

# Molecular phylogeny of the genus *Bikkia* (Rubiaceae) including a new endemic Philippine inland forest species *Bikkia montoyae*

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The genus *Bikkia* Reinw. (Rubiaceae) was formerly described as heterogeneous in terms of its habitat and corolla shape. Subsequently, a group of New Caledonian endemics was transferred to a genus of its own, *Thiollierea* Montrouz. (drooping flowers, inland habitats), leaving *Bikkia* (erect flowers, coastal habitats) with about 10 species worldwide. *Bikkia philippinensis* Valetou, the only species that occurs in the Philippines, is found in coastal areas of Cebu and the Siargao Islands. Recent observation of herbarium specimens at Central Mindanao University Herbarium revealed a divergent *Bikkia* species collected from the inland forests of Mt. Redondo, Dinagat Island. Comparative evaluation was conducted using morphological and molecular data. Nuclear internal transcribed spacer and chloroplast (*rps16* and *trnL-F*) regions were sequenced and analyzed from two isolates of *B. philippinensis* and four isolates of the *Bikkia* species from Mt. Redondo. Phylogenetic analyses showed that the inland

forest *Bikkia* species from Mt. Redondo was nested within a group of purely coastal species of *Bikkia* (BS=90%) but not with *B. philippinensis*. The two Philippine *Bikkia* species also differ morphologically, mainly in reproductive features. We propose to name this species *Bikkia montoyae* and provide a botanical illustration and discuss the conservation status of both Philippine species. We note that *Bikkia* can include both coastal and inland species.

## INTRODUCTION

The genus *Bikkia* Reinw. of the Rubiaceae (coffee family) was considered to comprise about 20 species (Darwin 1985) distributed throughout the Western Pacific. Most species were believed to be endemic to New Caledonia, with others found in New Guinea, the Philippines, Moluccas, Micronesia, Fiji, Tonga, and Niue, to the Wallis Islands (Barrabé et al. 2011). Motley et al. (2005) published the first molecular study of the Catesbaeae-Chiococceae Complex (CCC) which included seven species of *Bikkia* based on internal transcribed spacer (ITS) (nrDNA) and *trnL-F* (cpDNA) data. Their results suggested that the genus is not monophyletic, forming two well-supported groups: one

## KEYWORDS

*Bikkia*, conservation, cpDNA, nrDNA, Philippine endemic

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strictly coastal group with funnel-shaped corollas and the other comprising species from inland forests with campanulate corollas. Barrabé (2006) supported the findings of Motley et al. (2005) in a phylogenetic study of *Bikkia* including all New Caledonian species based on morphological data.

Earlier authors (e.g., Montrouzier 1860, Brongniart 1865, Brongniart and Gris 1871) previously suggested the exclusion of the New Caledonian endemics from *Bikkia*. Aside from the different corolla shape, the ten species of *Bikkia* endemic to New Caledonia are restricted to ultramafic soils from low to high elevations (Jaffré et al. 1994). This delimitation of *Bikkia* was supported by the phylogenetic studies conducted by Motley et al. (2005) and Barrabé (2006). Subsequently, Barrabé et al. (2011) transferred all New Caledonian species (drooping flowers and inland habitats) to a genus of their own, *Thiollierea* Montrouz., leaving the genus *Bikkia sensu stricto* (*s. str.*) with ten species (erect flowers and coastal habitats).

The poorly known Philippine endemic *Bikkia philippinensis* Valetton was not included in any of the previously studies. This species with erect flowers has been recorded in the coastal areas of Mualbual, Cebu (type specimen) and Siargao islands (Fig. 1A and B). Based on morphology and habitat, *B. philippinensis* is clearly part of *Bikkia s. str.* Interestingly, a recent observation of herbarium specimens at Central Mindanao University Herbarium (CMUH) revealed a morphologically aberrant species of *Bikkia* collected from the inland forest of Mt. Redondo, Dinagat island (Fig. 1C). The atypical location of collection did not correspond with the usual coastal habitats of *Bikkia s. str.*, raising questions on its identity.

In addition to morphological analyses, molecular markers are commonly used as a source of characters for more reliable phylogenetic and taxonomic studies. Oftentimes, combinations of multiple markers reveal better resolved phylogenetic trees. In Rubiaceae, the ITS (nrDNA), *rps16* intron and *trnL-F* regions (cpDNA) have been useful in delimiting species relationships (e.g., Alejandro et al. 2005, 2010, 2011). Therefore, we used multiple sequence data (ITS, *rps16* and *trnL-F*) to (1) determine the phylogenetic relationships of *B. philippinensis* and the *Bikkia* species found on Mt. Redondo with the currently recognized groups of *Bikkia* and *Thiollierea*, (2) provide a comprehensive morphological description of the *Bikkia* species and compare it with *B. philippinensis*, and (3) for the first time assess the conservation status of the two Philippine species of *Bikkia*.

