



Research article

Phylogeny of *Miliusa* (Magnoliales: Annonaceae: Malmeoideae: Miliuseae), with descriptions of two new species from Malesia

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Abstract. The molecular phylogeny of *Miliusa* (Annonaceae) is reconstructed, with 27 (of ca. 50) species included, using a combination of seven plastid markers (*rbcL* exon, *trnL* intron, *trnL-F* spacer, *matK* exon, *ndhF* exon, *psbA-trnH* spacer, and *ycf1* exon) constituting ca. 7 kb. In addition, two new species of *Miliusa* are described from the Malesian area: *M. butonensis* sp. nov. from Buton Island, Indonesia and *M. viridiflora* sp. nov. from Papua New Guinea. The former is included in the molecular phylogenetic analysis. The reconstructed phylogeny corresponds well to the informal morphological grouping proposed earlier. A revised key to 13 Austro-Malesian species of *Miliusa* is provided.

Keywords. Annonaceae, Buton Island, identification key, Papua New Guinea, molecular phylogeny.

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Introduction

The genus *Miliusa* Lesch. ex A.DC. (de Candolle 1832) (Annonaceae) comprises approximately 50 species of shrubs, or small to large trees, distributed from the Indian subcontinent, southern China and mainland Southeast Asia to Southeast Asian islands, New Guinea (including D'Entrecasteaux Islands and Louisiade Archipelago) and northern Australia (Chaowasku & Keßler 2006). It belongs to the tribe Miliuseae of the subfamily Malmeoideae (Chatrou *et al.* 2012). Members of the tribe Miliuseae are almost exclusively Asian (including New Guinea, Australia, and the western Pacific islands). Only two clades within this tribe consist of non-Asian members: one clade of four Neotropical genera and another clade of Afro-Madagascan species which are part of the recently described genus sister to *Miliusa*: *Hubera* Chaowasku (Chaowasku *et al.* 2012).

According to Chaowasku & Keßler (2006), *Miliusa* is circumscribed by having (1) equally-sized sepals and outer petals, both of which are much smaller than the inner petals; (2) a densely hairy torus; (3) miliusoid stamens (*sensu* Mols & Keßler 2003a), i.e. stamens that are loosely arranged without conspicuously dilated connective tissue covering the thecae; and (4) four-part-lamellate ruminations of the endosperm. Ten species were recognized according to the revision of the genus in the Austro-Malesian area (Mols & Keßler 2003b). One additional species, *M. lanceolata* Chaowasku & Kessler (Chaowasku

& Keßler 2006), was later described from D'Entrecasteaux Islands and Louisiade Archipelago, southeast off Papua New Guinea. New *Miliusa* species from southwestern India (Narayanan *et al.* 2010, 2012) as well as the Indian eastern Himalaya (Chaowasku 2013) have recently been described. Further, seven new species from Thailand are being added to this medium-sized genus by Chaowasku & Keßler (in press) who use floral/inflorescence morphology to elaborate the four informal groups within *Miliusa* first introduced by Chaowasku & Keßler (2006). These four morphological groups were the starting point to systematically study this genus further. In order to obtain additional evidence supporting the four mentioned groups, their pollen was investigated in detail (Chaowasku *et al.* 2008). The aim of the present study is to test whether each of the four morphological groups of *Miliusa* is monophyletic by means of molecular phylogenetic analysis.

In the course of selecting specimens for DNA extraction, we came across two collections, one from Buton Island (Indonesia) and another from Papua New Guinea, which are different from other described species in the Austro-Malesian area. After a thorough examination and comparison with similar species, we have concluded that they both represent undescribed species of *Miliusa*, which are herein formally described as *M. butonensis* sp. nov. and *M. viridiflora* sp. nov. The former is included in the molecular phylogenetic analysis. The number of *Miliusa* species in the Austro-Malesian area is thus increased to 13 and a key to these species is provided.

Materials and Methods

Molecular phylogenetic analysis

All accessions belong to the subfamily Malmeoideae (Appendix). Twenty-seven accessions of *Miliusa* covering the entire morphological variation known comprise the ingroup. The outgroups consist of 14 accessions, 11 of which were included as representatives of related genera in the tribe Miliuseae. Seven plastid markers, i.e. *rbcl* exon, *trnL* intron, *trnL-F* spacer, *matK* exon, *ndhF* exon, *psbA-trnH* spacer, and *ycfl* exon, were amplified (see Table 1 for the number of included, variable, and parsimony informative characters). In total, 7033 characters, including six separately coded indels were included in the analyses. Indel coding follows Simmons & Ochoterena (2000). The reverse complement of 15 continuous nucleotides in the *psbA-trnH* marker for roughly half of the accessions sequenced was present and altered into the reverse complement, following Pirie *et al.* (2006).

All methods of DNA extraction, amplification, and sequencing performed in Chaowasku *et al.* (2012) were used in the present study. Due to a poor quality of the extracted DNA or unavailability of leaf material, we could not produce seven markers for all accessions (see Appendix, Table 1). Sequences were edited using the program Staden version 1.7.0 (<http://staden.sourceforge.net/>) and subsequently manually aligned. Some sequences were obtained from previous studies (Mols *et al.* 2004a, 2004b; Pirie *et al.* 2006; Chaowasku *et al.* 2012). Maximum parsimony analyses were performed in TNT version 1.1 (Goloboff *et al.* 2008). All characters were equally weighted and unordered. Multiple most parsimonious trees were generated by a heuristic search of the combined data, with 6 000 replicates of random sequence additions, saving 10 trees per replicate, and using tree bisection and reconnection (TBR) branch swapping algorithm. Clade support was measured by symmetric resampling (SR), which is not affected by a distortion (resulting in incorrectly estimated percentages) as with some bootstrap and jackknife methods (Goloboff *et al.* 2003). A default change probability was used. Four hundred thousand replicates were run, each with two replicates of random sequence additions, saving one tree per replicate. Groups with SR of $\geq 85\%$, 70–84%, and $\leq 69\%$ were considered strongly, moderately, and weakly supported, respectively.

Bayesian analysis was performed in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Two independent runs were simultaneously run; each run comprised four Markov-chain-Monte-Carlo

Table 1. Important descriptive values of sequence data. NA = not applicable.

DNA region	No. of included characters	No. of accessions (out of all 41 accessions) lacking sequence data (%)	No. of variable characters (%)	No. of parsimony-informative characters (%)
<i>rbcL</i> exon	1380	31 (75.6)	48 (3.5)	16 (1.2)
<i>trnL</i> intron + <i>trnL-F</i> spacer	926	0	112 (12.1)	46 (5.0)
<i>matK</i> exon	828	20 (48.8)	78 (9.4)	31 (3.7)
<i>ndhF</i> exon	2033	0	225 (11.1)	105 (5.2)
<i>psbA-trnH</i> spacer	431	0	90 (20.9)	51 (11.8)
<i>ycf1</i> exon	1429	0	220 (15.4)	95 (6.7)
Combined data	7027	NA	773 (11.0)	344 (4.9)

(MCMC) chains and was set for 10^7 generations. The data matrix was divided into seven partitions [*trnL* intron and *trnL-F* spacer were included in the same partition (= *trnLF*)], including a set of binary indel coding. The most appropriate model of sequence evolution for each partition was selected by Akaike information criterion (AIC) scores, using FindModel (<http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>). The default model as well as the command ‘coding=variable’ were applied for the binary indel partition. The default prior settings were used except for the ratepr [=variable] and brlenspr [=unconstrained:exp(100)]. The latter prior setting was used to prevent the MCMC chains from being trapped in the areas of parameter space with unrealistically high values for the tree length parameter, resulting in a false convergence or a failure to reach convergence after hundreds of millions of generations (Marshall 2010). The temperature parameter was set to 0.1. Trees and all parameter values were sampled every 1000th generation. Convergence of the runs was checked by both the standard deviation of split frequencies and the values for effective sample sizes (ESS) using Tracer version 1.5 (Rambaut & Drummond 2009). The 50% majority-rule consensus tree was generated from the two runs combined, with 10% of the first trees removed as burn-in. Groups with posterior probabilities (PP) of ≥ 0.96 , 0.91–0.95, and ≤ 0.90 were considered strongly, moderately, and weakly supported, respectively.

