A MULTILOCUS PHYLOGENY OF *DAPHNIPHYLLUM* (DAPHNIPHYLLACEAE)[1]

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Abstract

The monogeneric Daphniphyllaceae contain 36 taxa in the genus *Daphniphyllum* Blume that are endemic to Southeast Asia. *Daphniphyllum* is a morphologically homogeneous group of evergreen shrubs to trees divided into three sections: *Daphniphyllum*, *Lunata* T. C. Huang, and *Staminodia* Hurusawa. While the medicinal value of the Daphniphyllaceae has been explored in many studies, the understanding of their evolutionary history and infrageneric classification is still limited. To test the infrageneric classification and to examine evolutionary and classification hypotheses proposed by previous taxonomic studies, we reconstructed multilocus phylogenies by sampling 55.6% (= 20/36) of taxa in the genus based on both chloroplast (*psb*A-*trn*H spacer and *trn*L intron) and nuclear ITS (ITS1, 5.8S rDNA, and ITS2) regions. Our data do not support the monophyly of the three sections. Our results indicate that some hybridization events might have occurred in the evolutionary history of the genus. Moreover, our results support the classification hypotheses of *D. glaucescens* Blume in a strict sense but not in a broad sense. In addition, we elevate *D.* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang, stat. nov., from varietal rank to species based on our results. Two putative natural hybrids, *D.* ×*lanyuense* and *D. teijsmannii* Zoll. ex Teijsm. & Binn., and their putative parental taxa are also revealed in our study. In sum, our results shed new insights into the sectional scope and understanding of evolutionary relationships among taxa in *Daphniphyllum*.

Key words: *Daphniphyllum*, natural hybrid, phylogeny, section *Lunata*, section *Staminodia*.

The Daphniphyllaceae is a monogeneric family that currently contains 36 taxa in the genus *Daphniphyllum* Blume (Bentham, 1854; Baillon, 1858; Müller Argoviensis, 1869; Hooker, 1890; Hayata, 1904; Rosenthal, 1919; Chien, 1933; Croizat & Metcalf, 1941; Hurusawa, 1942a, 1942b; Huang, 1965, 1966, 1992, 1993, 1996, 1997; Hatusima, 1971; Ming, 1980; Wang, 1981; Grierson & Long, 1987; Noshiro, 1999; Kiew & Rafidah, 2008; Ming & Kubitzki, 2008; Tang et al., 2009, 2012; Meng et al., 2020; Tagane et al., 2020) (Table 1). *Daphniphyllum* species are evergreen, glabrous trees or shrubs, characterized by axillary racemes, unisexual flowers, hypogynous ovaries, bistigmas, and oblique drupes (Fig. 1). They are widely distributed from the Himalayas east to China, Japan, Korea, and south to Indonesia, New Guinea, the Philippines, southern India, and Sri Lanka. Most taxa have very restricted distribution ranges (Table 1). A few *Daphniphyllum* taxa have been used for garden trees, street trees, furniture, construction, agricultural implements, and traditional herbal medicines for a long time in Asia (e.g., Kunwar, 2003; Tynsong & Tiwari, 2010), and some species are cultivated in the west (Boyce, 1999). Recently, an increasing number of studies have been working on understanding the anticancer and antioxidant functions of alkaloids and/or flavonoids extracted from *Daphniphyllum* plants (e.g., Gamez et al., 1998; Lu et al., 2013; Chen et al., 2018).

The historical classification of the Daphniphyllaceae has resulted in various placements in sometimes

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Table 1. Current infrageneric classification of genus *Daphniphyllum* Blume, the morphology of their pistillate flowers and fruits, and their distributions. Asterisks indicate the taxa sampled in this study. Abbreviations: A, absent; C, caducous; EA, East Asia; N/A, not available; P, persistent; SEA, Southeast Asia.

