Buxus sirindhorniana sp. nov. (Buxaceae), a bicarpellate species from Thailand

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A new bicarpellate *Buxus* species, *Buxus sirindhorniana* W. K. Soh, M. von Sternburg, Hodk. & J. Parn., is described from northern Thailand. This is the first reported bicarpellate species of *Buxus*. The pollen morphology and the degree of pollen viability of the species are reported. DNA was extracted from herbarium samples and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA sequenced. The palynological evidence and the phylogenetic reconstruction support the contention that this new species belongs to the genus *Buxus*. The morphological diversity of reproductive organs in the Buxaceae, in relation to this new species, is briefly discussed.

Buxaceae includes the genera *Buxus*, *Haptanthus*, *Notobuxus*, *Pachysandra*, *Sarcococca* and *Styloceras* (Köhler 2006, Shipunov and Shipunova 2011). Based on the study of nuclear and plastid (chloroplast) DNA (von Balthazar et al. 2000), two strongly supported major monophyletic groups are recognised, the first group is *Buxus* with *Notobuxus* and the second group consists of *Pachysandra*, *Sarcococca* and *Styloceras*. *Notobuxus* is now widely recognised as a section under *Buxus* (Tropicos 2013). African *Buxus*, American *Buxus*, Eurasian *Buxus* and *Sarcococca* each form strongly supported clades (von Balthazar et al. 2000) but the relationship within the *Buxus* and *Sarcococca* clades was not, however, fully resolved. *Haptanthus*, a monotypic taxon which was prviously placed in its own family Haptanthaceae, is now grouped in Buxaceae based on DNA evidence (Shipunov and Shipunova 2011).

The only genera of Buxaceae that occur in Thailand are *Buxus* and *Sarcococca*. In the course of a taxonomic revision of Buxaceae for the 'Flora of Thailand Project' (Parnell 2003), we discovered a new species which possessed characters of both genera. This is the second *Buxus* species for Thailand after *B. cochinchinensis* Pierre ex. Gagnep. This new species is similar to *Sarcococca* in having a bicarpellate ovary but it is also similar to *Buxus* in having a single terminal female flower, laterally inserted male flowers and decussate leaves. Herbarium specimens belonging to this new species have previously been identified either as *Sarcococca* or *Buxus*. Sealy (1986), who had revised *Sarcococca*, annotated the specimen Kerr 6131 as *S. coriacea* (Hook.) Sweet. The pollen of this new species, was studied by Brückner (1993) as an unidentified

Buxus species (Smitinand 4702, Hennipman 3194), and found to resemble the pollen type of the genus *Buxus*.

This paper determines the generic status of this new Buxaceae taxon and describes it as a new species.

Material and methods

Macro-morphology and taxonomy

Herbarium specimens of the new taxon were acquired from BKF, K and P. Vegetative and reproductive structures were measured and described following traditional herbarium taxonomy methods (Vogel 1987). Flowers and fruits were boiled and dissected under a Leica M50 stereo microscope. Specimens of *Buxus* and *Sarcococca* including types from BKF, K, KEP, P, TCD and US were compared to the new taxon. In addition to this, online type images were downloaded from A, GH, L and NY.

Pollen type and viability

We used a Tescan Mira XMU scanning electron microscopy (SEM) to determine pollen morphology. Unacetolyzed pollen (Maxwell 96-1672, BKF) was fixed on adhesive tape and coated with gold palladium. The descriptive terminology followed mainly Erdtman (1952).

The detection of decreased pollen viability in plants is commonly used along with other evidence, for the identification of hybrids. We assessed the pollen viability of three flowering herbarium specimens (Hennipman 3194, Maxwell 96-1672, Smitinand 4702) from BKF by staining

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with aniline blue in lactophenol (Dafni 1992). Inviable pollen was counted as that which remained unstained or was distorted. Therefore, pollen that stained blue and was not deformed was considered viable. The anthers were removed from a closed or newly opened young flower and dissected on a microscope slide in a drop of aniline blue–lactophenol solution. The anthers were macerated, the sacs removed and the preparation left for 30 min for the pollen to pick up the stain. The pollen was then observed at $200\times$ using an Olympus BX60 light microscope. Three to five samples were prepared from each herbarium specimen. The number of unstained and/or distorted grains in a sample of 80–100 pollen grains per preparation was counted to assess pollen inviability.

