Polyphyly of *Bikkia* Reinw. (Rubiaceae) based on multi-locus sequence analysis (ITS, *rps16*, *trnL-F*), with emphasis on the endemic *Bikkia philippinensis* Val. including its conservation status

Grecebio Jonathan D. Alejandro¹²*, Ian Kevin C. Balete¹, Julian Fabian C. Caagbay¹, Jana May Marie B. Cruz¹, Christine Joy C. Narciso¹, Dominic E. Nazareno¹, Cecilia I. Banag¹², and Millard M. Uy¹³

¹College of Science, ²Research Center for Natural and Applied Sciences, University of Santo Tomas, España Boulevard, 1015 Manila, Philippines, ³School of Science and Engineering, Ateneo de Manila University, Philippines

*Bikkia* Reinw. is a shrubby genus of the tribe Chiococceae s.s. (Rubiaceae) with ca. 20 species. A recent molecular phylogenetic study of the tribe using two markers showed that *Bikkia* is polyphyletic forming two separate clades, the bases of which are habitat and corolla shape. The endemic *Bikkia philippinensis* was excluded in their analysis due to the lack of plant material. The present study utilized three molecular markers (ITS, *rps16*, and *trnL-F*) to test the polyphyly of *Bikkia* as currently circumscribed, determine the phylogenetic position of the *B. philippinensis* in the previously identified two groups, and assess its conservation status. Our combined data tree showed that *Bikkia* is highly polyphyletic. The *B. philippinensis* is nested within the coastal group, together with the type species *Bikkia tetranda*. The result of the molecular analysis is also congruent with the habitat data and morphology wherein *B. philippinensis* was found to thrive in coastal areas and possess infundibular corolla. Finally, *B. philippinensis* was found to be a critically endangered species based on population size.

**Keywords:** *Bikkia*, conservation, endemic, ITS, Philippines, *rps16*, *trnL-F*

**INTRODUCTION**

The Rubiaceae (coffee family) is the fourth most diverse families among Angiosperms with ca. 13,000 species worldwide. Rubiaceous members are found mainly in the tropics and aside from being speciose, the family is known for its high endemicity. Such narrow or restricted distributions can be attributed to the ecologic sensitivity of its species [1]. In the case of the Philippine species, which constitute about 13% of total Rubiaceae worldwide [2], they are very much understudied taxonomically, and previously excluded in molecular phylogenetic studies due to lack

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*To whom correspondence should be addressed
Email: gdalejandro@mnl.ust.edu.ph / balejan@yahoo.com
of leaf material for DNA analyses. This is more evident in the case of endemic Philippine Rubiaceae, wherein it was only after the publication of an updated generic list [2] when significant taxonomic efforts started. Our country has the largest number of indigenous species and four endemic genera of Rubiaceae [3]: Antherostele Bremek. (ca. 5 spp.), Greeniopsis Merr. (6 spp.; [4], Sulitia (Merr.) Rids. (monotypic), and Villaria Rolfe (5 spp.; [5]).

The genus Bikkia Reinw. is a fascinating member of the Rubiaceae tribe Chiococceae sensu stricto (s.s.) with ca. 20 species of shrubs. Eleven species occur in New Caledonia, the remaining species are distributed from New Guinea, Philippines, the Moluccas, Micronesia, Fiji, Tonga, and Niue to the Wallis Islands. Recently, molecular data (ITS and trnL-F) showed that Bikkia is polyphyletic separated into two distinct clades [6]. One clade is formed by the species of New Caledonia with campanulate corollas found in inland forests; the other group consisted of species with funnel-shaped corollas typically coastal in habitat. However, Motley et al. [6] refrained to recommend any solution to resolve polyphyly in the genus. Probable reason is the limited sampling of Bikkia species. The imperfectly known Philippine endemic Bikkia species, B. philippinensis, was not included in the study of Motley et al. [6]. This raises questions as to the molecular phylogenetic placement of B. philippinensis in the two groups currently constituting the genus. Ironically, in spite of being an endemic and the only recorded species of Bikkia in the Philippines, B. philippinensis was not classified in any category in the National List of Threatened Philippine Plants, established by the Department of Environment and Natural Resources of the Philippines [7]. This study likewise provides the first assessment of the conservation status of B. philippinensis.

Modern systematics utilizes a combination of molecular markers to reconstruct robust phylogenies. Oftentimes, multiple markers resolve taxonomic conflicts with better resolution of phylogenetic trees. In Rubiaceae, the internal transcribed spacer (ITS) region of nuclear DNA, rps16 intron and trnL-F region of chloroplast DNA have been proven useful in delimiting relationships even at species level. Most members of the family are with available sequences of these three markers in the GenBank. Hence, we used in our study multiple sequence data (ITS, rps16 and trnL-F) to answer with more certitude the following objectives: (1) to test the polyphyly of Bikkia as currently circumscribed, (2) to determine the phylogenetic position of the Philippine endemic B. philippinensis, and (3) to assess conservation status of B. philippinensis.