Scientific name[1]	Section[2]	Pistillate flower[3]: Stigma		Calyx		Staminode	Fruit[4]	Distribution[1]
		Form at early stage of anthesis	Abscission	Form	Persistent/ Caducous/ Absent	Persistent/ Caducous/ Absent	Form of endocarp	
D. calycinum Benth.*	*Lunata*	punctiform	no	cleft	P	A	corniculate	EA
D. laurinum (Benth.) Baill.*	*Lunata*	punctiform	no	cleft	P	A	corniculate	SEA
D. majus Müll. Arg.*	*Lunata*	N/A	no	cleft	P	A	strumose	EA
D. peltatum Yan Liu & T. Meng	*Lunata*	punctiform	no	cleft	P	A	N/A	China: Guangxi
D. angustifolium Hutch.*	*Staminodia*	linear	yes	A	A	C	smooth	China: Hubei, Sichuan
D. buchananiifolium Hallier f.*	*Staminodia*	reniform	no	free	C	C	smooth	Philippines
D. celebense K. Rosenthal	*Staminodia*	reniform	no	free	C	C	smooth	Indonesia
D. chartaceum K. Rosenthal*	*Staminodia*	reniform	no	A	A	A	strumose	EA
D. himalense (Benth.) Müll. Arg.*	*Staminodia*	reniform	no	free	C	C	smooth or strumose	EA
D. longeracemosum K. Rosenthal*	*Staminodia*	reniform	no	A	A	A	strumose	EA
D. macropodum Miq.*	*Staminodia*	reniform	no	free or A	C or A	P or A	strumose	EA
D. macropodum var. *humile* (Maxim. ex Franch. & Sav.) K. Rosenthal*	*Staminodia*	reniform	no	A	A	P	strumose	Japan
D. parvifolium Quisumb. & Merr.*	*Staminodia*	N/A	N/A	N/A	C	N/A	smooth	Philippines
D. woodsonianum T. C. Huang	*Staminodia*	reniform	no	free	C	C	smooth	Indonesia: Sumatra
D. beddomei Craib	*Daphniphyllum*	oblong	no	parted	P or C	C	strumose	Indochina
D. borneense Stapf*	*Daphniphyllum*	oblong	no	parted	C	C	smooth	Borneo
D. ceramense (T. C. Huang) T. C. Huang	*Daphniphyllum*	oblong	no	free	C	C	smooth	Indonesia
D. dichotomum (T. C. Huang) T. C. Huang*	*Daphniphyllum*	oblong	no	parted	C	C	smooth	SEA
D. glaucescens Blume subsp. *oldhamii* (Hemsl.) T. C. Huang var. *kengii* (Hurus.) T. C. Huang*	*Daphniphyllum*	oblong	no	parted	P or C	A	strumose	Taiwan
D. glaucescens subsp. *oldhamii* var. *lanyuense* T. C. Huang*[,7]	*Daphniphyllum*	reniform	no	parted	C	A	strumose	Taiwan

D. glaucescens var. *blumeanum* (Baill. ex Müll. Arg.) J. J. Sm.*	*Daphniphyllum*	linear	yes	parted	C	C	strumose	Indonesia
D. glaucescens var. *glaucescens*	*Daphniphyllum*	linear	no	parted	C	C	strumose	Indonesia: Java
D. glaucescens var. *lancifolium* (Hook. f.) Rafidah	*Daphniphyllum*	linear	no	parted	C	C	strumose	Malay Peninsula
D. gracile Gage	*Daphniphyllum*	oblong	no	parted	C	C	smooth	New Guinea
D. gracile var. *newirelandum* T. C. Huang	*Daphniphyllum*	reniform	N/A	N/A	N/A	N/A	smooth	New Guinea
D. hongiaoense Yahara & Tagane	*Daphniphyllum*	oblong	N/A	A[6]	A	A	strumose	Vietnam
D. luzonense Elmer	*Daphniphyllum*	linear	no	parted	C	C	smooth	Philippines
D. neilgherrense (Wight) Thwaites	*Daphniphyllum*	oblong	no	parted	C	C	strumose	South of India and Sri Lanka
D. oldhamii (Hemsl.) K. Rosenthal*	*Daphniphyllum*	oblong	no	parted	C	A	strumose	EA
D. papuanum Hallier f.	*Daphniphyllum*	oblong	no	parted	C	C	smooth, strumose	New Guinea
D. paxianum K. Rosenthal*	*Daphniphyllum*	oblong	no	parted	P	A	strumose	EA
D. scortechinii Hook. f.*	*Daphniphyllum*	oblong	no	parted	C	C	smooth	Malay Peninsula
D. subverticillatum Merr.	*Daphniphyllum*	oblong	no	parted	C	A	strumose	China: Fujian, Guangdong
D. sumatraense (T. C. Huang) T. C. Huang	*Daphniphyllum*	reniform	no	parted	C	C	smooth	Indonesia: Sumatra
D. teijsmannii Zoll. ex Teijsm. & Binn.*	*Daphniphyllum*	oblong	no	parted	C	A[5]	strumose	Japan and South Korea: Jeju Island
D. timorianum (T. C. Huang) T. C. Huang	*Daphniphyllum*	oblong	no	parted	C	C	smooth	Indonesia

