Molecular Phylogeny of Rubiaceous Epiphytic Myrmecophytes Based on the *atpB-rbcL* Intergene Region of cpDNA

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ABSTRACT Various symbioses from facultative relationships to obligate mutualisms exist between the epiphytic myrmecophytes in 5 genera of Hydnophytinae (Rubiaceae) and the associated ants. However, the phylogenetic relationships and evolutionary processes have not been studied well. In the present study, a molecular phylogenetic relationship based on the DNA sequence of the *atpB-rbcL* intergene region was analyzed in 6 species of 4 genera in Hydnophytinae. The inferred phylogenetic relationship was shown as (*Myrmecodia* spp. + (*Myrmephytum* + (*Anthorrhiza* + *Hydnophytum* spp.))). It was suggested that the ancestral taxon of Hydnophytinae was *Myrmecodia* or *Myrmephytum* because these genera were settled in the basal position in Hydnophytinae used in the present study. In addition, we discuss the evolution of diverse symbioses of Hydnophytinae based on the inferred phylogenetic relationships, together with ecological and geographical information. It is possible that the ancestor evolved as an epiphyte which lived in the nutrient-poor habitat of Southeast Asia and had a specialization for efficient and relatively obligate mutualism with ants by increasing their complex cavities as ant nesting spaces. After *Myrmephytum* and *Anthorrhiza* were respectively evolved in the Philippines and Papua New Guinea, the ancestor of *Hydnophytum* evolved as a xeromorphic tuberous epiphyte in the dry environments of Southeast Asia and acquired facultative symbioses with arboreal ants.

Key words: Hydnophytinae / symbiosis / ant / mutualism / evolutionary process / molecular phylogeny

Epiphytic myrmecophytes in Hydnophytinae (Rubiaceae) have developed complicated symbiotic relationships with ants. The plants can absorb and obtain nutrients from the debris stored inside the cavities of plant tubers by the associated ants while supplying nesting space to the ants (Huxley, 1976; 1978; Rickson, 1979). Hydnophytinae consists of 88 species in 5 genera which are *Hydnophytum* (46 species), *Myrmecodia* (25), *Myrmephytum* (6), *Squamellaria* (3) and *Anthorrhiza* (8), and almost all species in these genera are epiphytic myrmecophytes (Huxley & Jebb 1990; 1991a; Jebb 1993). *Hydnophytum* and *Myrmecodia* are widely distributed in the Asia-Pacific region, and the other three genera are endemic, i.e. *Anthorrhiza* in Papua New Guinea, *Myrmephytum* in the Philippines and *Squamellaria* in Fiji (Jebb, 1991). The genera of ants associated with Hydnophytinae are mainly *Philidris* and *Dolichoderus* (Hymenoptera: Formicidae) (Maeyama *et al.*, 1997). The lower part of the stem forms a swollen tuber with a complex cavity system, in which the arboreal ants often live. The myrmecophytes show no host-specificity for any particular trees (Kiew & Anthonysamy, 1987). Our previous study revealed that symbioses between the myrmecophytes and their associated ants are

remarkably diverse, from facultative relationships to obligate mutualistic ones (Maeyama *et al.*, 1997; Maeyama, 1998). *Hydnophytum* spp. usually have facultative symbioses with the resident ants. Moreover, most species of this genus show xeromorphic adaptations. For example, they have small and leathery leaves and much succulent tuber tissue (Jebb, 1985). However, the species in *Myrmecodia*, *Myrmephytum* and *Squamellaria* tend to have complex mutualistic relationships with ants, and xeromorphism is less developed; the leaves are large and thin, they have many complex cavities used as nesting space for resident ants and less succulent tissue in their tubers. *Anthorrhiza* shows various morphologies, from *Hydnophytum*-like plants to *Myrmecodia*-like ones.

The evolutionary processes for such diverse associations between Hydnophytinae plants and ants is unclear. Discussing the initial stage of the evolution, Miehle (1911) suggested that mutualism requires adaptations from both participants which are too complex to have evolved without intermediate steps. He proposed that the function of cavities was originally trap of rain water and only later the cavities were invaded by ants. Janzen (1974) in contrast, based on a discussion of the physiology of epiphytic myrmecophytes and other plants, suggested that symbiosis with a nutrient-supplying effect for the epiphytic plant must have been the first function. Jebb (1985) stated that *Hydnophytum* was the most primitive genus in the Hydnophytinae genera, based on several morphological characters. In his hypothesis, the other genera *Myrmecodia*, *Myrmephytum*, *Squamellaria* and *Anthorrhiza* have arisen independently from *Hydnophytum* as a result of major evolutionary steps. Relationships among these genera, however, have not been evaluated using modern phylogenetic methodology, and the phylogeny of these groups is still ambiguous.