Taxonomy/morphology

Measurements/observations of the new species were made from herbarium specimens of A, BRI, CANB, E, K, L, U herbaria. The indumentum terminology follows Hewson (1988). The term ‘velvety’ is equivalent to densely hairy/with dense hairs, whereas ‘puberulous’ is equivalent to sparsely hairy/with sparse hairs. Morphological data of the 11 known species of *Miliusa* in the Austro-Malesian area were from Mols & Keßler (2003b), Chaowasku & Keßler (2006), and personal observations (= all specimens cited in Mols & Keßler 2003b). Morphological data of other species included in the molecular phylogenetic analysis were from Chaowasku (2013), Chaowasku & Keßler (in press), and personal observations (= voucher specimens for molecular phylogenetic analysis plus a few more specimens, see Appendix). The term ‘glandular structures’ in the present study means that such structures look like glands but further anatomical confirmation is required. When only a single measurement/observation was made, the word ‘circa (ca.)’ was added.

Results

Molecular phylogenetic analysis

The maximum parsimony analysis of combined data sets resulted in 135 most parsimonious trees with 980 steps (results not shown). The consistency and retention indices (CI, RI) were 0.86 and 0.88,

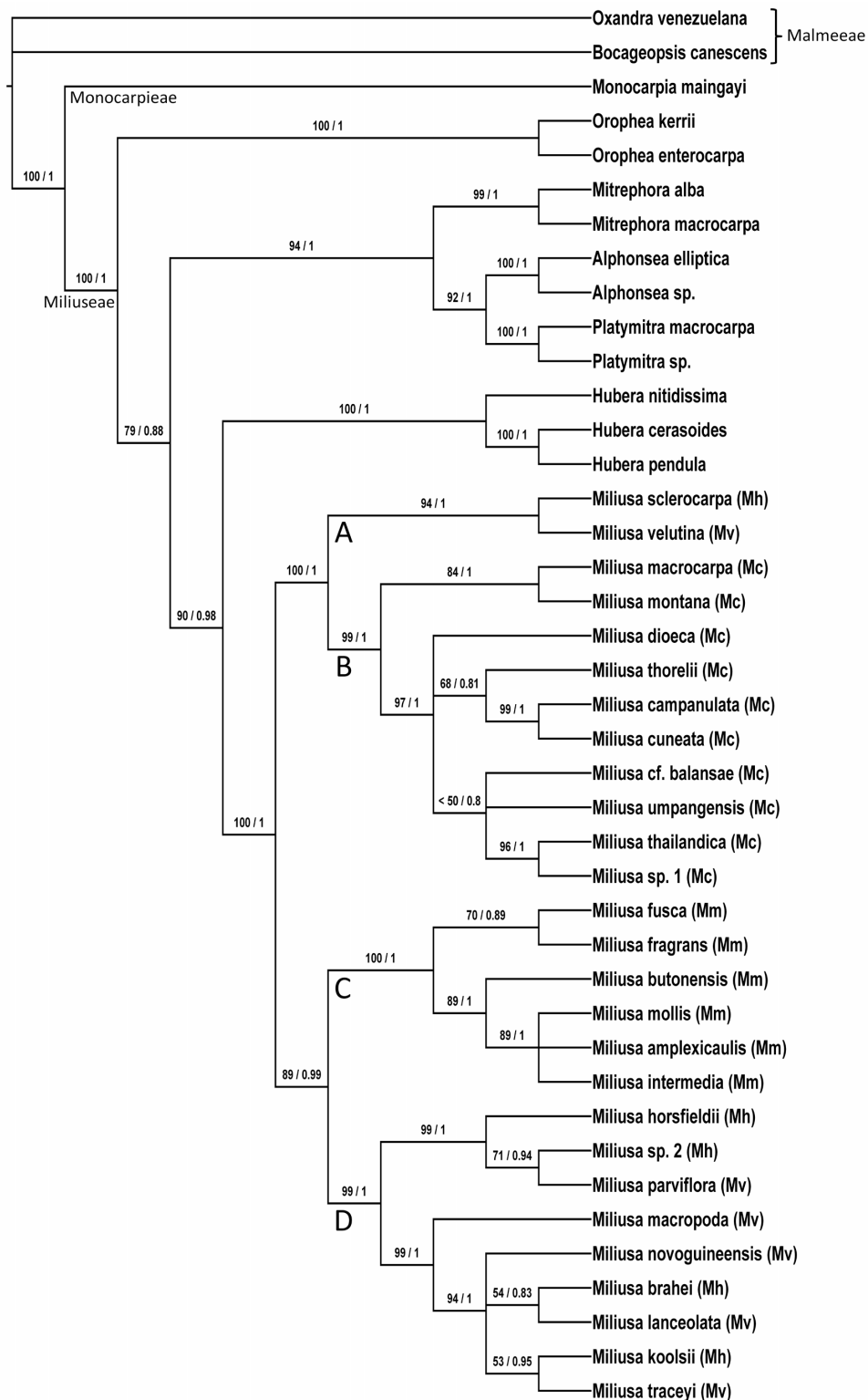


Fig. 1. 50% Bayesian majority-rule consensus cladogram of combined seven plastid markers. Clade support: left of slash - parsimony symmetric resampling values corresponding to clades recovered in Bayesian tree; right of slash - Bayesian posterior probabilities. Mc = *Miliusa campanulata* Pierre group; Mh = *Miliusa horsfieldii* (Benn.) Baill. ex Pierre group; Mm = *Miliusa mollis* Pierre group; Mv = *Miliusa velutina* (DC.) Hook.f. & Thomson group; all groups *sensu* Chaowasku & Keßler (in press).

respectively. For Bayesian analysis, the substitution model was generalized time-reversible plus gamma (GTR + G) for all partitions except the *trnLF* partition (= *trnL* intron + *trnL-F* spacer), which had Tamura-Nei plus gamma (TrN + G) model. The final standard deviation of split frequencies was < 0.002 and all ESS values after discarding the burn-in were > 1200, both indicating convergence of the runs.

Figures 1 (cladogram) and 2 (phylogram) show 50% majority-rule consensus trees derived from the Bayesian analysis, with support values indicated in Fig. 1. *Milium* is monophyletic with maximum support (SR 100%; PP 1.00). It is sister to *Hubera* with strong support (SR 90%; PP 0.98). Within *Milium*, four strongly supported (SR ≥ 85%; PP ≥ 0.96) clades have been identified (Fig. 1: clades A, B, C, D). Clade A is sister to clade B whereas clade C is sister to clade D, both with strong support. A clade comprising clades A and B is sister to a clade consisting of clades C and D.

Taxonomy

Order Magnoliales Juss. ex Bercht. & J.Presl (Berchtold & Presl 1820)

Family Annonaceae Juss. (Jussieu 1789), nom. cons.