[1] The list of the current accepted 36 *Daphniphyllum* taxa and distribution of each taxon were adopted from Huang (1965, 1993, 1996), Hatusima (1971), Ming and Kubitzki (2008), Kiew and Rafidah (2008), Meng et al. (2020), and Tagane et al. (2020).

[2] *Daphniphyllum peltatum* is assigned to section *Lunata* by Mo-Shih Tang based on its punctiform stigmas and cleft, 4- to 6-parted, persistent calyx (see the species description in Meng et al. [2020]). *Daphniphyllum hongiaoense* is assigned to section *Daphniphyllum* by Mo-Shih Tang based on its species description in Tagane et al. (2020). Sectional assignments for all other *Daphniphyllum* taxa were obtained from Tang et al. (2012).

[3] The pistillate flower morphology for each taxon was gained from Huang (1965, 1966), Tang et al. (2009, 2012), Meng et al. (2020), and Tagane et al. (2020).

[4] Fruit morphology was investigated by Mo-Shih Tang.

[5] The type specimen of *Daphniphyllum teijsmannii* has staminodes, while other *D. teijsmannii* plants have no staminode (Tang et al., 2012).

[6] For *Daphniphyllum hongiaoense*, the calyx is absent in pistillate flowers, and five calyces are shown in staminate flowers (Tagane et al., 2020).

[7] Note that *Daphniphyllum glaucescens* subsp. *oldhamii* var. *lanyuense* is elevated to *D.* ×*lanyuense* in this study.

