petiolar domatia and facultative associations*

Given that petioles form the primary domatia in *Piper* ant-plants, evolution of facultative myrmecophytism cannot be discussed separately from petiolar morphology. In fact, no close, species-specific relationship has developed in any ant-plant system studied thus far that only provides food for the ant partners, but no shelter (Fiala and Maschwitz 1992b). The petiole cavity of the obligate species is tightly closed throughout its length, and the petiole margins are pressed tightly to the stem such that little, if any, water running down the stem enters the cavity. This morphology is exceptionally constant among the five species of obligate ant-plants in *Piper*. The degree of petiole closure of the facultative myrmecophytes is more variable, but several petioles on a given plant are often closed enough so as to provide sufficient shelter for ant colonies.

The ITS phylogeny supports two to three independent origins of facultative myrmecophytism, based solely on the species that we have observed in the field with ants nesting in the petioles. However, we have only had the opportunity to study 14 species of *Macrostachys* in the field (Table 9.1). On the basis of the examination of herbarium specimens of species that we have not studied *in vivo*, we have observed that most species of sect. *Macrostachys* have large, sheathing petioles; consistent with the observed correlation of plant morphology and ant occupancy of species that we have observed in the field, we believe that facultative mutualisms are much more taxonomically and geographically widespread than we have reported here. If these potentially facultative ant-plants are mapped onto the phylogeny, then a single origin of facultative associations near the base of sect. *Macrostachys* is supported, with several independent losses, and with the obligate myrmecophytes derived from the facultative species (Fig. 9.6). Under this scenario, facultative myrmecophytes may represent evolutionary precursors to the obligate myrmecophytes. In fact, Risch *et al.* (1977) suggested that the mutualism between ants and *Piper* might have originated with the evolution of large, sheathing petioles. This suggestion and our predictive tree corroborate the findings of Fiala and Maschwitz (1992b) that domatia are the most important plant trait for the development of myrmecophytism. However, additional data and taxa are needed before this hypothesis can be more fully tested.

The ant genera that have been found nesting in the petioles of *P. biseriatum* and *P. imperiale* (e.g., *Crematogaster* spp., *Solenopsis* spp., *Wassmannia* spp., and other species of *Pheidole*) are opportunistically nesting, arboreal ants (Hölldobler and Wilson 1990, Orivel and Dejean 1999). These ants apparently nest in petioles of *Piper* species whenever they encounter one that provides sufficient protection from the environment.

Arboreal ants occasionally nest in cavities formed by other stem-boring insects in stems, petioles, and even the leaf midveins (E. Tepe, pers. obs.). Ward (1991) noted that a number of arboreal pseudomyrmecine ants have a tendency to nest in cavities in living plant parts, as opposed to dead, hollow twigs, as is typical of most opportunistic, arboreal pseudomyrmecines. He suggested that ant species that nest in living plant parts might lend insights into the evolution of obligate ant-plant associations (Ward 1991). However, phylogenetic studies of *Pheidole* are currently unavailable that would allow us to determine the nesting habits of species related to *Ph. bicornis*. Fiala *et al.* (1994) found that the presence of facultative ants in *Macaranga* can dramatically reduce damage by herbivores, and therefore may be important in driving plants toward more complex and mutually beneficial associations. This is likely the case in *Piper* as well.

9.6.2c. *Pearl Bodies*

Fischer *et al.* (2002) studied the chemical composition of pearl bodies of the four hollow-stemmed species of obligate myrmecophytes in *Piper* and found that, with the exception of slightly different levels of soluble carbohydrates and proteinaceous nitrogen in *P. sagittifolium*, pearl body composition did not vary significantly between species. This similarity in pearl body composition could be explained by common ancestry, but may also be explained by selective pressures exerted by the nutritional requirements of the ants. The ants derive the majority of their sustenance from the pearl bodies (Fischer *et al.* 2002). Food bodies are undeniably important, but in *Piper*, as in *Macaranga*, they appear to be second to domatia as the most important factor in the development of obligate ant-plant associations (Fiala and Maschwitz 1992b).