Molecular studies

Herbarium specimens from BKF (Hennipman 3194, Maxwell 96-113, Maxwell 96-1487, Maxwell 96-1672, Smitinand and Anderson 7228, Smitinand 4210, Smitinand 4702) were used for the DNA extraction. Total cellular DNA was extracted using the DNeasy Plant Mini Kit according to the manufacturer's protocol. The internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) were used in this study. Three sets of primers were used to amplify the entire ITS region of nrDNA: ITS5M (Liston et al. 1996) and ITS25R (Nickrent et al. 1994), ITS5/ITS2 (White et al. 1990) and ITS3/ITS4 (White et al. 1990). For the PCR amplification, each reaction mixture (25 μl) contained 14.38 μl of sterile water, 5 μl of $5\times$ Taq polymerase reaction buffer, 2.5 μl of 25 mmol l^{-1} MgCl$_2$, 0.5 μl of 10 mmol l^{-1} dNTPs, 0.25 μl of each of the two primers (100 ng μl^{-1}), 0.13 μl (5 units μl^{-1}) of Taq polymerase and 2 μl of genomic DNA template (20–50 ng). PCRs were performed using a Biometra T Professional Gradient thermal cycler under the conditions of 95°C for 90 s (premelting), 32 cycles of 95°C for 30 s (denaturation), 55°C for 1 min (annealing) and 72°C for 90 s (extension), and finally 72°C for 7 min (final extension). The PCR product was purified with the ExoSap Purification Kit. Cycle sequencing reactions were carried out using the BigDye Terminator Mix and the same primers as above. The sequencing protocol consisted of 28 cycles of 96°C for 1 min (premelting), 96°C for 10 s (denaturation), 50°C for 5 s (annealing) and 60°C for 4 min elongation. Products were run on ABI Prism 3130xl Genetic Analyser according to the manufacturer's protocol.

We were only able to amplify the ITS region from a single sample of the new taxon, Maxwell 96-1672. We obtained a 776 bp fragment, from which we constructed a phylogenetic tree using 25 other taxa from von Balthazar et al. (2000) (Table 1). We did not detect any polymorphic sites (multiple trace peaks) in the nucleotide sequence of the new taxon sequence. The sequences were analysed and assembled using Geneious ver. 6. The coding of hypothesised insertion and deletion events was not attempted. Parsimony analyses were performed using PAUP ver. 4.0b 10 (Swofford 2002). General heuristic search strategies were performed using 1000 replicates of random stepwise

Table 1. GenBank accession numbers for sequences used in the phylogenetic reconstruction.

Taxon	Accession no.
Buxus balearica Wild.	AF245423
Buxus citrifolia (Wild.) Spreng.	AF245433
Buxus glomerata (Griseb.) Müll. Arg.	AF245426
Buxus gonoclada (Wright ex Griseb.) Müll. Arg.	AF245427
Buxus harlandii Hance	AF245410
Buxus henryi Mayr	AF245409
Buxus hildebrandtii Baill.	AF245415
Buxus liukiuensis Makino	AF245428
Buxus macowanii Oliv.	AF245411
Buxus microphylla Siebold & Zucc. subsp. sinica Hatusima	AF245414
Buxus microphylla Siebold & Zucc. var. japonica	AF245412
Buxus riparia Makino	AF245413
Buxus sempervirens L.	AF245429
Buxus sirindhorniana W. K. Soh, M. von Sternburg, T. Hodkinson & J. Parn.	KF447777
Notobuxus acuminata (Gilg) Hutch.	AF245434
Notobuxus natalensis (Oliv.) Hutch.	AF245425
Pachysandra axillaris Franch. subsp. *axillaris*	AF245420
Pachysandra axillaris subsp. *stylosa* Dunn	AF245424
Pachysandra procumbens Michx.	AF245421
Pachysandra terminalis Siebold & Zucc.	AF245430
Sarcococca confertiflora Sealy	AF245416
Sarcococca hookeriana var. *humilis* Baill.	AF245419
Sarcococca ruscifolia var. *ruscifolia* Stapf	AF245417
Sarcococca saligna (D. Don) Müll. Arg.	AF245418
Sarcococca wallichii Stapf	AF245422
Styloceras brokawii A. Gentry & R. Foster	AF245431
Didymeles perrieri Olivier Leandri	AF245432