In the present study, we attempted a molecular phylogenetic analysis using the intergene region between the ATP synthetase β -subunit (*atpB*) gene and the ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) gene of the chloroplast DNA of several species in 4 genera (*Hydnophytum*, *Myrmecodia*, *Myrmephytum* and *Anthorrhiza*) of Hydnophytinae and inferred the evolutionary processes.

MATERIALS AND METHODS

Samples

The 4 genera, *Hydnophytum*, *Myrmecodia*, *Myrmephytum* and *Anthorrhiza*, in Hydnophytinae (Rubiaceae) were used in this study. *Squamellaria* was not analyzed because no samples of the species were available. Taxa used in this study are shown in Table 1. The sequence of *Hydnophytum formicarum* (sample 3) was obtained from the GenBank Data Library (Manen *et al.*, 1994). Manen *et al.* (1994) showed that *Psychotria bacteriophila* (Rubiaceae: Psychotrieae) was the sister taxon of *H. formicarum*. Thus, we used both *P. bacteriophila* and distantly related taxon *Galium verrucosum* (Rubiaceae: Rubieae) as outgroups.

Total DNA extraction and purification

Fresh leaves (about 500-1000 mg) were collected and stored at -80°C until DNA extraction. They were pulverized into a fine powder in liquid nitrogen. DNA was extracted using the CTAB method (Hasebe & Iwatsuki, 1990). After extraction, the DNA was purified using the ultracentrifuge method (Hasebe *et al.*, 1994).

Table 1. List of taxa used in this study.

Species	Location of origin	Accession No.
<i>Anthorrhiza caerulea</i> Huxley & Jebb	Wau, Morobe Pr., Papua New Guinea	AB044146
<i>Hydnophytum formicarum</i> Jack sample 1	Bako, Sarawak Pr., Malaysia	AB044147
<i>Hydnophytum formicarum</i> Jack sample 2	Gaya Island, Sabah Pr., Malaysia	AB044148
<i>Hydnophytum formicarum</i> Jack sample 3*	Botanical Garden, Geneva, Switzerland	X76480
<i>Hydnophytum</i> sp.	Danao, Ormoc, Leyte Pr., The Philippines	AB044149
<i>Myrmecodia longifolia</i> Valetton	Jayapura, Irian Jaya (Western New Guinea), Indonesia	AB044150
<i>Myrmecodia platytyrea</i> Beccari	Wau, Morobe Pr., Papua New Guinea	AB044151
<i>Myrmephytum beccarii</i> Elmer	Danao, Ormoc, Leyte Pr., The Philippines	AB044152
<i>Psychotria bacteriophila</i> Valet.*	Botanical Garden, Geneva, Switzerland	X76481
<i>Galium verrucosum</i> Hudson*	Bonifacio, Corsica Island, France	X76471

*Manen *et al.* (1994)

DNA amplification of the *atpB-rbcL* intergenic spacer of cpDNA

The *atpB-rbcL* intergene region of the chloroplast DNA was amplified by PCR. A pair of primers were used for the amplification. The primer sequences used for the amplification were forward: 5'-GAA GTA GTA GGA TTG ATT CTC-3', and reverse: 5'-TAC AGT TGT CCA TGT ACC AG-3' (Manen *et al.*, 1994). The reaction was performed in an ASTEC PC-700 programmable temperature control system and a GeneAmp 2400 thermal cycler (Perkin-Elmer). The temperature profile was 94°C for 1 min, followed by 35 cycles at 94°C for 30 sec, 45°C for 1 min and 60°C for 3 min (Manen *et al.*, 1994).

Purification of PCR products and sequencing

PCR products were purified using a Prep-A-Gene DNA Purification Kit (Bio-Rad). Purified products were used as a template for the sequencing reaction. The sequencing reaction was performed using the dideoxy-nucleotide cycle sequencing procedure with a Dye-Terminator Cycle Sequencing Kit (Perkin-Elmer) and a GeneAmp 2400 thermal cycler. Electrophoresis and data collection were performed using an automatic DNA sequencer (Perkin-Elmer, model 373S) with a 6% polyacrylamid gel (TOYOBO, Super Reading DNA Sequence Solution), following the recommended procedure. Both strands of the amplified PCR product were sequenced.