9.6.2d. *Origin of ant-associated plant structures*

In all plant genera that have obligate mutualisms with ants, very few, if any, of the plant parts implicated in the associations evolved completely *de novo*. With the possible exceptions of the Beltian bodies in *Acacia* and the collagen-containing Müllerian bodies of *Cecropia*, all ant-associated plant traits are modifications of preexisting structures (Janzen 1966, Rickson 1973). In *Piper*, the petioles of the obligate species are not fundamentally different from those of many species of sect. *Macrostachys*, except that they are more tightly folded and more consistently closed. In fact, Fiala and Maschwitz (1992b) noted that only *Macaranga* species with a predisposition for domatia developed into obligate myrmecophytes, and this appears to hold true for *Piper* as well. Stem anatomy of hollow-stemmed species appears to differ in that the medulary vascular bundles do not extend as far into the pith as in species with solid stems (Fig. 9.2). The arrangement of vascular bundles is novel, but again, are no more than modifications of preexisting structures.

Although no pearl bodies were found in the petioles of the facultative ant-plant species, we have frequently observed structures resembling pearl bodies on leaf and young shoot surfaces of *P. aduncum*, *P. nigrum*, and *P. tuberculatum* growing in greenhouses and even on leaves of *P. auritum* for sale in a Mexican market in Nashville, TN (E. Tepe, pers. obs.). Furthermore, they have been observed on young shoots and leaves on a number of *Piper* species in the field (L. Dyer, pers. comm.). It is possible that these structures function as generalized ant attractants in some species of *Piper*, in lieu of extrafloral nectaries, but may not have been reported from plants in the field because they are removed continuously by ants and leave no macroscopically visible trace. The difficulty of detecting food bodies on exposed plant surfaces has also been reported in *Macaranga* (Fiala and Maschwitz 1992b); however, microscopically visible traces of food bodies have been observed (Hatada *et al.* 2001). These extrapetiolar bodies have a somewhat different appearance than those found in the petioles of obligate myrmecophytes in that they are recognizably larger and more translucent. However, they also contain lipids and proteins as do the pearl bodies of obligate myrmecophytes (Sudan IV and Bromphenol Blue spot staining respectively; methods from Baker and Baker 1975), and around 2% carbohydrates when freshly extracted contents were measured with a refractometer (E. Tepe, unpubl. data; method from Kearns and Inouye 1993). If, in fact, these bodies are homologous with the pearl bodies found in the petioles of

the obligate species, then pearl bodies, which are so important to the maintenance of ant-plant mutualisms (Fiala and Maschwitz 1992b), are also preexisting structures modified only in size and location.

9.7. CONCLUSIONS

The ITS phylogeny presented here further supports the monophyly of *Piper* sect. *Macrostachys*. Several ambiguities remain, however, due to a lack of variation in ITS. Further examination of the systematic relationships is currently under way, with more variable molecular markers and the addition of the remaining species of sect. *Macrostachys*. Examination of William Burger's hypotheses regarding evolutionary trends and relationships in sect. *Macrostachys* using our phylogenetic analyses reveal that his ideas are astonishingly insightful and are, for the most part, supported by our data. For example, although the *P. obliquum* complex and its allies are not monophyletic, they do represent all of the Costa Rican species of *Macrostachys*. Furthermore, the affinities that he suggested between many species are supported. Burger appeared to be misled by several homoplasious morphological characters, however, in his proposed affinities of *P. sagittifolium* to the rest of the *P. obliquum* complex. He believed that *P. sagittifolium* was rather isolated and "primitive" among Costa Rican *Piper* species. Instead, it appears to be nested well within *Macrostachys* and has retained plesiomorphic characters. Obligate myrmecophytism appears to have evolved independently two to four times. Observed facultative myrmecophytes have evolved one to three times, but the association is predicted to be much more widespread and common than we have observed. The introduction of DNA sequencing and phylogenetic analysis has provided additional data and new insights into relationships and evolutionary patterns, and allows us to revisit previously proposed hypotheses with a renewed perspective.