Tribe Miliuseae Hook.f. & Thomson (Hooker & Thomson 1855)

Genus *Milium* Lesch. ex A.DC. (de Candolle 1832)

Milium butonensis Chaowasku & Kessler, sp. nov.

urn:lsid:ipni.org:names:77131155-1

Figs 3–4

Diagnosis

Milium butonensis sp. nov. belongs to clade C. It is best characterized by (1) dense hairs on the apical half inside the inner petals and (2) glandular structures that cover almost the whole basal half of the inner side of the inner petals.

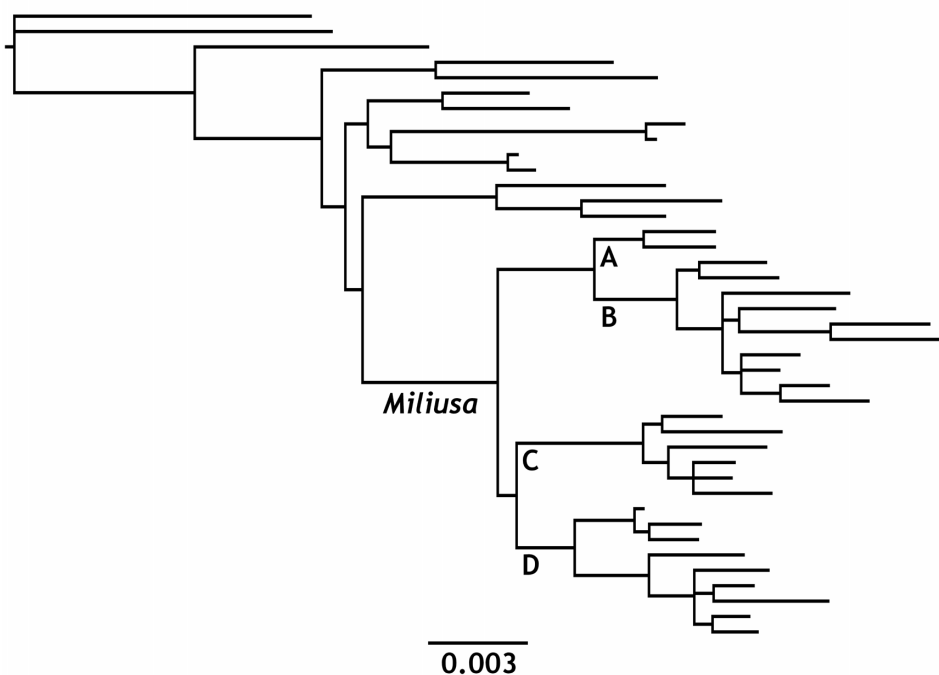


Fig. 2. 50% Bayesian majority-rule consensus phylogram of combined seven plastid markers, showing branch length proportional to amount of lineage sequence divergence. Scale bar unit: substitution per site.

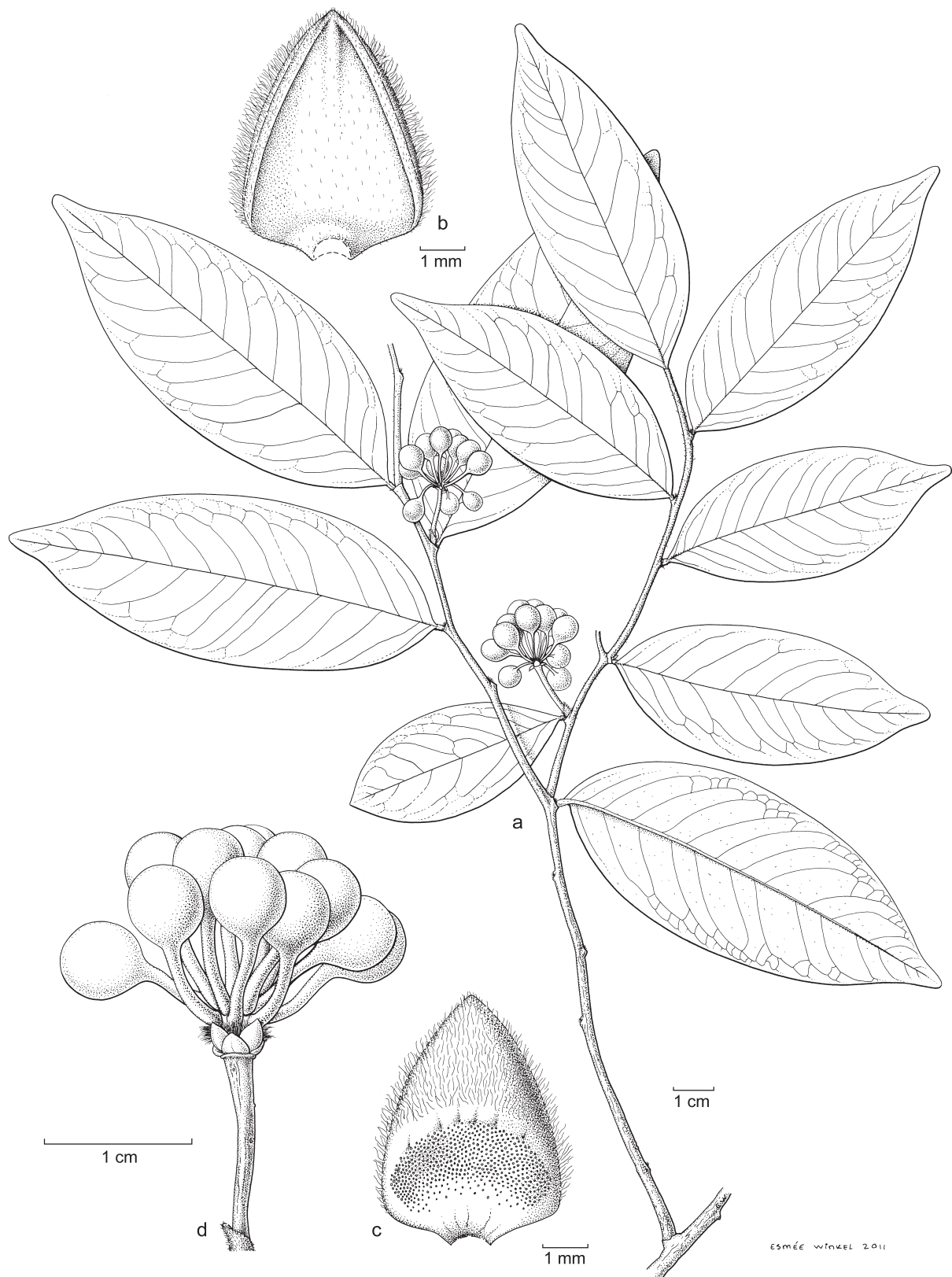


Fig. 3. *Miliusa butonensis* sp. nov. **a.** Habit. **b.** Outside (abaxial surface) of a persistent inner petal. **c.** Inside (adaxial surface) of a persistent inner petal. **d.** Fruit. (All from *Coode 6279*). Drawn by E. Winkel.

Etymology

Named after the Buton Island (Indonesia) where this species is endemic.

Type

INDONESIA: Southeast of Sulawesi, North Buton Is., Jismil camp inland from Labuan Tobelo, Nov. 1989, *Coode 6279* [holo-: U; iso-: A, K, L], in fruit (with a few persistent inner petals).