addition, saving no more than 100 trees per replicate (to avoid time swapping on large islands of trees), and tree–bisection–reconnection (TBR) branch-swapping with MULPARS on (keeping multiple equally most parsimonious trees). Gaps were treated as missing, multi-state characters were interpreted as uncertainty, all characters were unordered and had equal weight. Strict consensus trees were obtained. For clade support, bootstrap support (Felsenstein 1985) was estimated based on 1000 replicates with the same settings as in the heuristic search, but with only 10 replicates of random stepwise additions per bootstrap replicate.

Bayesian analyses were performed using MrBayes ver. 3.2.1 (Ronquist and Huelsenbeck 2003). The best-fit evolutionary model was estimated using Akaike information criterion test (AIC) and Bayesian information criterion test (BIC) implemented in jModelTest ver. 2. (Darriba et al. 2012). The transition model of substitution with invariable sites and gamma distribution $(TIM + I + G)$ was selected. However, we used a general time reversible model of substitution with gamma distribution and invariable sites $(GTR + I + G)$ because this is the best matching model available in MrBayes. The Monte Carlo Markov chain was run for 500 000 generations with four chains, at the temperature of 0.2 and sampling at every 100th cycle. Convergence was achieved when the standard deviation of split frequencies was less than 0.01. The stationary phase was also confirmed by plotting generations vs log likelihoods using Tracer ver. 1.4.1 (Rambaut and Drummond 2007). The first 25% of the 5000 trees
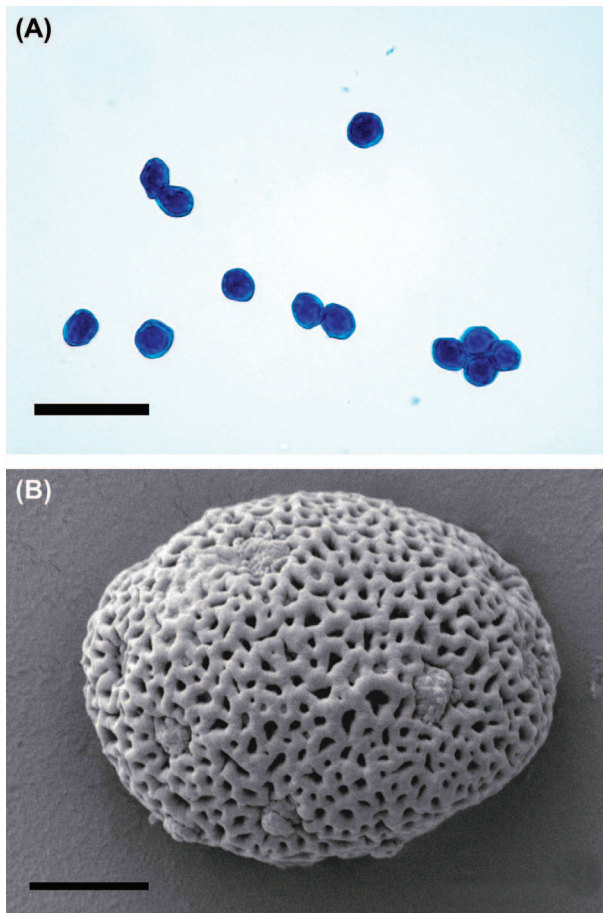
Figure 1. *Buxus sirindhorniana* sp. nov. (Maxwell 96-1672). (A) pollen grains stained with aniline blue–lactophenol, scale bar = 50 μm, (B) a whole pollen grain, SEM × 8800, scale bar = 5 μm.

was discarded as burn-in, and the posterior distribution of trees was summarised using the 'allcompat' consensus.

Results

The pollen type of Maxwell 96-1672 is similar to Hennipman 3194 and Smitinand 4702 as shown in Brückner (1993) (Fig. 1B). The pollen type of our unknown species clearly places it in the genus *Buxus* as it has a reticulate sexine rather than the crotonoid sexine of *Sarcococca* (Erdtman 1952). The following pollen description of our new species is based on this study and Brückner (1993)

Table 2. Pollen size of *Buxus sirindhorniana* sp. nov.