**MATERIALS AND METHODS**

**Plant material.** Two plant samples of B. philippinensis were collected in 2010 from Dako Island and Magpupungko, Pilar, Surigao del Norte. The collection sites were based on the type locality indicated on original publications and on previous collections indicated in the herbarium specimens from different herbaria (NY, K, MO, A, L, PNH, CAHUP). Such field survey allowed for a detailed morphological study and the assessment of the conservation status of B. philippinensis. For the molecular analysis, leaf samples were air-dried for 1 day, cut into 2”×2” pieces and then placed in a zip-lock bag containing silica gel [8]. The zip-lock bags were properly labeled with the corresponding codes (BP01 and BP02).

**DNA extraction and amplification.** The Qiagen DNeasy Plant Mini Kit (Germany) was used for genomic DNA extraction following the manufacturer’s protocol.
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DNA samples were amplified using a PCR cocktail with the following proportions in 25 μL: 15.3 μL of water, 2.5 μL of 10× buffer, 2.0 μL of MgCl, 1.5 μL of 2 mM dNTP, 1 μL of 5 pmol forward primer, 1 μL of 5 pmol reverse primer, 0.2 μL of Taq polymerase and 1.5 μL of DNA sample. For the amplification of the ITS region, primers p17F (5’-CTA CCG ATT GAA TGG TCC GGT GAA-3’) and 26S–28R (5’-TCC CGG TTC GCT CGC CGT TAC TA-3’) [9] were used. For the rps16 intron, the primers rps16F-1f (5’-GTG GTA GAA AGC AAC GTG CGA CTT-3’) and rps16-2r (5’-TCG GGA TCG AAC ATC AAT TGC AAC-3’) were used [10]. The trnL-F region was amplified using primer pair primer pair c (5’- CGA AAT CGG TAG ACG CTA CG-3’) and f (5’- ATT TGA ACT GGT GAC ACG AG-3’) [11].

PCR reactions were run using a Biometra T personal cycler. PCR reactions for ITS started with initial denaturation for 90 sec at 97°C, followed by 35 cycles of 20 sec at 97°C, 90 sec at 72°C for the annealing of primers and, 30 sec at 72°C for the primer extension and ended with a final extension phase of 7 min at 72°C.

PCR parameters for rps16 intron and trnL-F amplification started with the initial denaturation of 5 min at 80°C. Thirty five cycles at 94°C for 30 sec; 50–55°C for 30 sec; 72°C for 1 min was done for the secondary denaturation, annealing and extension, respectively. The whole amplification terminated at 75°C for 5 min.

**Agarose gel electrophoresis.** To confirm the presence of amplicons, samples were subjected to 1% agarose gel electrophoresis. The agarose gel was prepared by adding 0.8 g of Sigma Type VII agarose powder to 80 mL of 1× TAE buffer. The solution was then cooled without allowing it to solidify at room temperature. On a parafilm, 4 μL of gel loading dye was placed and 4 mL of the amplified DNA was mixed. The electrophoresis current was set at 80 V for 40 min. Subsequently, the gel loaded with samples was left in a solution with ethidium bromide. The gel was viewed using a UV Transilluminator (UVP DigiDoc-ItTM) and the bands were recorded using digital gel documentation (LaunchDoc).

**DNA purification and sequencing.** The PCR products were cleaned following the protocols of QIAquick Purification Kit (Germany). Each purified DNA sample totals to about 35 μL. Purified DNA were then subjected to agarose gel electrophoresis (Fig. 1). Purified DNA samples were sent to Macrogen (Seoul, Korea) and Department of Plant Systematics, University of Bayreuth for sequencing.

![Figure 1](image-url). Purified DNA bands of *B. philippinensis* (A) ITS, (B) rps16, and (C) trnL-F (BP01 and BP02)
**DNA sequence analysis.** CodonCode Aligner v.3.0.1 was used to assemble and manually edit the forward and reverse sequences. Subsequently, the sequences were assembled using MacClade v.4.0 [12] for alignment and the excision of unnecessary bases. Additional DNA sequences were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) and post-assembled in MacClade v. 4.0. Sequences gathered represent taxa from Chiococceae s.s. and two outgroup taxa, *Cinchona* and *Guettarda*, were included for character polarity [13]. Parsimony analysis of the separate and combined sequence data sets were performed using Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0b [14] on a Power Macintosh G3 computer using heuristic searches, with the MULTREES option on, tree-bisection-reconnection (TBR) branch swapping, swap on best only in effect, and 10,000 random addition sequences. In all the analyses, characters were given equal weight, and gaps were treated as missing data. The length of the tree (L), consistency index (CI) [15] and retention index (RI) [16] were calculated to estimate the levels of homoplasy. Bootstrap (BS) [17] analysis was performed to assess relative statistical support for identified clades, using 5000 replicates, the MULTREES option off, nearest neighbor interchanges (NNI) branch swapping, and five random addition sequence. Clades that received the bootstrap support of 86–100% were treated as strongly supported, 70–85% as moderately supported, and 50–69%, weakly supported [18].