Description

Trees, ca. 8 m tall, ca. 10 cm in diameter. Young twigs appressed-puberulous. Petioles 2.0–3.0 mm long, appressed-puberulous. Leaves elliptic to slightly ovate, 4.7–11.1 × 2.2–4.5 cm, base (broadly) cuneate, sometimes slightly unequal, apex acute; lamina glabrous both sides; midrib usually slightly raised to flat above, sometimes slightly sunken, glabrous, raised below, appressed-puberulous; secondary veins 15–18 pairs, angle with midrib 42°–50°. Flowers partially known; fruiting peduncles or fruiting pedicels axillary; fruiting peduncles up to 0.4 cm long; fruiting pedicels 0.9–2.1 cm long; sepals and sometimes outer petals persistent in fruit, both ovate-triangular. Inner petals a few persistent ones fallen on herbarium sheet near the torus bearing monocarps seen, ovate-triangular, ca. 6.0 × 4.0 mm; outside appressed-puberulous, mostly on the middle part, margin puberulous, inside (curly-)velvety on the apical half; glandular structures observed on almost the whole basal half, slightly raised, between crescent-shaped and semicircular, slightly finely warty; base not saccate. Monocarps 3–21, subglobose-ellipsoid, 0.7–0.9 × 0.6–0.8 cm; surface smooth, glabrous; apex not apiculate; stipe ca. 6.0 mm long, glabrous. Seed(s) 1 (rarely 2), subglobose(-ellipsoid), 0.6–0.8 × 0.4–0.7 cm.

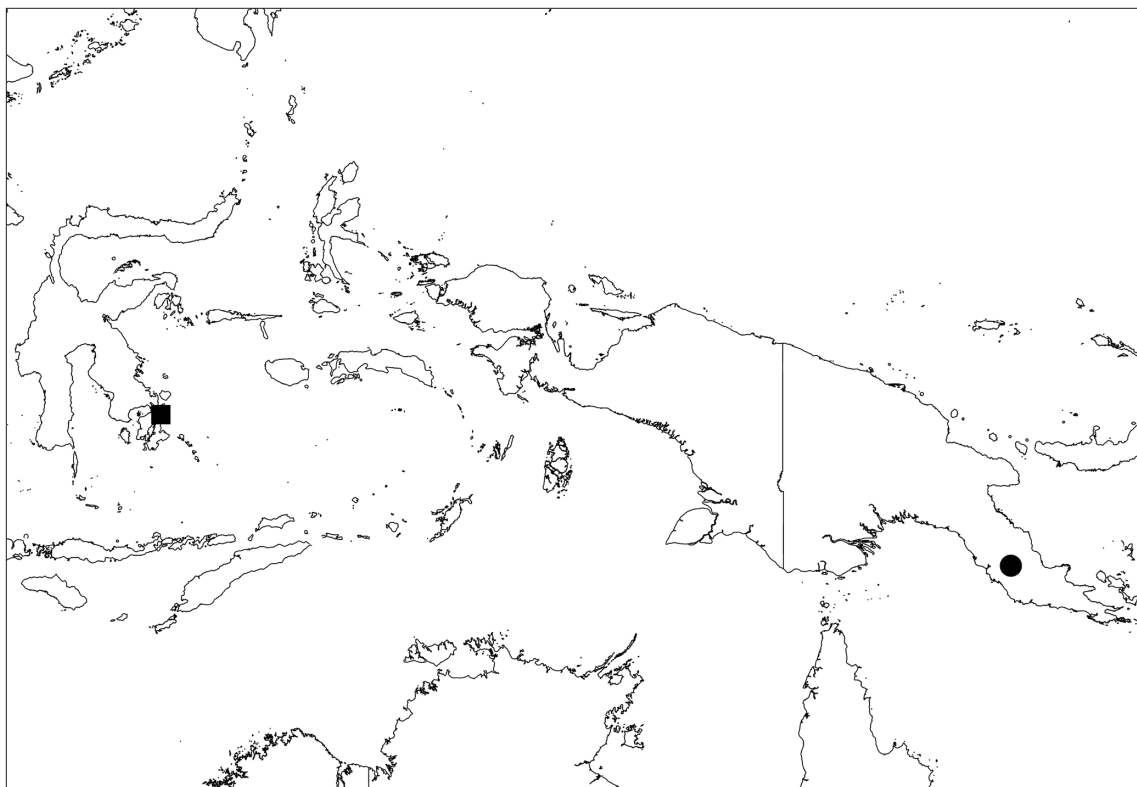


Fig. 4. Distribution of *Miliusa butonensis* sp. nov. (filled square) and *Miliusa viridiflora* sp. nov. (filled circle).

Distribution, habitat and phenology

Indonesia (Buton Island, Fig. 4), occurring in forests on flat ridge-tops, with slightly broken canopy due to rocky terrain of raised coralline limestone. Elevation: ca. 300 m. Fruiting: November.

Field notes

Branches horizontal and foliage in flat sprays. Bark grey-brown, ca. 4 mm thick overall, \pm smooth with fine vertical cracks and rows of low lenticels, wood straw-colored, cut bark and wood pleasantly aromatic. Young leaves \pm yellow-green, mature leaves mid green on both sides.

Miliusa viridiflora Chaowasku & Kessler, sp. nov.

urn:lsid:ipni.org:names:77131156-1

Figs 4–5

Diagnosis

Miliusa viridiflora sp. nov. is most morphologically similar to the recently described *M. lanceolata*. The new species chiefly differs in having much sparser indumentum on young twigs and lower surface of the leaf midrib, usually (broadly) elliptic leaves (narrowly ovate in *M. lanceolata*), and greater number of stamens (ca. 56 vs. ca. 32 in *M. lanceolata*) and carpels (ca. 28 vs. ca. 14 in *M. lanceolata*) per flower.

Etymology

The epithet refers to the light green flowers (probably also at anthesis).

Type

PAPUA NEW GUINEA: Central District, Port Moresby Subdistrict, on ridge below Boridi Village, Oct. 1973, *Foreman & Vinas LAE 60222* [holo-: L; iso-: A, BRI, CANB, E], in flower and fruit.

Description

Trees. Young twigs (almost glabrous to) appressed-puberulous. Petioles ca. 1.0 mm long, almost glabrous to appressed-puberulous. Leaves usually (broadly) elliptic, sometimes slightly ovate, 4.5–13.3 \times 2.1–4.9 cm, base (broadly) cuneate to obtuse, often slightly unequal, apex acute to acuminate; lamina glabrous above, (glabrous to) appressed-puberulous below; midrib flat to slightly sunken above, almost glabrous, raised below, almost glabrous; secondary veins 11–15 pairs, angle with midrib 48°–65°. Flowers usually in \geq 7-flowered inflorescences, terminal developing to internodal; peduncles 2.3–4.4 cm long, glabrous; rachis 2.3–4.5 cm long, glabrous; pedicels 5.0–13.0 cm long, glabrous, basal articulation usually observed; bracts of peduncles and inflorescence axes triangular, number depending on the number of flowers per inflorescence; pedicel bract 1 for each flower, triangular. Sepals (broadly) triangular, 0.6–0.7 \times 0.7–0.8 mm, persistent in fruit; both sides glabrous, margin puberulous. Outer petals triangular, ca. 1.3 \times 1.0 mm; both sides glabrous, margin puberulous. Inner petals ovate, 7.0–8.0 \times 5.0–6.0 mm; both sides glabrous, margin puberulous; surface of the inner side somewhat raised on the basal half compared to the apical half; base slightly saccate. Torus shortly cylindrical. Stamens ca. 56, 1.0–1.4 mm long. Carpels ca. 28, ca. 1.3 mm long; stigmas capitate-globose; ovaries almost glabrous; ovules 2, lateral, uniseriate. Monocarps 2–7, subglobose-ellipsoid to slightly irregular-shaped, 0.9–1.2 \times 0.8–1.1 cm, slightly constricted between seeds when two seeds present in the monocarps; surface slightly verruculose, glabrous; apex not apiculate; stipe 10.0–17.0 mm long, glabrous, obliquely attached to the monocarps. Seed(s) 1–2, subglobose(-ellipsoid), 0.8 \times 0.4–0.7 cm.