## MATERIALS AND METHODS

### Taxon Sampling

Two isolates of *B. philippinensis* (Fig. 1A and B) from Dako Island and Magpupungko, Pilar, and four isolates of the *Bikkia* species (Fig. 1C and D) found on Mt. Redondo, Dinagat island, Surigao del Norte, were collected. The sites were chosen based on examined previous collections indicated on specimens

**Table 1.** Nucleotide sequence database accession numbers of the new Philippine *Bikkia* species used in this study.

Taxon	EMBL accession number		
	ITS	<i>rps16</i>	<i>trnL-F</i>
<i>Bikkia philippinensis</i> 1	HG810911	HG810905	HG810899
<i>Bikkia philippinensis</i> 2	HG810912	HG810906	HG810900
<i>Bikkia montoyae</i> 1	HG810913	HG810907	HG810901
<i>Bikkia montoyae</i> 2	-	HG810908	HG810902
<i>Bikkia montoyae</i> 3	-	HG810909	HG810903
<i>Bikkia montoyae</i> 4	HG810914	HG810910	HG810904

from different herbaria [Nationaal Herbarium Nederland, Leiden University branch; CMUH; and New York Botanical Garden]. The field survey allowed for a detailed morphological study and herbarium preparations including assessment of the conservation status of both species. The reproductive parts were preserved in 70% ethanol. For the molecular analysis, leaf samples were air-dried for 1 day, cut into 2" x 2" pieces and then placed in a zip-lock bag containing silica gel (Chase and Hills 1991) and properly labeled with corresponding codes.

### Morphological Analysis

Comparative morphological examinations of all collected *Bikkia* specimens were done. The reproductive parts of the plant were dissected to examine the inner parts. For the scanning electron microscopy examinations (Philips XL-30), preserved pollen structures were dehydrated in alcohol series and FDA (formaldehydedimethylacetal) and subsequently critical-point-dried. The dried samples were mounted on aluminium stubs which were covered with PVA glue and then sputter-coated with gold (Alejandro et al. 2010).

### DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted from silica gel-dried leaf tissues using a DNeasy Plant Mini kit (Qiagen, Germany). One nuclear region (ITS) and two chloroplast regions (*rps16* and *trnL-F*) for which sequences were available for representatives of CCC (Motley et al. 2005) were selected for amplification. The primer pair used for amplification and sequencing of the ITS region was P17F and 26S-28R (Popp and Oxelman 2001). The *rps16* intron was amplified and sequenced using *rps16-1F/rps16-2R* (Oxelman et al. 1997) while the *trnL-F* region was done using the primer pair *c/f* (Taberlet et al. 1991, Bremer et al. 2002). PCR reactions were performed following methods in previous studies (Alejandro et al. 2005, 2010, 2011). Amplified DNA was cleaned with the QiaQuick PCR purification kit (Qiagen). All sequences were retrieved by the commercial services of Macrogen, Korea. EMBL accession numbers of all new ITS, *rps16* and *trnL-F* sequences of all Philippine *Bikkia* specimens generated are listed in Table 1.