**RESULTS AND DISCUSSION**

**Sequence characteristics and variation.** Separate (ITS, *rps16* and *trnL-F*) and combined analyses included 30 sequences each of taxa. Six sequences of *B. philippinensis* from the three molecular markers are newly published here. Although the ITS data set has the shortest matrix length (Table 1), this marker yielded the highest number of parsimony informative characters (118 bp), followed by the *trnL-F* (71 bp) and *rps16* intron (47 bp). The aligned combined data set consisted of 2502 bp and a total of 259 parsimony informative characters (Table 1). Statistics of PT, L, CI and RI are summarized in Table 1. Genetic variation across the two cpDNA regions is low allowing alignment without difficulty. Few ambiguous parts in the aligned ITS

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<th>Table 1. Dataset matrix characteristics and tree information</th>
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<td><strong>Matrix Length</strong></td>
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<td>No. of informative characters</td>
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<td>No. of most parsimonious trees (PT)</td>
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<td>Tree Length (L)</td>
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<td>Consistency Index (CI)</td>
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<td>Retention Index (RI)</td>
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**Figure 2.** Scanning electron micrograph of the spinulose pollen of *B. philippinensis*, characteristic of tribe Chiococceae s.s. (A) Whole pollen grain and (B) Details of microspines (SEM photo taken by Meve U & Alejandro GJD)
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were observed but excluding them did not change the ITS tree topology. Hence, we decided to include all characters from the three markers.

**Polyphyly of *Bikkia***. The phylogenetic relationships within Chiococceae s.s. in our combined tree (Fig. 3) are almost congruent
Bikkia is clearly polyphyletic in its current circumscription because the New Caledonian species (labeled A) are found in a clade together with the monotypic Morierina, while the other species are found in another clade (labeled B) as sister to Badusa (Fig. 3). In contrast to Motley et al. [6], Morierina is here placed as sister to the New Caledonian Bikkia with strong support (BS=100). Moreover, the two clades of Bikkia gained more support in our combined tree compared to that of Motley et al. [6].

The tribe Chiococceae was defined by a combination of two synapomorphic features: stamens inserted at the base of corolla and spinulose pollen [6]. However, stamen insertion at the base of corolla is also found in the tribe Hamelieae. Thus, spinulose pollen (Fig. 2) should be retained as the only synapomorphy of Chiococceae s.s.

**Phylogenetic position of B. philippinensis.** Our separate trees and combined tree (Fig. 3) showed that B. philippinensis is nested within the coastal Bikkia group. These molecular results are congruent with the habitat and morphology of the Philippine Bikkia species. Similar to other Bikkia species found in this clade, B. philippinensis was found to thrive in coastal environments, having solitary white flowers and infundibular (funnel-shaped) corollas (Fig. 4). Furthermore, the widespread B. tetrandra, the type species of the genus is also found in this clade.

**Proposed taxonomic solution.** As the genus Bikkia was found to be polyphyletic based on our three-gene analysis and that of Motley et al. [6], we propose the coastal species to be retained as Bikkia since it includes the type species B. tetrandra. The New Caledonian inland forest Bikkia should be transferred to another name, since they also differ with the coastal group in terms of flower morphology (Fig. 4).

**Conservation status of B. philippinensis.** Field work based on type localities indicated on herbarium specimens, photographs from online databases and original publications provided us information to assess the conservation status of the only Bikkia species in the Philippines. Here, we used the IUCN Red List Categories and Criteria [19] in classifying the species. Criterion E was not used because no quantitative analysis was performed on the taxon.

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**Figure 4.** Bikkia philippinensis, (A) the habitat of the species is coastal and (B) infundibular corolla of B. philippinensis distinct of coastal Bikkia species.
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B. philippinensis Val. The species is restricted along the coasts of Dako island and Magpupungko, Surigao del Norte, Mindanao. Critically Endangered [CR C2a(i)]; C2, population size estimated to number fewer than 250 mature individuals (B. philippinensis: <100 individuals in all areas surveyed); a(i), no subpopulation estimated to contain more than 50 mature individuals (B. philippinensis: <50 mature individuals in each subpopulation).

REFERENCES