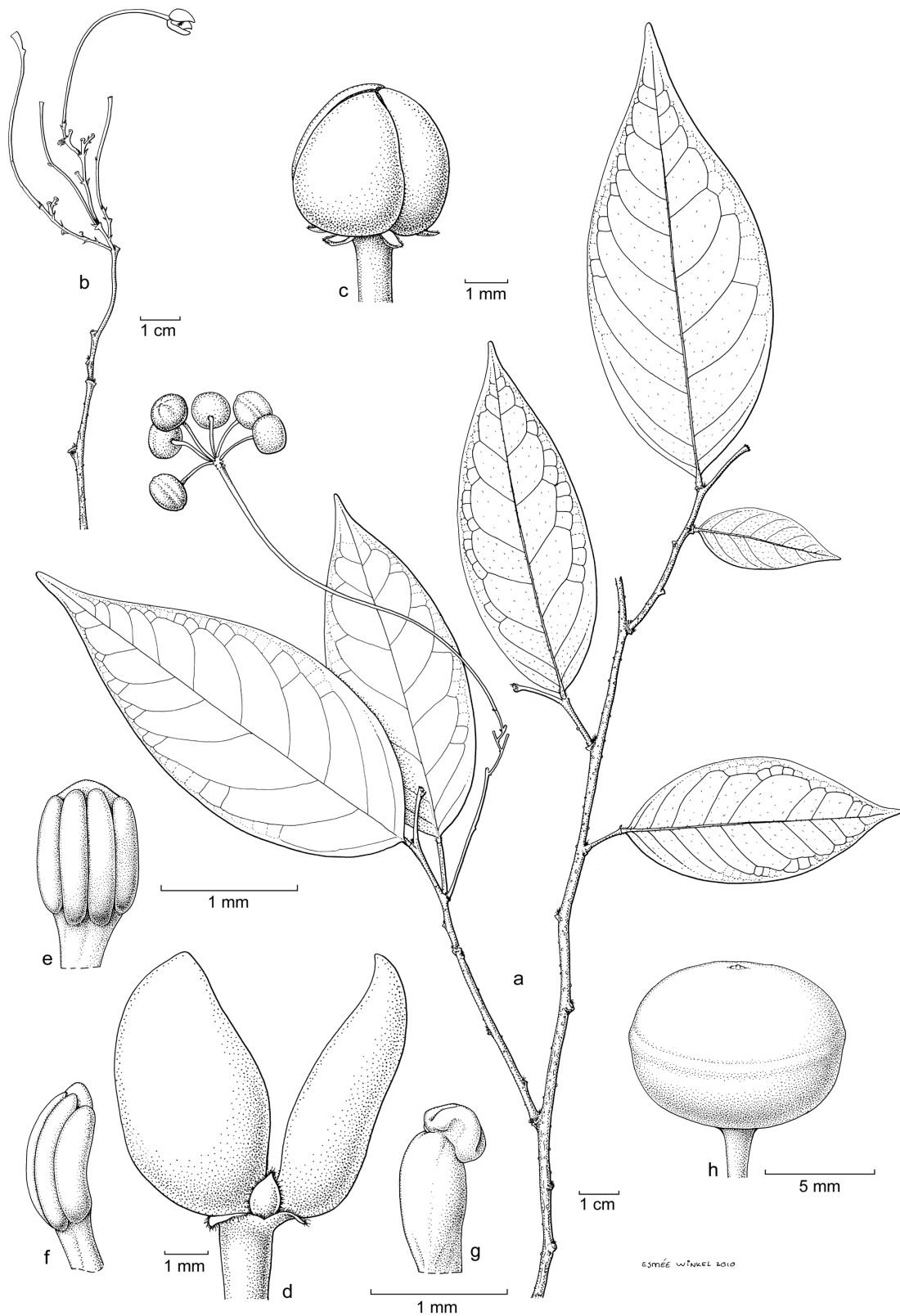


Fig. 5. *Miliusa viridiflora* sp. nov. **a.** Habit. **b.** Flowering twig. **c.** Flower bud. **d.** Flower with one inner petal, stamens, and carpels removed. **e.** Stamen, abaxial view. **f.** Stamen, side view. **g.** Carpel. **h.** Monocarp. (All from Foreman & Vinas LAE 60222). Drawn by E. Winkel.

Distribution, habitat and phenology

Papua New Guinea (Central Province, Fig. 4), occurring in secondary forests; on ridges. Elevation: ca. 1220 m. Flowering and fruiting: October.

Field notes

A small tree. Leaves mid green. Flowers light green.

Key to the Austro-Malesian species of *Milium*

Notes

In some couplets differences are small, therefore when there is any ambiguity, it is advised to consult the descriptions of relevant species.

1. Inner petals with crescent-shaped to semicircular glandular structures inside, at base or covering almost the whole basal half.....2
 - Inner petals without pronounced glandular structures inside or with narrow glandular structures running inside along their bilateral midline.....3
2. Leaves 4.7–11.1 × 2.2–4.5 cm, base (broadly) cuneate, sometimes only slightly unequal, not clasping the twigs. Inner petals with dense hairs inside on the apical half; glandular structures inside between crescent-shaped and semicircular, located on almost the whole basal half. BUTON ISLAND (INDONESIA).....*Milium butonensis* sp. nov.
 - Leaves 9.3–26.0 × 4.0–11.0 cm, base (sub-)cordate, always (moderately to notably) unequal, ± clasping the twigs. Inner petals glabrous inside; glandular structures inside ± crescent-shaped, located at base. THAILAND, PENINSULAR MALAYSIA.....*Milium amplexicaulis* Ridl. (Ridley 1910)
3. Inner petals reflexed at anthesis, stamens ≥ 125 per flower. BORNEO.....*Milium macropoda* Miq. (Miquel 1865)
 - Inner petals not reflexed at anthesis but sometimes the apex/apical half recurved at anthesis, stamens ≤ 65 per flower.....4
4. Stamens ≤ 9 per flower.....5
 - Stamens ≥ 18 per flower.....6
5. Sepals and outer petals ≥ 2.0 mm long, staminodes 4–5 per flower, carpels 12–14 per flower. AUSTRALIA.....*Milium traceyi* Jessup (Jessup 1988)
 - Sepals and outer petals ≤ 1.5 mm long, staminode(s) up to 3 per flower, carpels 3–11 per flower. NEW GUINEA.....*Milium novoguineensis* Mols & Kessler (Mols & Kessler 2003b)
6. Ovules ≥ 3 per ovary, biseriate.....7
 - Ovules 2(–3) per ovary, uniseriate.....11
7. Inner petals without pronounced glandular structures inside. ANDAMAN AND NICOBAR ISLANDS (INDIA), THAILAND, PENINSULAR MALAYSIA, SUMATRA.....*Milium parviflora* Ridl. (Ridley 1911)
 - Inner petals with narrow glandular structures running inside along their bilateral midline.....8