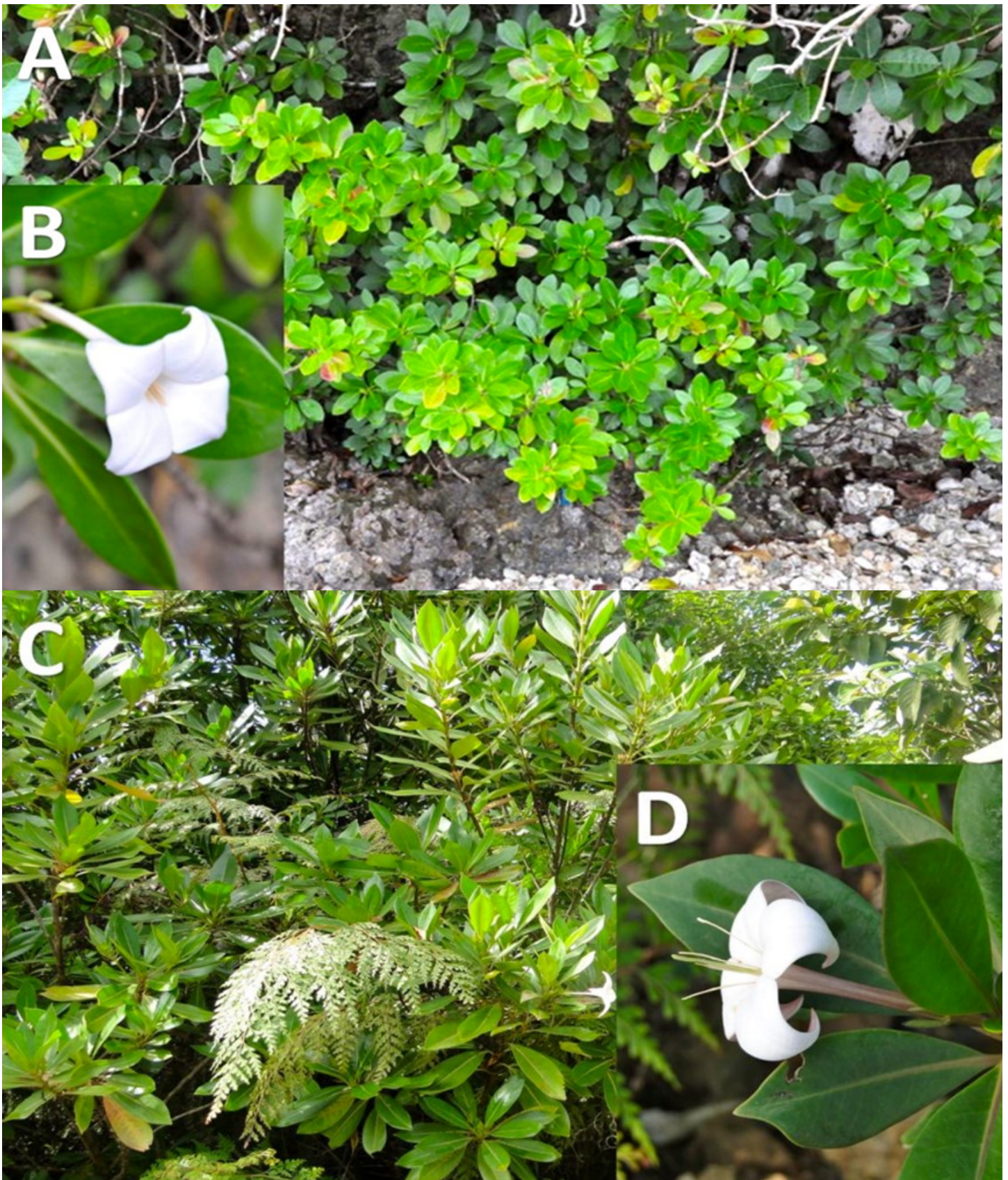


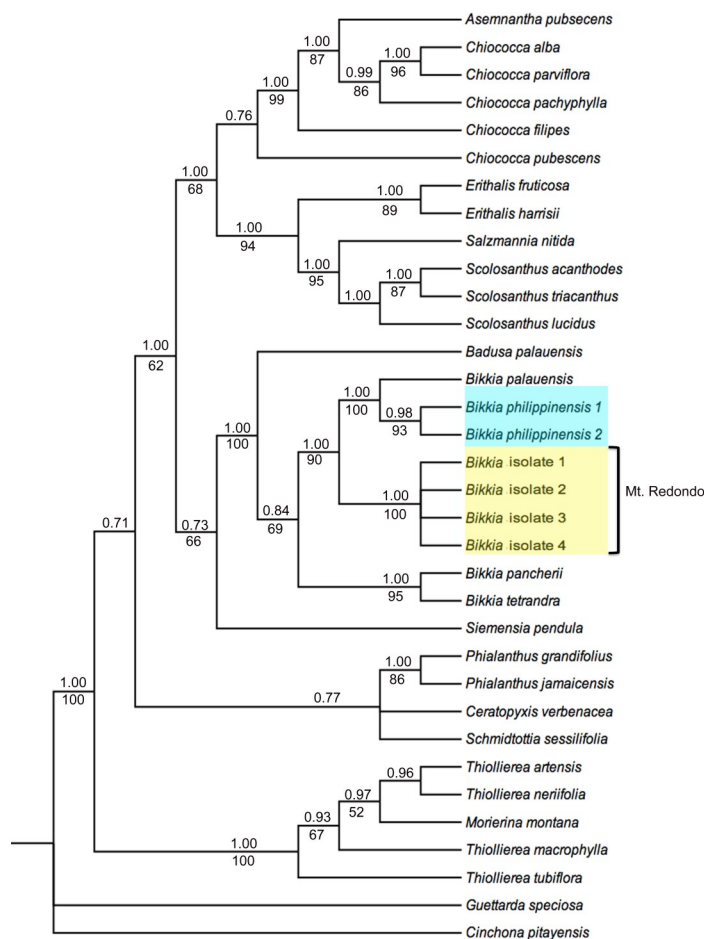
Figure 1. Habit and enlarged flowers of Philippine *Bikkia* species. A, *B. philippinensis*; B, flower of *B. philippinensis*; C, *Bikkia* species (Mt. Redondo); E, flower of *Bikkia* species. Photos taken by I Balete (A,B) and GJD Alejandro (C,D).