Phylogenetic analysis

Sequences were aligned using the Clustal W program package (Thompson *et al.*, 1994), and confirmed with the aligned DNA sequences of Rubiaceae (Manen *et al.*, 1994). Distance-based neighbor-joining tree (Saitou & Nei, 1987) was computed and assessed using Clustal W. Kimura's (1980) 2-parameter distances were computed (Table 2), and 1000 bootstrap replicates were utilized to assess the strength of suggested associations.

RESULTS AND DISCUSSION

The available sequences of the *atpB-rbcL* intergene region were (including gaps) 489 bp (Fig. 1). Nevertheless, the analyzed DNA were not coding regions, there are not so many variant sites among Hydnophytinae (Fig. 1) and both uncorrected and Kimura's (1980) 2-parameter distances among

Table 2. Uncorrected pairwise differences (below diagonal) and Kimura's (1980) 2-parameter distances (above diagonal) in *atpB-rbcL* intergene region

	1	2	3	4	5	6	7	8	9	10
<i>A. caerulea</i>		0.0086	0.0107	0.0129	0.0108	0.0127	0.0149	0.0127	0.0171	0.0661
<i>H. formicarum</i> 1	0.0086		0.0022	0.0043	0.0022	0.0108	0.013	0.0108	0.0153	0.0691
<i>H. formicarum</i> 2	0.0107	0.0021		0.0021	0	0.0129	0.0151	0.0129	0.0174	0.071
<i>H. formicarum</i> 3	0.0128	0.0043	0.0021		0.0021	0.0151	0.0173	0.0151	0.0196	0.0735
<i>H. sp.</i>	0.0107	0.0021	0	0.0021		0.0129	0.0151	0.0129	0.0174	0.0712
<i>Myrmec. longifolia</i>	0.0126	0.0107	0.0128	0.0149	0.0128		0.0021	0.0127	0.0171	0.066
<i>Myrmec. platytyrea</i>	0.0147	0.0129	0.0149	0.0171	0.015	0.0021		0.0149	0.0193	0.0683
<i>Myrmec. beccarii</i>	0.0126	0.0107	0.0128	0.0149	0.0128	0.0126	0.0147		0.0171	0.0709
<i>P. bacteriophila</i>	0.0169	0.0151	0.0171	0.0193	0.0172	0.0169	0.019	0.0169		0.0639
<i>G. verrucosum</i>	0.0629	0.0655	0.0672	0.0694	0.0674	0.0629	0.0651	0.0672	0.0609	

	10	20	30	40	50	60
<i>A. caerulea</i>	ATAGACAATCCCATCTAT-----TCTATGGAATTCGAACCCGAGCTCTATTTACA					
<i>H. formicarum</i> 1	N.....					
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3	G.....					
<i>H. sp.</i>	N.....					
<i>Myrmec. longifolia</i>						
<i>Myrmec. platytyrea</i>						
<i>Myrmec. beccarii</i>						
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>	A.....C....ATTATCTATG....A.....					
	70	80	90	100	110	120
<i>A. caerulea</i>	TTACGATTTCATTAATTTTATCCCATTCAAAAA---GGGTAAATGGGTTGCGTTATAT					
<i>H. formicarum</i> 1	..-T.....					
<i>H. formicarum</i> 2	C..T.....					
<i>H. formicarum</i> 3	C..T.....					
<i>H. sp.</i>	C..T.....					
<i>Myrmec. longifolia</i>						
<i>Myrmec. platytyrea</i>						
<i>Myrmec. beccarii</i>	G.....					
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>	G..C....G.C....AAAA-....G.....					
	130	140	150	160	170	180
<i>A. caerulea</i>	ATATGAAAGGGTATACAAATGATGTATTGGCAAATCGCATGGTTTAATAATCAAACA					
<i>H. formicarum</i> 1						
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3						
<i>H. sp.</i>						
<i>Myrmec. longifolia</i>						
<i>Myrmec. platytyrea</i>	C.....					
<i>Myrmec. beccarii</i>	...C.....					
<i>P. bacteriophila</i>	A...T.					
<i>G. verrucosum</i>	A.....AT....C...G...T....					
	190	200	210	220	230	240
<i>A. caerulea</i>	TTCTGATTAGTTGATAATATTAGTATTCATATTGGTATTAGTTGGGAATTTTGTGAAAG					
<i>H. formicarum</i> 1	G.....T-----					
<i>H. formicarum</i> 2	G.....T-----					
<i>H. formicarum</i> 3	G.....T-----					
<i>H. sp.</i>	G.....T-----					
<i>Myrmec. longifolia</i>	G.....TG.....					
<i>Myrmec. platytyrea</i>	G.....TG.....					
<i>Myrmec. beccarii</i>	TG.....					
<i>P. bacteriophila</i>	GC-TG.....					
<i>G. verrucosum</i>	T..T-----G.....C.....					