Voucher	Pollen diameter (μm)	Aperture		Sculpture diameter (μm)	
		Number	Diameter (μm)	Lumina	Muri
Smitinand 4702	22 (19–25)	12–14	1.5–2.5	0.5–1.5	0.5–1.0
Hennipman 3194	24.8 (21–28)	12–16	1.5–2.0	0.3–1.0	0.5–1.5
Maxwell 96–1672	18.4 (17–20)	10–15	1.4–2.3	0.3–0.9	0.4–0.8

(Table 2): Pollen pantoporate, 17–28 μm diameter. Pori 10–16, 1.4–2.5 diameter, more or less rounded, and partly covered with reticulum. Exine coarsely reticulate. Muri 0.4–1.5 μm, more or less equal in width, thick, rounded, smooth, with thick or sunken murus at places. Lumina variable 1.4–2.5 μm diameter, irregularly shaped, usually angular. The pollen viability of all three herbarium specimens was between 90–95%. This suggests that the new species has high pollen viability (Fig. 1A).

A single most parsimonious tree was generated from the parsimony analysis of the ITS data set (Fig. 2). The tree length was 1636 with consistency index (CI) of 0.652 and retention index (RI) of 0.748. The tree topology was congruent with that of von Balthazar et al. (2000). The new taxon belongs to the Eurasian *Buxus* clade with strong support (bootstrap and posterior probability value of 100%).

Buxus sirindhorniana W. K. Soh, M. von Sternburg, Hodk. & J. Parn. sp. nov. (Fig. 3, Supplementary material Appendix A1)

Buxo rupicola Ridl. foliis sine venis intramarginalibus et inflorescentia pubescente similis, sed ab ea foliis lanceolatis apice acuto, floribus bicarpellatis differt.

Type: Thailand, Chiang Mai, Doi Chieng Dao, Hennipman 3194 (holotype: BKF!, isotypes: K!, C).

Etymology

The specific epithet is chosen to honour Her Royal Highness Princess Maha Chakri Sirindhorn of Thailand, who is a strong advocate for the preservation of biodiversity in the country.