**Table 2.** Dataset matrix characteristics and tree information.

	ITS	<i>rps16</i>	<i>trnL-F</i>	Combined
Matrix Length	649	821	1,032	2,502
No. of informative characters	157	45	96	298
No. of most parsimonious trees (PT)	180	45,045	1,764	65
Tree Length (L)	499	104	383	1,004
Consistency Index (CI)	0.66	0.90	0.93	0.78
Retention Index(RI)	0.74	0.91	0.91	0.78

## DNA Sequence Analysis

CodonCode Aligner v.3.0.1 (CodonCode Corporation, USA) was used to assemble and manually edit the forward and reverse sequences. The sequences were assembled using MacClade v.4.0 (Maddison and Maddison 1992) for alignment and the excision of unnecessary bases, and subsequently edited manually. Additional DNA sequences were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>) to represent taxa from Chiococceae (Motley et al. 2005) and post-assembled in MacClade v.4.0. Two outgroups (*Cinchona* and *Guettarda*) were included for character polarity (Bremer et al. 1999). Bayesian inference (BI) was used to estimate phylogenetic positions of the Philippine endemic *Bikkia* species. The analysis was carried out using the MrBayes v.3.1.2p software (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Altekar et al. 2004). Model selection for the best-performing evolutionary models was determined under three model selection criteria: (a) Akaike Information Criterion (AIC) (Akaike 1974), (b) AICc (second order criterion of AIC, necessary for smaller samples), and (c) the Bayesian Information Criterion (BIC) (Schwartz 1978). The selected models used the method of Hasegawa et al. (1985) for ITS and the generalized time reversible + gamma method of Evans and Sullivan (2012) for *rps16* and *trnL-F*. In analyzing a single marker, the best performing model was selected and a one-million generation was considered with a sample frequency of 1000 and four parallel chains. For combined analyses, model selection, as well as the settings, were similar to that of the single-marker analysis; however, there were a total of three million running generations. Clades with posterior probability (PP) exceeding 0.95 were regarded as strongly supported. To compare the topology of the BI, Parsimony analysis of the separate and combined sequence data sets was performed using Phylogenetic Analysis Using Parsimony (PAUP\*) version 4.0b (Swofford 2000) 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) (Kluge and Farris 1969) and retention index (RI) (Farris 1989) were calculated to estimate the levels of homoplasy. Bootstrap (BS) analysis (Felsenstein 1985) was performed to assess relative statistical support for identified



**Figure 2.** Majority rule consensus tree inferred from the combined ITS-*rps16*-*trnL-F* sequence data. Numbers above nodes are Bayesian posterior probabilities and below branches indicate bootstrap values >50%. Blue and yellow boxes indicate Philippine *Bikkia* species.

clades using 5,000 replicates, the MULTREES option off, nearest neighbor interchanges (NNI) branch swapping, and five random addition sequence replicates. Clades that received the bootstrap support of 86-100% were treated as strongly supported, 70-85% as moderately supported, and 50-69% as weakly supported (Alejandro et al. 2005).

## RESULTS AND DISCUSSION

### Sequence Characteristics and Variation

The separate (ITS, *rps16*, and *trnL-F*) and combined analyses included 34 sequences. Sixteen sequences of *B. philippinensis* and the *Bikkia* species (Mt. Redondo) from the three molecular markers are newly published here. Although the ITS data set has the shortest matrix length (Table 2), this marker yielded the highest number of parsimony informative characters (157 bp), followed by the *trnL-F* (96 bp) and the *rps16* intron (45 bp). The aligned combined data set consisted of 2,502 bp and a total of 298 parsimony informative characters (Table 2). Statis-

tics of parsimonious trees (PT), L, CI, and RI are summarized in Table 1. Genetic variation across the two cpDNA regions was low, allowing alignment without difficulty. Few ambiguous parts in the aligned ITS were observed, but excluding them did not change the ITS tree topology. Hence, we decided to include all characters from the three markers.