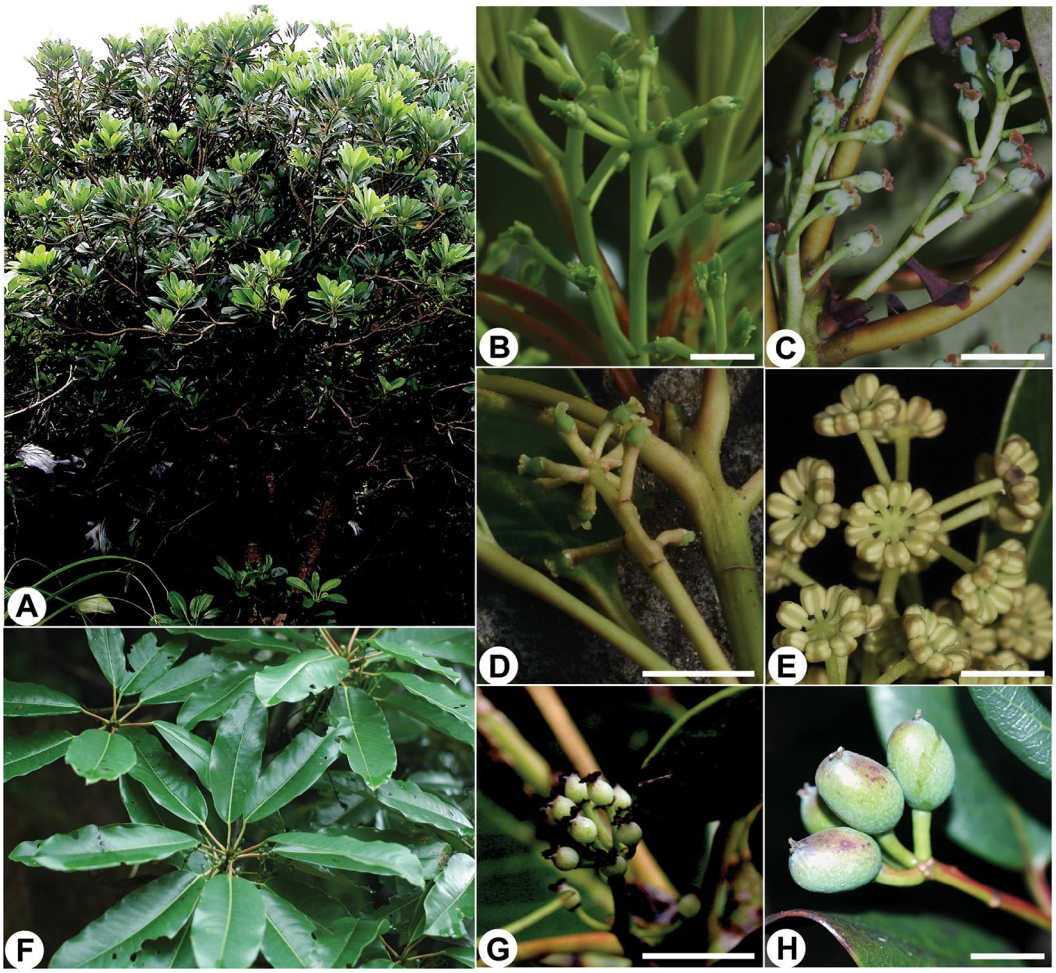

Figure 1. Representatives of *Daphniphyllum* Blume taxa. —A. Habit of *D.* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang. —B. Female inflorescences and flowers with linear stigma and parted calyx in *D. glaucescens* var. *blumeanum* (Baill. ex Müll. Arg.) J. J. Sm. —C. Female inflorescences and flowers with reniform stigma and staminodia in *D. macropodum* Miq. var. *humile* (Maxim. ex Franch. & Sav.) K. Rosenthal, scale bar = 1 cm. —D. Female inflorescences and flowers with oblong stigma and parted calyx in *D. oldhamii* (Hemsl.) K. Rosenthal, scale bar = 1 cm. —E. Male inflorescences and flowers in *D. glaucescens* subsp. *oldhamii* var. *kengii* (Hurus.) T. C. Huang, scale bar = 0.5 cm. —F. Leaves of *D. macropodum*. —G. Female inflorescences and flowers with reniform stigma and free sepal in *D. buchananiifolium* Hallier f., scale bar = 1 cm. —H. Fruits of *D. borneense* Stapf, scale bar = 1 cm. All photos were taken by Mo-Shih Tang.

distantly related families. *Daphniphyllum glaucescens* Blume was the first species of the genus and was considered to belong in the Euphorbiaceae (Blume, 1826). Baillon (1858) placed *Daphniphyllum* in the Rhamnaceae. Based on the morphology of sepals, stamens, and fruits. Müller Argoviensis (1869) assigned *Daphniphyllum* to its own family Daphniphyllaceae. Studies focusing on palynology (Huang, 1965; Bhatnagar & Garg, 1977), floral morphology (Tiffney, 1986; Kapil & Bhatnagar, 1994), embryology (Endress & Igersheim, 1999), wood anatomy (Huang, 1965; Cronquist, 1983), and dispersal mechanisms (Tiffney, 1986) suggested that *Daphniphyllum* is close to Hamamelids. Based on recent molecular phylogenetic studies, the family Daphniphyllaceae is placed in the Saxifragales and is closely related to Altingiaceae, Cercidiphyllaceae, and Hamamelidaceae (Feng et al., 1998; Shi et al., 2001; Davis & Chase, 2004; Dong et al., 2013a; Soltis et al., 2013; Tank et al., 2015; APG IV, 2016; Folk et al., 2019; Li et al., 2019).

Sectional classification of *Daphniphyllum* is difficult and has varied based on the morphological characters that have been used to delimit species into sections. Hurusawa (1942a, 1942b) investigated four Japanese