8. Inner petals 17.0–40.0 mm long. CHINA, LAOS, THAILAND, MYANMAR, ANDAMAN AND NICOBAR ISLANDS (INDIA), PENINSULAR MALAYSIA, SOUTHEAST ASIAN ISLANDS (INCLUDING NEW GUINEA), AUSTRALIA.....
*Miliusa horsfieldii* (Benn.) Baill. ex Pierre (Pierre 1881; basionym: Bennett 1840)
 – Inner petals \leq 15.0 mm long.....9
9. Carpels 21–27 per flower, ovules 5–7 per ovary. AUSTRALIA.....
*Miliusa brahei* (F.Muell.) Jessup (Jessup 1986; basionym: Mueller 1874)
 – Carpels \leq 18 per flower, ovules \geq 8 per ovary.....10
10. Leaf base usually equal. Pedicels 0.9–2.5(–4.5) cm long. Monocarps (1.3–)2.0–3.2 cm wide, stipe (11.0–)16.0–29.0 mm long, seeds 1.1–1.7 \times 0.7–1.5 cm. NEW GUINEA.....
*Miliusa koolsii* (Kosterm.) J.Sinclair (Sinclair 1955; basionym: Kostermans 1952)
 – Leaf base unequal. Pedicels 2.9–14.5(–21.5) cm long. Monocarps 0.6–1.3 cm wide, stipe (4.0–)10.0–16.0(–32.0) mm long, seeds 0.6–0.8 \times 0.3–0.5 cm. THE PHILIPPINES.....
*Miliusa vidalii* J.Sinclair (Sinclair 1955)
11. Flowers and/or inflorescences axillary, inner petals usually tightly appressed from the base to \pm the midpoint at anthesis. THAILAND, PENINSULAR MALAYSIA, SUMATRA, JAVA.....
*Miliusa longipes* King (King 1892)
 – Flowers and/or inflorescences terminal developing to internodal, inner petals completely open at anthesis.....12
12. Young twigs with dense hairs (visible by naked eye). Leaves narrowly ovate, lower surface of midrib with dense hairs. Stamens ca. 32 per flower, carpels ca. 14 per flower. D'ENTRECASTEAUX ISLANDS AND LOUISIADE ARCHIPELAGO (PAPUA NEW GUINEA).....
*Miliusa lanceolata* Chaowasku & Kessler (Chaowasku & Keßler 2006)
 – Young twigs (almost glabrous to) sparsely hairy (visible only under a binocular). Leaves usually (broadly) elliptic, lower surface of midrib almost glabrous. Stamens ca. 56 per flower, carpels ca. 28 per flower. PAPUA NEW GUINEA.....*Miliusa viridiflora* sp. nov.

Discussion

Two clades, B and C, recovered in the phylogenetic trees (Figs 1, 2) correspond to *Miliusa campanulata* Pierre (Pierre 1881) and *M. mollis* Pierre (Pierre 1881) groups *sensu* Chaowasku & Keßler (in press), respectively. The important features characterizing the *M. campanulata* group: inner petals that are usually tightly appressed from the base to \pm the midpoint at anthesis (Fig. 6D–F), and the *M. mollis* group: inner petals with crescent-shaped to semicircular glandular structures inside at the base or (a bit) higher (Figs 3c; 6G–I), are thus the synapomorphies of clades B and C, respectively. In contrast, *M. horsfieldii* (Benn.) Baill. ex Pierre (Pierre 1881; basionym: Bennett 1840) and *M. velutina* (DC.) Hook.f. & Thomson (Hooker & Thomson 1855; basionym: Dunal 1817) groups *sensu* Chaowasku & Keßler (in press) each does not form a clade; clades A and D each consists of members of both groups (see Fig. 1). The *M. velutina* group seems to have been characterized principally by a likely symplesiomorphy: an absence of conspicuous glandular structures inside the inner petals (Fig. 6B, K), whereas the main feature characterizing the *M. horsfieldii* group [inner petals with narrow glandular structures running inside along their bilateral midline (Fig. 6A, J, L)] seems to have evolved multiple times in *Miliusa*. Detailed ancestral character reconstructions in combination with a denser taxon sampling, however, are needed before any solid conclusion on character evolution occurred in *Miliusa* can be drawn.

Besides the inner petal morphology, flower and/or inflorescence position also corresponds to the phylogenetic results, i.e. all species recovered in clade C and most species recovered in clade B possess