### ***Bikkia* vs. *Thiollierea***

Our results (Fig. 2) agree with the phylogenetic analysis of Motley et al. (2005) and Barrabé (2006) that *Bikkia s. str.* should not include the New Caledonian species, prompting Barrabé et al. (2011) to transfer all *Bikkia* endemic to New Caledonia to a genus of its own, the *Thiollierea*. *Bikkia* and *Thiollierea* differ from each other by the texture of abaxial lamina, form and shape of stipules, growth direction of flowers, marginal form of calyx lobes, appearance of anthers, indumentum of filaments, orientation of ovules, number of locules, and form and shape of seeds (Barrabé et al. 2011).

### **Habitat of *Bikkia***

Motley et al. (2005) and Barrabé et al. (2011) restricted *Bikkia s. str.* to comprise only those species belonging to the coastal *Bikkia* clade. In contrast, the inclusion of the *Bikkia* species from Mt. Redondo shows that *Bikkia s. str.* includes both coastal and inland forest species. The *Bikkia* species from Mt. Redondo has a habitat that is similar to that of *Thiollierea* (inland habitats on ultramafic substrates) and shows that *Bikkia s. str.* is not exclusively found on calcareous substrates derived from coral. Hence, our findings delimit the habitat of *Bikkia*. All other features of *Bikkia* sensu Barrabé et al. (2011) are applicable to the *Bikkia* species from Mt. Redondo: shrubs; stipules free and acuminate; leaves granular when dried; inflorescences erect, flowers 4-merous, calyx lobes with involute and free margins, corolla with indument to the base on the inner surface; anthers straight, filaments basally pilose; ovules with quincuncial arrangement, 7 or 8 series of globose ovules per placental arm, more than 500 ovules per locule; and seeds rhomboid, laterally compressed.

### **Phylogenetic Positions of the Philippine *Bikkia* Species**

Similar to the molecular study by Motley et al. (2005), our combined tree (Fig. 2) suggests that *Bikkia s. str.* is closely related to *Badusa* A. Gray with high support (PP=1.0, BS=100%), a genus that is likewise associated with calcareous substrates, often in coastal areas. The monotypic genus *Siemensia* Urb. was found sister to the *Badusa-Bikkia* group in our combined tree (PP=0.73, BS=66%) probably due to their similar habitat. More data are needed to confirm the phylogenetic position of *Siemensia* which has been recorded in limestone haystack mountains of the Pinar del Rio province, Cuba (Liogier 1962).

Our separate trees (not shown here but available from the first author) and combined tree (Fig. 2) showed that *B. philippinensis* (shaded blue) and the *Bikkia* isolates 1-4 from Mt. Re-

dondo (shaded yellow) are nested within the coastal *Bikkia* group. These molecular results are congruent with the morphology of the Philippine *Bikkia* species. *Bikkia philippinensis* and *Bikkia* from Mt. Redondo possess erect solitary white flowers and infundibular corollas (Fig. 1B and D) like other *Bikkia* species found in this clade. However, the two Philippine *Bikkia* species were not sister species. Rather, *B. philippinensis* was recovered as sister to *B. palauensis* from Palau with strong support (PP=1.0, BS=100%). This subgroup is sister to all the sampled *Bikkia* species from Mt. Redondo with high support (PP=1.0, BS=90%). This clade in turn was recovered as sister (PP=0.84, BS=69%) to *B. pancherii* (distributed in New Caledonia, New Guinea, Solomon Island, and Vanuatu) and *B. tetrandra* from the Western Pacific, and the type species of the genus. Only half of *Bikkia s. str.* representatives are included here and inclusion of more species is required to fully understand the phylogeny of the genus.