and Taiwanese taxa and divided those *Daphniphyllum* into two sections: *Staminodia* Hurusawa and *Calycifera* Hurusawa. Huang (1966) treated all *Daphniphyllum*—nine species, 19 subspecies, 16 varieties, and three forms—in three sections: *Calycifera* (assigned as *Daphniphyllum* [after Huang, 1966]); *Lunata* T. C. Huang; and *Staminodia*. Later, Huang (1996, 1997) described 16 Malaysian *Daphniphyllum* taxa and relegated section *Staminodia* to be a subsection of section *Daphniphyllum*. Tang et al. (2009, 2012) investigated leaf anatomy and pistil morphology in 19 taxa of *Daphniphyllum* that supported classification into three sections proposed by Huang (1965, 1966). *Daphniphyllum buchananiifolium* Hallier f. and *D. woodsonianum* T. C. Huang were found to belong to section *Staminodia* based on their pistil characters and leaf morphology (Tang et al., 2009, 2012) (see Table 1). Section *Lunata* now comprises four taxa characterized by a persistent, cleft calyx and punctiform stigma; section *Daphniphyllum* consists of 22 taxa with a persistent or deciduous calyx and oblong or linear stigma; and section *Staminodia* contains 10 taxa with asepalous or two (to four) free deciduous sepals (four taxa are asepalous; four taxa have two [to four] free sepals; one taxon has both asepalous flowers or two [to four] free sepal flowers; and information of another taxon is unavailable) and a reniform stigma (Huang, 1965, 1966; Tang et al., 2012) (Table 1). The limited morphology that defines sections and delimitation of species may also be further complicated by cryptic differences; thus, a densely sampled molecular phylogeny is needed to better inform relationships and classification schemes.

No comprehensive phylogeny has tested the evolutionary history of *Daphniphyllum*. Huang's (1965) classification scheme was based on six traits including long/short sepals, free/fused sepals, present/absent calyx, long/short style, colpate/colporoidate-colporate pollen, and multiseriate (2[to 3]-)/uniseriate wood rays. Based on these characteristics, Huang (1965, 1996) proposed that sections *Lunata* and *Daphniphyllum* were the earliest diverging lineages, and section *Staminodia* is sister to section *Daphniphyllum*. However, based on pistillate flower morphology, Tang et al. (2012) proposed different hypotheses on the relationships among the sections of *Daphniphyllum*. They proposed a hypothetical phylogeny that section *Staminodia* with the sepals absent or free was the earliest lineage sister to sections *Lunata* and *Daphniphyllum* with connate sepals (Tang et al., 2012). In a broad study of the Saxifragales based on 301 low-copy and 24 multicopy regions (Folk et al., 2019), *D. macropodum* Miq. (section *Staminodia*) was placed as the earliest branch of the Daphniphyllaceae, which supported the hypotheses of Tang et al. (2012), and the low resolution of sections and species in Daphniphyllaceae implied the need of a more comprehensive phylogenetic study in this family.

High morphological similarity among taxa in *Daphniphyllum* has led to the difficulty in intrageneric classification and recognition of species. Delimitation of *D. glaucescens* has varied in rank classification from subspecies to a variety. For example, Huang (1965, 1966, 1992, 1993) proposed a classification hypothesis with a broad species concept of *D. glaucescens* and assigned a list of subspecies and varieties in his early *Daphniphyllum* studies. Later morphological investigation did not support Huang's earlier classification of *D. glaucescens* and resulted in the subspecies and/or varieties being treated at a specific status (Ming, 1980; Huang, 1996, 1997; Ming & Kubitzki, 2008; Tang et al., 2009, 2012). However, no analyses had been provided to test *D. glaucescens* s.l. in these studies.

Molecular phylogenetic analyses have been successfully applied to provide insights into species delimitation and to investigate infraspecific relationships for many plant groups (e.g., Koch et al., 2003; Gutiérrez & Garbino, 2018; Liu et al., 2018; Vigalondo et al., 2019). Here, we aim to (1) examine the sectional classification of *Daphniphyllum*, (2) elucidate the evolutionary relationships among taxa, and (3) test the classification hypothesis of *D. glaucescens* s.l.