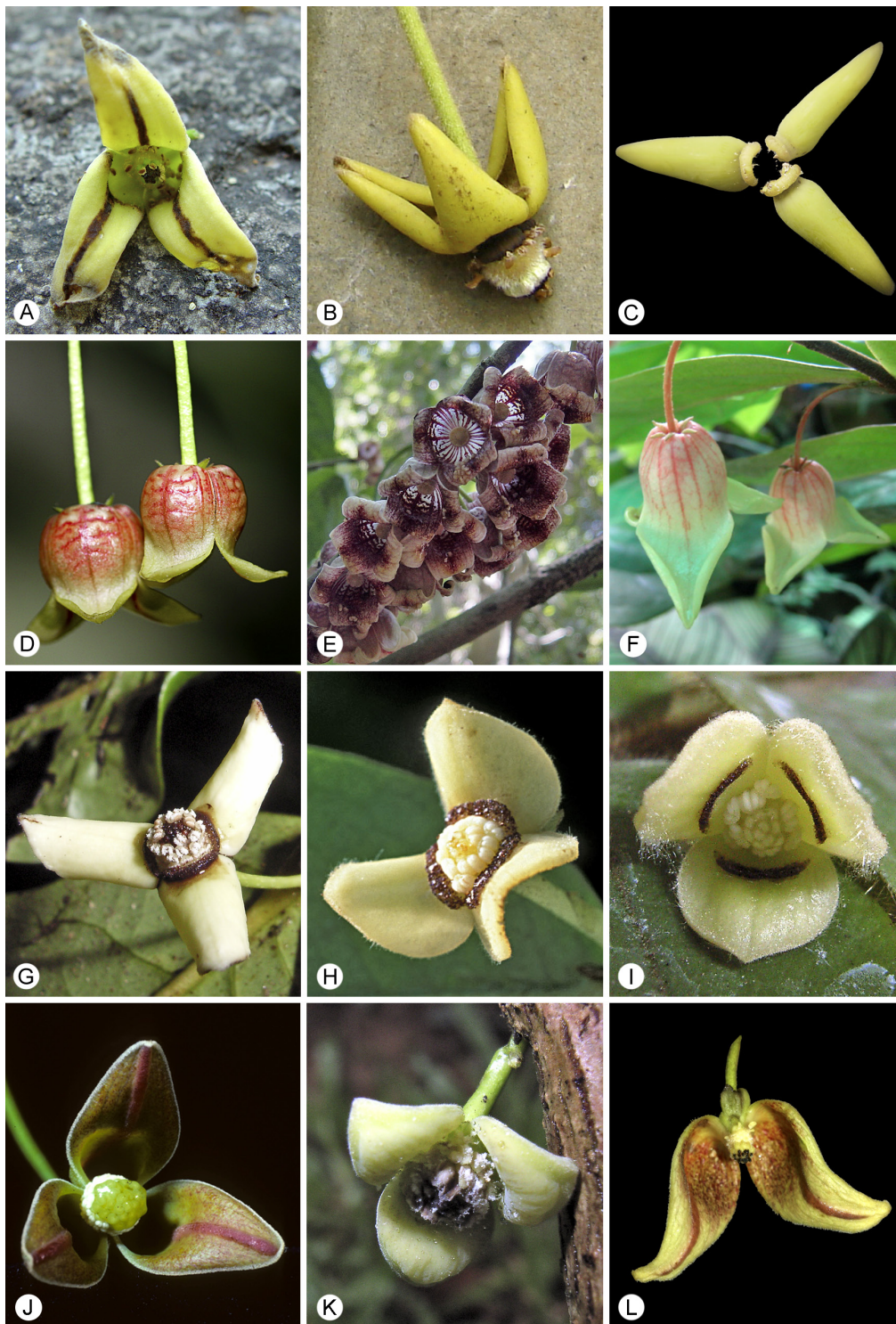


Fig. 6. Flowers/inner petals of (representatives of) *Miliusa* species recovered in each clade. **A-C.** Clade A. **A.** *Miliusa sclerocarpa* (A.DC.) Kurz. **B, C.** *Miliusa velutina* (DC.) Hook.f. & Thomson. **D-F.** Clade B. **D.** *Miliusa campanulata* Pierre. **E.** *Miliusa thorelii* Finet & Gagnep. **F.** *Miliusa thailandica* Chaowasku & Kessler. **G-I.** Clade C. **G.** *Miliusa amplexicaulis* Ridl. **H.** *Miliusa intermedia* Chaowasku & Kessler. **I.** *Miliusa mollis* Pierre. **J-L.** Clade D. **J.** *Miliusa brahei* (F.Muell.) Jessup. **K.** *Miliusa parviflora* Ridl. **L.** *Miliusa* sp. 2. (Photographs taken by: **A:** P. Keßler; **B, G, I, K:** T. Chaowasku; **C:** A. Rodphitak; **D, L:** B. Siriphiphat; **E:** S. Pongcharean; **F:** S. Thanapathomsinchai; **H:** S. Gardner; **J:** L. Jessup).

axillary flowers and/or inflorescences, while all species recovered in clades A and D possess terminal (developing to internodal) flowers and/or inflorescences. This correlation shows predictive significance, e.g. species exhibiting completely open inner petals at anthesis and terminal (developing to internodal) flowers and/or inflorescences (= belonging to the *M. horsfieldii* or *M. velutina* group; see Chaowasku & Keßler in press), but having not been included in the phylogenetic analyses are likely to belong to clade A or D.

After a thorough examination on the floral morphology of *Miliusa velutina*, a member of clade A (Fig. 1), peculiar structures have been observed. At the base inside the inner petals, there are thickened structures (Fig. 6C) hidden at female anthesis, but as male anthesis begins and continues, these structures are gradually becoming exposed. These structures seem to be non-glandular; however, anatomical comparisons with the glandular structures observed in the species of clade C (Figs 3c; 6G–I) are likely to shed light on whether they are homologous.

Miliusa butonensis sp. nov. is only known from the type specimens. Results of the molecular phylogenetic analysis, nonetheless, assure the new species status. *Miliusa butonensis* sp. nov. is sister to a clade comprising three mainland Asian species (Fig. 1). These three species share one remarkable feature: (sub-)cordate leaf base, whereas *M. butonensis* sp. nov., and the remaining species of clade C, *M. fusca* Pierre (Pierre 1881) and *M. fragrans* Chaowasku & Kessler (Chaowasku & Keßler in press), do not. The occurrence of this species on Buton Island is unexpected since all other species (expected to be part) of clade C thus far known occur on mainland Asia only. Phytochemically, it is worthwhile to note that neolignans have been found as principal secondary metabolites in two species of clade C: *M. mollis* (Sawasdee *et al.* 2010, 2013a) and *M. fragrans* (Sawasdee *et al.* 2013b), but have not been reported to occur in any species (expected to be part) of clade A, B or D so far investigated (see Sawasdee *et al.* 2010). If it is eventually proved that neolignans really occur only in the species (expected to be part) of clade C, including *M. butonensis* sp. nov., this class of natural product could be developed as a chemotaxonomic marker.

Miliusa viridiflora sp. nov. is also only known from the type specimens; however, it is readily distinguishable from its most morphologically similar species, *M. lanceolata*, by both vegetative and generative features (see diagnosis). Additionally, the elevation where both species occur is considerably different [ca. 1220 m in *M. viridiflora* sp. nov. vs. 2–20 m in *M. lanceolata* (Chaowasku & Keßler 2006)]. These differences convince us to recognize the former as new species.

So far neither macromorphological nor pollen morphological (see Chaowasku *et al.* 2008) features have been found to be able to distinguish species of clade A from those of clade D. Nevertheless, *Miliusa viridiflora* sp. nov. is more likely to belong to clade D because (1) clade A thus far known contains only continental Asian species and (2) all species known to occur in New Guinea belong to clade D (Fig. 1). The peduncles plus rachis (if present) plus pedicels of *M. viridiflora* sp. nov. (Fig. 5a, b) and of *M. lanceolata* (Chaowasku & Keßler 2006) are notably long. This trait may be associated with bat dispersal syndrome since the fruits eventually set will be clearly separated from the foliage, and hence can be more easily detected (Marshall 1983). In Miliuseae, this feature has been earlier reported in some New Guinean species of *Pseuduvaria* Miq. (Miquel 1858) (Su & Saunders 2006; Su *et al.* 2008).

It is worth conducting an anatomical investigation of the inner side of the inner petals of *Miliusa* to reveal the ontogeny and possible functions of the glandular [and seemingly non-glandular (e.g. Fig. 6C, E)] structures. Probably these differentiations are associated with the different pollination strategies. So far there has been no detailed study on pollination biology of *Miliusa*; however, according to Mols & Keßler (2003b), fruit flies were observed to visit the flowers of *M. horsfieldii*. A detailed pollination biological study is required to determine if this kind of insect is potential pollinators for this species.

Certain species of clade B exhibit \pm transparent, window-like structures at the base of the inner petals (Chaowasku & Keßler in press), e.g. *M. campanulata* and *M. thorelii* Finet & Gagnep. (Finet & Gagnepain 1907; see Fig. 6E). Flies are likely to be the potential pollinators for these species as they are lured to crawl inside to the stamens/stigmas by light (Dafni 1984).

There are correlations between clades and habitat preferences in *Miliusa*. Most species (expected to be part) of clades A and D prefer drier habitats (e.g. deciduous/dipterocarp forests), resulting in a various degree of deciduous lifecycle exhibited by a number of species (Mols & Keßler 2003b). In contrast, the majority of the species (expected to be part) of clades B and C prefer more humid habitats, e.g. dry/moist evergreen forests (Mols & Keßler 2003b; Chaowasku & Keßler in press). It is apparent that habitat shifts occurred in *Miliusa*; these shifts might correspond to the paleoclimate.

More species of *Miliusa*, especially the Indian, Philippine, and Vietnamese ones, need to be included in order to reconstruct a more robust molecular phylogeny, which will be the ground for the study in, for example, biogeography/molecular dating and character evolution.

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Appendix

Appendix. Voucher specimens for molecular phylogenetic analysis (with GenBank accession numbers indicated) and morphological observations.

--- = sequence not available for this study.