### **A New Species of Philippine *Bikkia***

The *Bikkia* found on Mt. Redondo formed a group of its own not directly related to *B. philippinensis* (Fig. 2). Our molecular results are supported by morphology. The two *Bikkia* species differ mainly in their reproductive structures. The *Bikkia* from Mt. Redondo is characterized by long calyx lobes (1.5–2 cm), square (with 4 ridges) calyx tube, large corollas (tube 7.2–10.7 cm long, lobes 3.5–4.5 x 2–2.5 cm) with purple shade externally, long and well exerted stamen and style (Fig. 1D), and shows longitudinal cells in the arils of seeds (Fig. 3H). Moreover, the pollen of *Bikkia* from Mt. Redondo shows more spines that are longer (Fig. 4C and D). Therefore, we propose a new species of *Bikkia* endemic to the Philippines. The nomenclature, diagnosis, description, and full illustration of this new species are found below.

### ***Bikkia montoyae* Mejillano, Santor and Alejandro, sp. nov.**

**Type:** Philippines, Surigao del Norte, Dinagat Island, Loreto, Mt. Redondo [10°35'N, 125°64'E], May 22 2011, Mejillano, Santor and Alejandro 11-082 (holotype, USTH!; isotypes, PNH!, CMUH!). Fig. 3.

**Diagnosis:** This inland forest species of *Bikkia* differs from coastal *B. philippinensis* by having longer (1.5–2 cm) calyx lobes, bigger corollas (tube 7.2–10.7 cm long, lobes 3.5–4.5 x 2–2.5 cm) shaded purple externally, longer and well exerted stamen and style, and presence of longitudinal cells in the arils of seeds.

Shrubs or small trees, 1–4 m; stems herbaceous, braches glabrous. Leaf blades isophyllous, lanceolate to elliptic or obovate, 6.5–14.5 x 1.5–4.2 cm, thick, leathery, glabrous, broadly acute to acuminate at apex, attenuate at base, 5–15 lateral veins; petioles 1.2–2 cm long, green or deep purple, glabrous. Stipules free, widely triangular to widely ovate, 3–4 x 4–5 mm, green, glabrous.