Fig. 1. Aligned sequences of the *atpB-rbcL* intergene region (489 bp) of the Hydnophytinae used in this study. Dots indicate identity to the sequence from *Anthorrhiza caerulea*. Dashes indicate gaps.

	250	260	270	280	290	300
<i>A. caerulea</i>	ATTCTCTGTGCAAAATTGCATTAACAATGAATTCGTGTCGAGTAGACCTTGTGTGTGAG					
<i>H. formicarum</i> 1	A.....					
<i>H. formicarum</i> 2	A.....					
<i>H. formicarum</i> 3	A.....					
<i>H. sp.</i>	A.....					
<i>Myrmec. longifolia</i>	A.....					
<i>Myrmec. platytyrea</i>	A.....					
<i>Myrmec. beccarii</i>	A.....					
<i>P. bacteriophila</i>	A.....C.....					
<i>G. verrucosum</i>	C...A.....CA.....					
	310	320	330	340	350	360
<i>A. caerulea</i>	AATTCTTAATTCATGAGTTGTAGGGAGGGATTATGTACACCACAAACAGAGACTAAAGCA					
<i>H. formicarum</i> 1						
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3						
<i>H. sp.</i>						
<i>Myrmec. longifolia</i>						T
<i>Myrmec. platytyrea</i>						T
<i>Myrmec. beccarii</i>						
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>						G
	370	380	390	400	410	420
<i>A. caerulea</i>	AGTGTGGATTCAAAGCTGGTGTAAAGAGTACAAATTGACTTATTATACTCCTGAATAC					
<i>H. formicarum</i> 1						
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3						
<i>H. sp.</i>						
<i>Myrmec. longifolia</i>	TT.....					
<i>Myrmec. platytyrea</i>	TT.....					
<i>Myrmec. beccarii</i>						
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>	G.....A.....					
	430	440	450	460	470	480
<i>A. caerulea</i>	CAAACCAAGATACTGATATCTTGGCAGCATTCCGAGTAACCTCCCAACCTGGAGTTCCG					
<i>H. formicarum</i> 1						
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3						
<i>H. sp.</i>						
<i>Myrmec. longifolia</i>						
<i>Myrmec. platytyrea</i>						
<i>Myrmec. beccarii</i>						
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>	G.....T.....T.....G.....					
	489					
<i>A. caerulea</i>	CCGGAAGAA					
<i>H. formicarum</i> 1						
<i>H. formicarum</i> 2						
<i>H. formicarum</i> 3						
<i>H. sp.</i>						
<i>Myrmec. longifolia</i>						
<i>Myrmec. platytyrea</i>						
<i>Myrmec. beccarii</i>						
<i>P. bacteriophila</i>						
<i>G. verrucosum</i>						

Fig. 1. Continued.

Hydnophytinae are relatively small (<2%; Table 2). Thus, it is suggested that speciation in Hydnophytinae was due to recent and rapid radiation. The phylogenetic tree based on the neighbor-joining method after 1000 bootstrap replicates is shown in Fig. 2. The Hydnophytinae taxa used in this study were shown to be monophyletic, with two Rubiaceae species as outgroups. *Hydnophytum* spp.