Species	Origin/voucher	<i>rbcL</i>	<i>trnL</i> F	<i>matK</i>	<i>ndhF</i>	<i>psbA-trnH</i>	<i>ycf1</i>
<i>Alphonsea elliptica</i>	Bogor Bot. Gard., Indonesia/ <i>Van Balgooy 5141</i> (L)	AY318966	AY319078	AY519907	JQ690401	JQ690402	JQ690403
<i>Alphonsea</i> sp.	Thailand/ <i>Keßler PK 3186</i> (TISTR, Bangkok)	---	AY319082	AY518808	JQ690404	JQ690405	JQ690406
<i>Bocageopsis canescens</i>	Brazil/ <i>Maas et al. 9243</i> (U)	JQ690407	JQ690408	JQ690409	JQ690410	JQ690411	JQ690412
<i>Hubera cerasoides</i>	Thailand/ <i>Chalermglin 440214-4</i> (L)	AY319017	AY319131	AY518854	JQ889985	JQ889980	JQ889975
<i>Hubera nitidissima</i>	Australia/ <i>Ford AF 4967</i> (L)	---	JQ889988	JQ889989	JQ889986	JQ889981	JQ889976
<i>Hubera pendula</i>	Madagascar/ <i>Rabevohitra 2386</i> (WAG)	---	AY319144	AY518852	JQ889987	JQ889982	JQ889977
<i>Miliuma amplexicaulis</i>	Thailand/ <i>Chaowasku 54</i> (L)	---	JQ690478	---	JQ690479	JQ690480	JQ690481
<i>Miliuma</i> cf. <i>balansae</i>	Vietnam/ <i>Harder et al. 7233</i> (MO) [additional specimen morphologically observed = <i>Van der Werff & Dao</i> <i>14261</i> (L)]	---	JQ690482	---	JQ690483	JQ690484	JQ690485
<i>Miliuma brahei</i>	Australia/ <i>Ford AF 5005</i> (CNS)	---	JQ690430	---	JQ690431	JQ690432	JQ690433
<i>Miliuma butonensis</i>	Buton Is., Indonesia/ <i>Coode 6279</i> (L)	---	JQ690434	---	JQ690435	JQ690436	JQ690437
<i>Miliuma campanulata</i>	Thailand/ <i>Chaowasku 23</i> (L)	---	JQ690486	---	JQ690487	JQ690488	JQ690489
<i>Miliuma cuneata</i>	Thailand/ <i>Keßler PK 3277</i> (L)	---	JQ690490	---	JQ690491	JQ690492	JQ690493

Species	Origin/voucher	<i>rbcL</i>	<i>trnL</i> F	<i>matK</i>	<i>ndhF</i>	<i>psbA-trnH</i>	<i>ycf1</i>
<i>Miliusa dioeca</i>	Assam, India/ <i>Thakur Rup Chand</i> 4565 (L) [additional specimen morphologically observed = <i>Thakur Rup Chand</i> 8353 (L)]	---	JQ690494	---	JQ690495	JQ690496	JQ690497
<i>Miliusa fragrans</i>	Thailand/ <i>Chaowasku</i> 38 (L)	---	JQ690438	---	JQ690439	JQ690440	JQ690441
<i>Miliusa fusca</i>	Thailand/ <i>Chaowasku</i> 46 (L)	---	JQ690442	---	JQ690443	JQ690444	JQ690445
<i>Miliusa horsfieldii</i>	Thailand/ <i>Chaowasku</i> 109 (L)	---	JQ690446	---	JQ690447	JQ690448	JQ690449
<i>Miliusa intermedia</i>	Thailand/ <i>Chaowasku</i> 26 (L)	---	JQ690450	---	JQ690451	JQ690452	JQ690453
<i>Miliusa koolsii</i>	Papua New Guinea/ <i>Hoogland</i> 4927 (CANB)	---	JQ690454	---	JQ690455	JQ690456	JQ690457
<i>Miliusa lanceolata</i>	Papua New Guinea/ <i>Brass</i> 28198 (L)	---	JQ690458	---	JQ690459	JQ690460	JQ690461
<i>Miliusa macrocarpa</i>	Bhutan/ <i>Grierson & Long</i> 4095 (E)	---	JQ690498	JQ690499	JQ690500	JQ690501	JQ690502
<i>Miliusa macropoda</i>	Borneo, Indonesia/ <i>Ambriansyah & Arifin</i> AA 272 (L)	---	JQ690462	---	JQ690463	JQ690464	JQ690465
<i>Miliusa mollis</i>	Thailand/ <i>Keßler</i> PK 3207 (L)	---	AY319102	AY518851	JQ690503	JQ690504	JQ690505
<i>Miliusa montana</i>	Sri Lanka/ <i>Hladik</i> 1039 (US) [additional specimen morphologically observed = <i>Wirawan</i> 621 (AAU, UC)]	---	JQ690506	JQ690507	JQ690508	JQ690509	JQ690510

Species	Origin/voucher	<i>rbcL</i>	<i>trnLF</i>	<i>matK</i>	<i>ndhF</i>	<i>psbA-trnH</i>	<i>ycf1</i>
<i>Miliusa novoguineensis</i>	Papua New Guinea/ <i>Womersley NGF. 24845</i> (NSW)	---	JQ690466	---	JQ690467	JQ690468	JQ690469
<i>Miliusa parviflora</i>	Thailand/ <i>Chaowasku 98</i> (L)	---	JQ690470	---	JQ690471	JQ690472	JQ690473
<i>Miliusa sclerocarpa</i>	Thailand/ <i>Chaowasku 19</i> (L)	---	JQ690474	---	JQ690475	JQ690476	JQ690477
<i>Miliusa thailandica</i>	Thailand/ <i>Chaowasku 70</i> (L)	---	JQ690515	---	JQ690516	JQ690517	JQ690518
<i>Miliusa thorelii</i>	Thailand/ <i>Keßler PK 3184</i> (L)	---	AY319104	AY518846	JQ690519	JQ690520	JQ690521
<i>Miliusa traceyi</i>	Australia/ <i>Ford AF 4778</i> (L)	---	JQ690531	JQ690532	JQ690533	JQ690534	JQ690535
<i>Miliusa umpangensis</i>	Thailand/ <i>Chaowasku 89</i> (L)	---	JQ690522	---	JQ690523	JQ690524	JQ690525
<i>Miliusa velutina</i>	Thailand/ <i>Pholsena &</i> <i>Koonkhunthod 2842</i> (L)	AY318993	AY319105	AY518847	JQ690536	JQ690537	JQ690538
<i>Miliusa</i> sp. 1	Thailand/ <i>Chaowasku 110</i> (L)	---	JQ690511	---	JQ690512	JQ690513	JQ690514
<i>Miliusa</i> sp. 2	Thailand/ <i>Nakorn-</i> <i>Thiemchan NTC 7</i> (L)	---	JQ690526	JQ690527	JQ690528	JQ690529	JQ690530
<i>Mitrephora alba</i>	Thailand/ <i>Chalermglin 440304-1</i> (TISTR, Bangkok)	AY318994	AY319106	AY518855	JQ889983	JQ889978	JQ889973
<i>Mitrephora macrocarpa</i>	Bogor Bot. Gard., Indonesia/ <i>Mols 8</i> (L)	---	AY319107	AY518859	JQ889984	JQ889979	JQ889974
<i>Monocarpia maingayi</i>	Thailand/ <i>Kaewruang 1</i> (L)	JQ690395	JQ690396	JQ690397	JQ690398	JQ690399	JQ690400
<i>Orophea enterocarpa</i>	Thailand/ <i>Chalermglin</i> <i>440403</i> (TISTR, Bangkok)	AY319006	AY319119	AY518815	JQ690416	JQ690417	JQ690418
<i>Orophea kerrii</i>	Thailand/ <i>Chalermglin 440416-1</i> (L)	AY319008	AY319121	AY518818	JQ690419	JQ690420	JQ690421

Species	Origin/voucher	<i>rbcL</i>	<i>trnLF</i>	<i>matK</i>	<i>ndhF</i>	<i>psbA-trnH</i>	<i>ycf1</i>
<i>Oxandra venezuelana</i>	Costa Rica/ <i>Chatrou et al. 120 (U)</i>	AY841645	AY841723	JQ690413	JQ690414	AY841495	JQ690415
<i>Platymitra macrocarpa</i>	Bogor Bot. Gard., Indonesia/ <i>Okada 3457 (L)</i>	AY319013	AY319127	AY518812	JQ690422	JQ690423	JQ690424
<i>Platymitra</i> sp.	Thailand/ <i>Chaowasku 100 (L)</i>	---	JQ690425	JQ690426	JQ690427	JQ690428	JQ690429