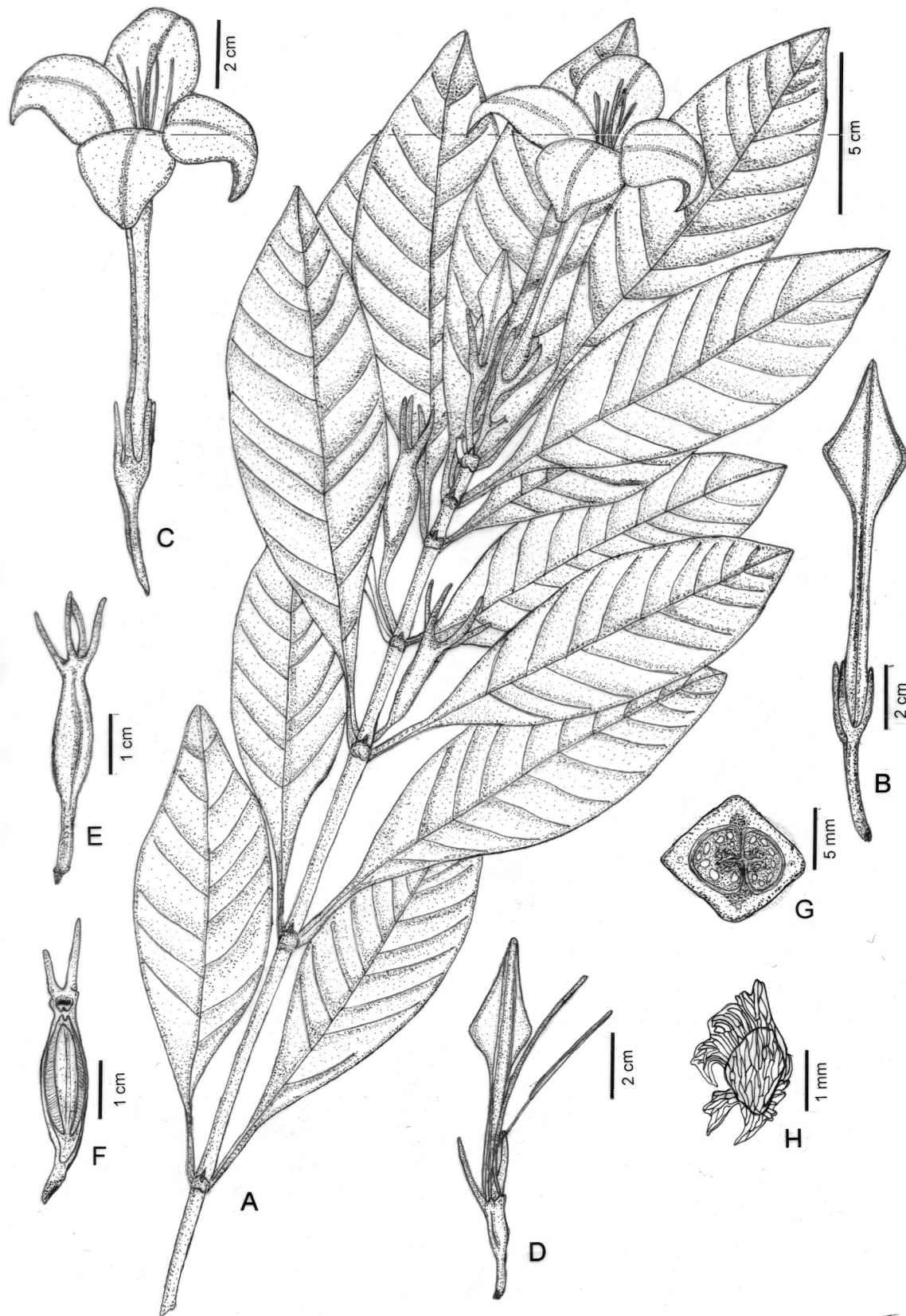
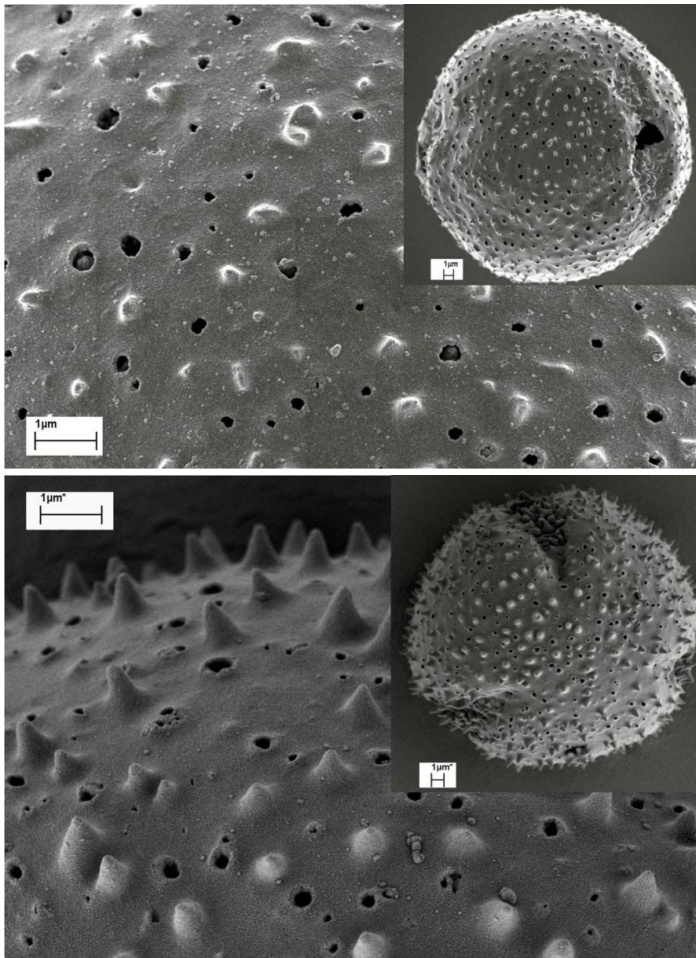


Figure 3. *Bikkia montoyae* Mejillano, Santor & Alejandro. A, flowering branch; B, corolla bud; C, flower; D, opened corolla showing anther and style; E, fruit; F, longitudinal section of fruit; G, cross section of ovary; H, seed. Drawn by P. Santor. From Mejillano, Santor & Alejandro 11-082 (USTH).



**Figure 4.** Pollen morphology of *Bikkia philippinensis* (above) and *B. montoyae* (below). Photos taken by U. Meve & GJD Alejandro.