Fig. 2. Phylogenetic relationships of the Hydnophytinae based on the neighbor-joining method (1000 replications of bootstrapping). *Psychotria bacteriophila* and *Galium verrucosum* were used as outgroups. The numbers above and below the branches correspond to the percentage of 1000 bootstrap replicates. A.: *Anthorrhiza*, H.: *Hydnophytum*, Myrmec.: *Myrmecodia*, Myrmep.: *Myrmephytum*.

and *Anthorrhiza* were found to be more closely related to each other than either was to *Myrmephytum*. The monophyletic group of *Myrmecodia* spp. was joined to the above three genera. All these relationships were supported by 50% or more of bootstrap values. The inferred phylogenetic relationships suggest that *Hydnophytum* spp. are the most apical taxa in the Hydnophytinae used in this study. Jebb (1985) suggested that *Hydnophytum* is a paraphyletic genus, certain species will be more closely related to the species in other genera than they are to the other species within *Hydnophytum*. To confirm the monophyly of *Hydnophytum*, analysis of numerous species belonging to this genus is needed.

The resultant tree suggests that *Myrmecodia* or *Myrmephytum*, which have relatively complex mutualistic relationships with ants, are basal groups in the Hydnophytinae. If Hydnophytinae's ancestor was *Myrmecodia*/*Myrmephytum* or a *Myrmecodia*/*Myrmephytum*-like tuberous epiphyte, the ancestral plant would have lived in a nutrient-poor habitat and have a specialization for efficient and relatively obligate mutualism with ants by increasing their complex cavities for ant nesting spaces. Considering the ecological information on current *Hydnophytum*, the ancestor of *Hydnophytum* would advance into a habitat with a dry environment, exhibiting the xeromorphic adaptations, in addition the facultative symbiotic relationship with ants, such as that between the present *Hydnophytum* species and its associated ants, would be acquired. Moreover, the phylogenetic relationships presented here

are consistent with the findings that *Anthorrhiza* show various morphologies (Huxley & Jebb, 1991b). Unfortunately, we could analyze only one species of this genus. If more species of *Anthorrhiza* are collected and analyzed, this hypothesis (suggested above) on the evolutionary direction of *Hydnophytum* can be precisely confirmed.

The geographical distribution and inferred phylogeny of Hydnophytinae suggest that Hydnophytinae's ancestor arose in Southeast Asia. Today, species of *Myrmecodia* are widely distributed in this region (Jebb, 1985; Huxley & Jebb, 1991a). The ancestors of *Myrmephytum* and *Anthorrhiza* evolved in the Philippines and Papua New Guinea, respectively. *Myrmephytum* and *Anthorrhiza* are genera mainly defined by corolla lobing and inflorescence character (Huxley & Jebb, 1991b; 1991c). Since the characteristics of the flowers and inflorescence are generally resistant to change (Beattie, 1985), it is suggested that speciation of *Myrmephytum* or *Anthorrhiza* required radical changes over a short time in the evolutionary process.

In the present study, the numbers of analyzed species in Hydnophytinae were insufficient. However, Hydnophytinae are relatively difficult to collect and this study is an important first step to elucidate the phylogenetic relationships and to infer the developmental process of various symbioses between the rubiaceous epiphytic myrmecophytes and their associated ants. Phylogenetic analyses of numerous taxa, including *Squamellaria* and based on molecular findings of other genes are required to understand the whole evolutionary process regarding the complicated symbiotic relationships.

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前山智宏、前川清人、松本忠夫 クロロプラスト *atpB-rbcL* intergene の塩基配列
に基づくアカネ科着生性アリ植物の分子系統学的研究

アカネ科アリノスダマ亜科に属するアリノスダマ類 (5 属 88 種) は、ニューギニア島を中心に分布する着生性のアリ植物であり、共生するアリ類との間に緊密な相利共生関係をもつものから共生関係の弱いものまで、様々な共生関係を示すことが知られている。本研究では、アリノスダマ類の系統関係を明らかにした上で、その進化プロセスの推定を行うことを目的とし、アリノスダマ類 4 属に属する 6 種のクロロプラスト DNA・*atpB-rbcL* intergene の塩基配列を決定し、分子系統樹の構築を試みた。その結果、アリとの高度な共生関係をもつ *Myrmecodia* 属や *Myrmephytum* 属は偽系統群となり、アリとの共生関係が弱く、また乾性適応が進んだ *Hydnophytum* 属が派生的分類群である可能性が示唆された。従って、アリノスダマ類の進化を考えると、多雨林で祖先型の着生性植物が、まずアリ類との共生関係を進化させ、その後一部が乾燥地へと進出し、乾燥適応型の形態を持つ種が出てきたと考察される。