APPENDIX 9.1

DNA was isolated from silica gel-dried and herbarium leaf material for species of sect. *Macrostachys* and representative outgroup species from other subgenera, using a modified mini-prep CTAB procedure (Doyle and Doyle 1987). DNA was PCR-amplified for the internal transcribed spacers (ITS) of nuclear ribosomal DNA using the published primers of Blattner (1999). PCR products for ITS were sequenced on an ABI 310 or ABI 3100 automated DNA sequencer using ET terminator chemistry, or downloaded from GenBank for four species (Table 9.1). Sequences analyzed included sequences of ITS 1 and 2 and the 5.8s nrDNA. The extreme ends of ITS 1 and 2 were excluded because of dubious sequence quality in some accessions. The aligned matrix was 551 base pairs (bp) long with raw sequences ranging from 525bp in *P. spoliatum* to 538bp in *P. pseudonobile*. Within *Piper* sect. *Macrostachys*, sequences ranged from 93.1% similar between *P. arboreum* and *P. pseudonobile* to 100% similar between *P. cenocladum* and *P. subglabribracteatum*, with a mean of 97.5% similarity. In the aligned matrix, 134 characters were variable and 54 were parsimony informative. Thirteen gaps were coded (see below), seven of which were parsimony informative.

Sequence data were aligned using Clustal W (Thompson *et al.* 1996), and analyzed with PAUP* 4.0 (Swofford 2001) for Parsimony and Maximum Likelihood (ML) analyses

and MrBayes (Huelsenbeck and Ronquist 2001) for Bayesian analyses to construct phylogenies of sect. *Macrostachys*. For the ML and Bayesian analyses, the appropriate model parameters were selected using a Hierarchical Likelihood Ratio Test (HLRT) in Modeltest (Posada and Crandall 1988). The model that best fit the data corresponded to the K80 (K2P) +G model. Gaps were treated as missing data and coded as separate presence/absence characters following the guidelines of Simmons and Ochoterena (2000) and analyzed with Maximum Parsimony. Trees were rooted with outgroups from outside Sect. *Macrostachys* using Fitch Parsimony. Maximum Likelihood and Maximum Parsimony analyses were run employing the following options: 100 random addition sequences, tree bisection reconnection (TBR), MULTREES in effect (Swofford 2001). Bayesian trees were generated using MrBayes (Huelsenbeck and Ronquist 2001), applying the same model used for the ML analysis. The analysis was run for 1,000,000 generations using four Markov Chain Monte Carlo chains and randomly generated starting trees. Trees were sampled every 100 generations, resulting in 10,000 saved trees. To avoid artifacts from the nature of the analysis, the first 2,500 trees were discarded to account for burn-in, i.e., the generations required for the analysis to reach optimality. To retrieve a single tree and the posterior probability values, the remaining 7,500 trees were used to construct a majority rule consensus tree. Branch support was evaluated using bootstrap values (Felsenstein 1985) and Bayesian posterior probabilities (Larget and Simon 1999, Huelsenbeck and Ronquist 2001). Bootstrap support for recovered nodes was estimated from analysis of 1,000 pseudoreplicate data sets using maximum parsimony. All polytomies are considered soft polytomies.

Alternate topologies were evaluated by constraining analyses to correspond to several hypotheses (see text). Because the null hypothesis of the Shimodaira–Hasegawa test is that all trees are equally good explanations of the data, this test is most appropriate for comparing alternate tree topologies (Shimodaira and Hasegawa 1999, Goldman *et al.* 2000). The log-likelihood scores of these constraint analyses were compared to the score of the unconstrained tree using the Shimodaira–Hasegawa test option in PAUP* 4.0 using RELL bootstrap with 1,000 pseudoreplicates and the same model parameters as above. MacClade 4.0 (Maddison and Maddison 2000) was used to map the ant-related traits onto the molecular phylogenies (Cunningham 2001, Omland 2001).

The morphological and anatomical data are based on observations and collections of 79 populations of all of the 15 Costa Rican species of *Piper* sect. *Macrostachys*. In addition, 1,067 herbarium specimens from throughout the range of sect. *Macrostachys* were examined for the presence of hollow stems, ants, pearl bodies, and petiole morphology. Micrographs are hand sections of FAA-fixed stems and petioles collected from living specimens in the field. Unless otherwise indicated, all stem sections are taken from the third internode, and the petiole sections are taken from the midpoint between the stem and the end of the sheathing margins. Sections were stained with Toluidine Blue and photographed with a SPOT High Resolution Digital Camera through a Nikon SMZ-2T dissecting microscope.

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