Description

Shrub or small tree, to 8 m tall, to 26 cm dbh, bark whitish, cracked and corky; twigs terete or subangular, striate and lenticellate, 2–3 mm diameter. Leaves decussate; petiole 0.5–1.0 cm long; blade coriaceous, lanceolate, 7.5–13 × 1.5–2.8 cm, attenuate at base, acute at apex; margin slightly revolute; midrib prominent, raised above, sunken below; intramarginal veins absent; secondary veins faint, 25–30 pairs, 0.5–3.0 mm apart, ca 45 degrees from midrib, arcuate (eucamptodromous), sometimes branched; tertiary veins obscure, reticulate. Inflorescence axillary, hairy, botryoid, to 1.3 cm long, flowers greenish, male flowers 8–12, inserted laterally, female flowers solitary, terminal; bract ovate, acuminate, 2.0–2.5 mm long, fimbriate. Male flowers pedicellate, ca 4 mm long (excluding stamen); pedicel 1.5–2.0 mm long; bract ovate, acuminate, 2.0–2.5 mm long, fimbriate; tepals 4, opposite in two pairs, ovate, concave, ca 2.5 × 1.0 mm, fimbriate; stamens 4, 2–3 mm long; pistillode wider than high, ca 1.5 × 1.0 mm, 4-angled. Female flowers pedicellate, ca 3 mm long, bicarpellate; tepals 4, ovate, ca 1.0–1.5 mm long, opposite in two pairs, fimbriate; ovary 1.5–2.0 mm long; interstylar nectaries present, bulged; styles 2, ca 1 mm long, recurved; stigma decurrent along ventral fold. Fruit a loculicidal capsule, dark green when young, dark purplish when ripe (fide Smitinand 4210), globose, ca 5 mm diameter, persistent
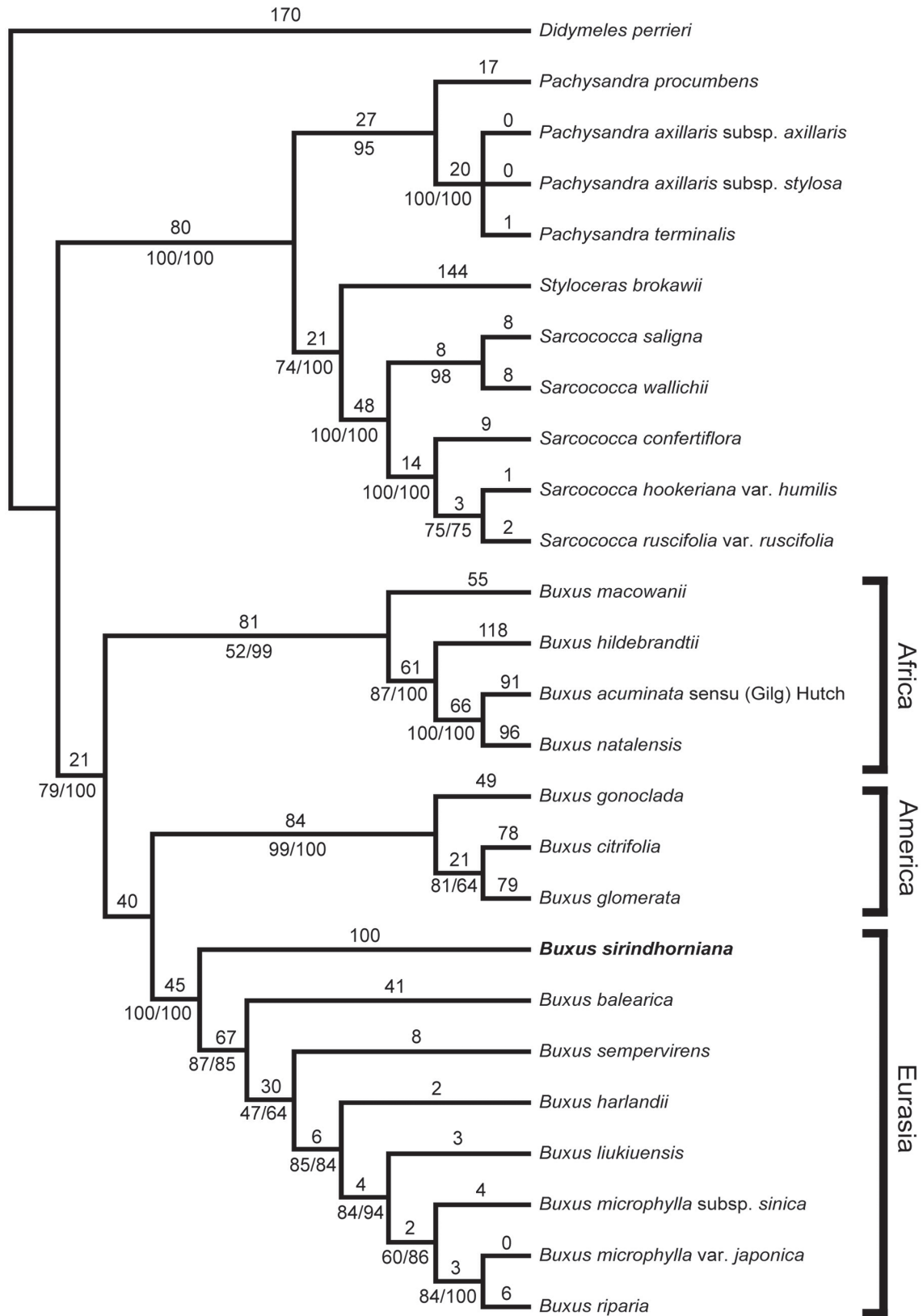
Figure 2. The single most parsimonious tree from the ITS data. Numbers above branches indicate branch lengths, numbers below branches are bootstrap values > 50% and numbers after slashes are posterior probability values > 50%.
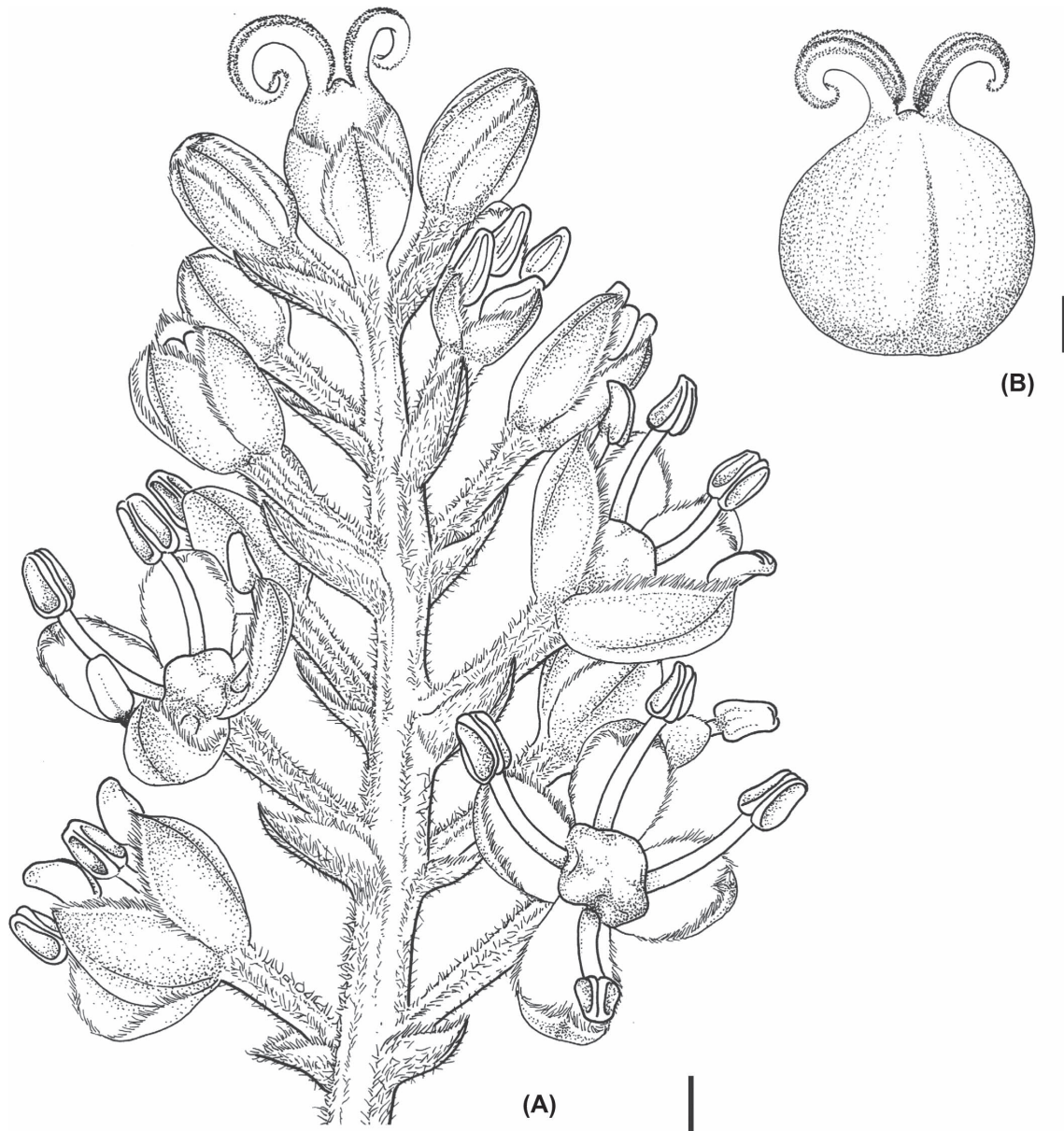
Figure 3. *Buxus sirindihorniana* sp. nov. (A) inflorescence, (B) fruit. Scale bar = 1 mm. Drawn by Wuu Kuang Soh.