Inflorescences axillary to terminal, solitary; bracts small. Flowers actinomorphic, bisexual, glabrous; pedicels 1–1.5 cm long, glabrous. Calyx tube narrow, square, 9–13 x 3–4 mm, green, glabrous; lobes 4, linear to lanceolate, connate at the base, 1.5–2.5 cm long, 2 mm wide, green, glabrous, colleters present at the base. Corolla tube infundibular, square, 7.2–10.7 cm long, 3–4 mm wide, purple shaded, glabrous; lobes 4, connate at the base, widely trullate, 3.5–4.5 cm long, 2–2.5 cm on the widest part, glabrous on both sides. Stamens 4, yellow; anthers didymous, basifixed, exserted, 2–2.9 cm long, glabrous; filaments extending up to the base of corolla tube, 4.7–6.7 cm long; glabrous on the upper ¼, hairy on the lower ¾. Style cylindrical, exserted, 7–8 cm long, 1 mm wide, glabrous; stigmatic lobes 2, linear, 3–5 x 1 mm, glabrous. Ovary bilocular, placentation axile; ovules numerous, 0.3–0.5 mm long. Fruits ellipsoid, capsular with 2 locules, dehiscent by 4 sutures, 2 septicidal and complete, 2 loculicidal and partial, with 4 ridges, calyces persistent, 2–2.5 cm long, 8–10 mm wide, green, glabrous; stalk 1–1.5 cm long, glabrous. Seeds numerous, laterally compressed, angled, 1–1.5 mm x 1 mm, aril longitudinal, glabrous.

**Distribution and habitat:** Restricted to Paragua and Mt. Redondo, Dinagat Island, Surigao del Norte, and Mt. Hamiguitan Range, Davao Oriental; from low to high elevations, on ultramafic soils.

**Etymology:** The specific epithet is coined in honor of Academician Jaime C. Montoya.

**Phenology:** Flowering May–June; fruiting May–August.

**Additional specimens examined:** Surigao del Norte: Dinagat Island, Paragua, May 2011, Mejillano, Santor and Alejandro 11-069 (PNH, USTH); Loreto, Mt. Redondo, May 2011, Mejillano, Santor and Alejandro 11-070 (CAHUP, USTH <2 sheets>, PNH), 11-071 (CAHUP, USTH). Mt. Hamiguitan, May 29, 2005, Amoroso, Polizon and Opiso (CMUH, 2 sheets). Loreto, Redondo, May 23, 2011, Amoroso and Coritico (CMUH # 00008154, 2 sheets). Loreto, Mt. Redondo, May 2, 2011, Amoroso and Coritico (CMUH # 8158, 6 sheets).

**Discussion:** *Bikkia montoyae* and *B. philippinensis* are very similar vegetatively except for minor variations in the shape of leaf blades including apex and number of secondary veins. However, the two species differ significantly in reproductive features. This species growing on ultramafic soils is restricted to Mt. Hamiguitan and Dinagat Island, particularly in Paragua and Mt. Redondo. These sites have recently yielded other new plant species, as well as site endemic species such as the fern *Lindsaea hamiguitanensis* (Karger et al. 2012), pitcher plants *Nepenthes micramphora* and *N. hamiguitanensis* (McPherson and Amoroso 2011), and *N. peltata* (Kurata 2008). Clearly, these isolated habitats with unusual soils harbour a unique flora and site endemic species that are of special conservation concern (Amoroso and Aspiras 2011).

#### Conservation Status of Philippine *Bikkia*

Previously collected herbarium specimens and recent fieldwork conducted provided information with which to assess the conservation status of *Bikkia* in the Philippines. Here, we used the IUCN Red List Categories and Criteria (IUCN 2001) in classifying the species. Criterion E was not used because no quantitative analysis was performed on the taxon.

***Bikkia philippinensis*:** The species is restricted to the coasts of Dako Island and Magpupungko, Surigao del Norte and Mualbual, Cebu. **Critically Endangered** [CR C2a(i)]; C2, population size estimated to number fewer than 250 mature individuals (*B. philippinensis*: <100 individuals each in all areas surveyed); a(i), no subpopulation estimated to contain more than 50 mature individuals (*B. philippinensis*: <50 mature individuals in each subpopulation).

***Bikkia montoyae*:** This species is distributed from low to high elevations on ultramafic soils of Paragua and Mt. Redondo, Dinagat Island, Surigao del Norte, and Mt. Hamiguitan, Davao

Oriental. **Critically Endangered** [CR C2a(i)]; C2, population size estimated to number fewer than 250 mature individuals (*B. montoyae*: <200 individuals in all areas surveyed); a(i), no subpopulation estimated to contain more than 100 mature individuals (*B. montoyae*: <100 mature individuals in each subpopulation).

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## CONFLICTS OF INTEREST

All authors have declared no conflict of interests.

## CONTRIBUTIONS OF INDIVIDUAL AUTHORS

GJDA conceptualized the study and drafted the manuscript; LARS, HWCH, MSSM and PJRS conducted the molecular work and morphological assessment; all authors participated in the field collection and taxonomic treatment.

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