tepals appressed to base, orbicular, style persistent, recurved; pericarp thin, ca 0.2 mm thick; seeds 4 (2 per carpel), oblong, with testa black, shiny, and caruncle present.

Phenology
Flowering: July, November, December. Fruiting: January, February, June, July, November, December.

Distribution, habitat and conservation status
Buxus sirindhorniana is found in northeastern Thailand, Chiang Mai Province (Doi Chieng Dao National Park), Lumpang Province (Jae Sawn National Park) and Tak Province (Kao Hua Mot). It grows on exposed limestone ridges at 775–1525 m a.s.l. The species has been collected several times from Doi Chiang Dao, twice from Jae Sawn National Park and once from Kao Huat Mot. All these localities are protected areas and therefore the species does not strictly qualify for any of the IUCN threatened categories, and is thus provisionally assessed as 'Least Concern' (LC) under IUCN (2001).

Notes
A fruiting specimen, Kerr 6131, was identified by Sealy (1986) as *S. coriacea* based on the bicarpellate flower despite its decussate leaf arrangement which resembled *Buxus*. In *Sarcococca*, the inflorescence may be androgynous with the female flowers always at the distal part or wholly male or female. It is evident from the scars at the basal part of the inflorescence in Kerr 6131 that the male flowers may have fallen off, leaving behind the female flowers at the apex. This therefore gives a false impression that its inflorescence is wholly female.

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Additional specimens examined (paratypes)

Chiang Mai Province: Doi Chiang Dao, Chiang Dao Wildlife Sanctuary, 27 Jan 1996, Maxwell 96-113 (BKF); Doi Chiang Dao, Payap, 4 Dec 1965, Hennipman 3194 (holotype: BKF, isotype: K); Doi Chiang Dao, 1 Dec 1961, Smitinand and Anderson 7228 (K, BKF); Doi Chiang Dao, east slope, 16 Feb 1968, Smitinand 4210 (BKF); Doi Chiang Dao, 15 Jul 1958, Smitinand 4702 (BKF, K). Lampang Province: Jae Sawn National Park, northern part, 28 Jan 1996, Maxwell 96-1487 (BKF); Jae Sawn National Park, northern part, 19 Dec 1996, Maxwell 96-1672 (BKF). Tak Province: Kao Hua Mot, 13 Jun 1922, Kerr 6131 (K).

Discussion

In the ITS phylogeny, *Buxus sirindhorniana* was positioned at the base of the Eurasian *Buxus* clade within the larger *Buxus* clade (Fig. 2). A more extensive taxon sampling from an estimated total of 10 species in southeast Asia (Hatusima 1942) should further improve our understanding of the phylogenetic relationships and evolution of Eurasian *Buxus*.

Buxus sirindhorniana was considered allied to *B. rupicola* in having the same pollen type which is pantoporate and reticulate with smooth muri (Brückner 1993). In addition, *Buxus rupicola* is similar to *B. sirindhorniana* in having a hairy inflorescence and lacking intramarginal veins while differing in having an oblong-ovate leaves (3–6 × 1.2–2.5 cm) with rounded apecies (vs lanceolate with acute apex, 7.5–13 × 1.5–2.8 cm) and tricarpellate flowers (vs bicarpellate flowers). *Buxus rupicola* is a rare limestone species that is confined to the Langkawi Islands in northern Peninsular Malaysia bordering southern Thailand while *B. sirindhorniana* is confined to northern Thailand. The pollen type of *B. rupicola* and *B. sirindhorniana* is considered distinctive from other Eurasian *Buxus* (Brückner 1993) because of the smooth muri texture. The smooth and spatially branched muri on *B. rupicola* and *B. sirindhorniana* pollen resembled that of *B. macrophylla* from the neotropics (Köhler and Brückner 1989) while its eucamptodromous venation is similar to *B. madagascarica* from Madagascar and the Comoro Islands. In the revision of the Asiatic *Buxus*, Hatusima (1942) placed *B. rupicola* under the section *Eubuxus* in its own subgroup (Group 1).

The monoecious genera in the Buxaceae have a diverse inflorescence structure (von Balthazar and Endress 2000a, b). The *Buxus* clade (Fig. 2) consistently shares similar morphological characters including decussate leaves, inflorescence type, tricarpellate flowers, interstylodial nectaries and dehiscent 3-horned capsules (Köhler 2006). *Buxus sirindhorniana* is the first recorded solely bicarpellate *Buxus* species, though an anonymous referee has indicated that Madagascaran *Buxus* species are occasionally bicarpellate or 4-carpellate. Its fruit is interpreted as a dehiscent capsule, based on the putative dehiscing line on intact fruits in herbarium specimens. We have not, however, yet encountered specimens with mature dehiscent fruits. The pericarp is thin (ca 0.2 mm) and unlike *Buxus*, the exocarp is not leathery. Based on herbarium labels, young fruits are dark green (Maxwell 96-113, Maxwell 96-1487, Maxwell 96-1672, Smitinand and Anderson 7228) maturing to dark purplish (Smitinand 4210). Though blackish fruits shaded red to purple occur in *Buxus* sect. *Notobuxus*, *Pachysandra* and *Sarcococca,* we are not aware of Eurasian *Buxus* species with dark purplish fruits. Other morphological characters in *B. sirindhorniana*, such as the leaf arrangement, inflorescence type and interstylodial nectaries, are the same as *Buxus*.

In the *Pachysandra–Sarcococca–Styloceras* clade, the reproductive characters are variable. In these three genera, the flowers can be bicarpellate and tricarpellate. Also, whilst the fruits of *Sarcococca* and *Styloceras* are drupaceous to subdrupaceous, those in *Pachysandra* can be either an indehiscent capsule or subdrupaceous (Köhler 2006) (Fig. 4). Our description of *B. sirindhorniana* means that the morphological diversity in the reproductive organs of the *Pachysandra–Sarcococca–Styloceras* clade is now paralleled in *Buxus*. Exceptionally, *Buxus conzatii* Standley and *Styloceras laurifolium* are similar to *Sarcococca* in having an alternate leaf arrangement, bicarpellate flowers and subdrupaceous to drupaceous fruits that lack interstylar nectaries but differ in having a terminal female flower (Fig. 4). The morphological diversity in the reproductive organs of the *Pachysandra–Sarcococca–Styloceras* clade can also be found in *Buxus* as demonstrated in *B. sirindhorniana*.

Interspecific hybrids among cultivars of *Buxus* are well known, for example between *B. sempervirens* and *B. microphylla* (Larson 1999) but natural hybrids among Buxaceae species including intergeneric hybrids have
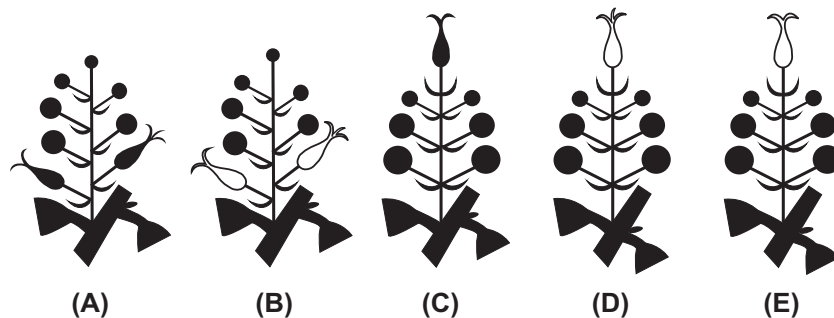

Figure 4. A schematic diagram of the monoecious inflorescence types in Buxaceae. The shaded female flower develops into a drupaceous or sub-drupaceous fruit. The hollow female flower develops into a capsule. (A) *Pachysandra* and *Sarcococca*, (B) *Pachysandra*, (C) *Sarcococca conzattii* and *Styloceraslaurifolium*, (D) *Buxus*, (E) *Buxus sirindhorniana*.

not, to our knowledge, been documented. We are confident that *B. sirindhorniana* is not an intergeneric or interspecific hybrid because, firstly the pollen viability in *B. sirindhorniana* is high. Secondly, there are no polymorphic sites in the nucleotide sequences of the ITS ribosomal DNA which indicates that there are no different ITS tandem repeats present. An F1 hybrid or hybrid would inherit ITS types from each of the parental taxa (Hodkinson et al. 2002) and there is no evidence of this. Thirdly, the new species is morphologically distinct from other Asian Buxaceae species. Fourthly the species distribution only overlaps in one locality with *Sarcococca* species (*S. balansae* and *S. coriaceae*) in Doi Chieng Dao but not at all with *Buxus*. This implies that natural hybridisation from sympatric species of *Buxus* and *Sarcococca* is highly improbable. Finally, intergeneric hybridization is rare for distinct genera and is very unlikely for *Buxus* and *Sarcococca* since both genera are phylogenetically far apart.

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Supplementary material (Appendix NJB-00314 at < www.oikosoffice.lu.se/appendix >). Appendix A1.