Materials and Methods

Plant materials and taxon sampling

To provide a comprehensive phylogeny of *Daphniphyllum*, we sampled as many *Daphniphyllum* taxa as possible to represent all three sections and included 20/36 taxa. Species were identified by investigating the leaf, pistillate flower, and fruit morphology (see Table 1), reviewing earlier systematic works on *Daphniphyllum* (Bentham, 1854; Baillon, 1858; Müller Argoviensis, 1869; Hooker, 1890; Hayata, 1904; Rosenthal, 1919; Chien, 1933; Croizat & Metcalf, 1941; Hurusawa, 1942a, 1942b; Huang, 1965, 1966, 1992, 1993, 1996, 1997; Hatusima, 1971; Ming, 1980; Wang, 1981; Grierson & Long, 1987; Noshiro, 1999; Ming & Kubitzki, 2008; Tang et al., 2009, 2012), and examining specimens from BM, BO, CDBI, HAST, IBSC, K, KEP, KUN, L, MEL, PE, PNH, PPI, PYU, SAPS, SING, SNP, SZ, TAI, TAIF, TCF, TI, TNM, and TNS herbaria (Thiers, 2016). Most material was collected during field trips and preserved in silica by us in China, Indonesia, Japan, Malaysia, the Philippines, and Taiwan, and some additional samples collected and preserved in silica were provided by other botanists. Herbarium materials were used when no fresh material was available. The details of the sampled taxa and collections are shown in Supplementary Table S1. Seven outgroup

taxa (*Liquidambar formosana* Hance, *Cercidiphyllum japonicum* Siebold & Zucc., *Corylopsis glabrescens* Franch. & Sav., *Corylopsis pauciflora* Siebold & Zucc., *Corylopsis spicata* Siebold & Zucc., *Eustigma oblongifolium* Gardner & Champ., and *E. balansae* Oliv.) representing the three most closely related families Altingiaceae, Cercidiphyllaceae, and Hamamelidaceae (Tank et al., 2015; APG IV, 2016; Li et al., 2019) were also selected. The details of outgroup taxa are provided in Supplementary Table S1.

DNA ISOLATION, DNA REGION SELECTION, AMPLIFICATION, CLONING, AND SEQUENCING

Genomic DNA was isolated from dried leaf materials using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The ITS (ITS1, 5.8S rDNA, and ITS2), *psb*A-*trn*H spacer, and *trn*L intron regions were chosen for the study for their broad application across plant families and utility. The primers and amplification protocols described in Taberlet et al. (1991), Azuma et al. (1999), and Tsai et al. (2005) were employed in the present study for the three selected regions (Supplementary Table S2). The amplified products were purified by cutting the gel after gel separation on 1.0% agarose, and then DNA was dissolved by the Gel/PCR DNA Isolation System (Viogene, New Taipei City, Taiwan) according to the manufacturer's protocol. The purified DNA was sequenced commercially on an ABI PRISM 3700 Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.A.) at the Genomics BioSci & Tech. Co. Ltd. (New Taipei City, Taiwan) with the polymerase chain reaction (PCR) primers (Supplementary Table S2).

Our initial sequencing results showed that some *Daphniphyllum* samples have a few ambiguous bases in their ITS sequences. For these *Daphniphyllum* samples (see Results), TA cloning was carried out using pGEM-T Easy Vector Systems (Promega, Madison, Wisconsin, U.S.A.) with the purified PCR products of the region. Positive colonies were double-checked using PCR amplifications with the T7 and SP6 primers. Three to five positive double-checked clones were selected for each sample and were sequenced commercially with the primers T7 and SP6 at the Genomics BioSci & Tech. Co. Ltd.

Contigs were assembled with BioEdit 7.2.6.1 (Hall, 1999). The newly generated sequences have been submitted to the NCBI database, and the accession numbers are reported in Supplementary Table S1.

PHYLOGENETIC ANALYSES

Sequence alignments were conducted using MAFFT 7 (Katoh et al., 2002; Katoh & Standley, 2013), and alignments were viewed in Mesquite 3.6 (Maddison & Maddison, 2018). Alignments were adjusted to reduce misalignment of poly-A or poly-T repeats. An earlier plastome comparison study showed no recombination in plastomes of order Saxifragales plants (Dong et al., 2013b). Hence, the alignments of our *psb*A-*trn*H spacer and *trn*L intron regions were concatenated as the chloroplast (cp) alignment. Indels in the alignment were coded using simple gap-coding (Simmons & Ochoterena, 2000) implemented using FastGap 1.2 (Borchsenius, 2009). Studies have shown that gap-coding provides support to branch nodes in analyses (Dessimoz & Gil, 2010; Goh et al., 2020).

The ITS and cp alignments were inferred separately using maximum likelihood (ML) and Bayesian inference (BI) using RAxML 8.2.12 (Stamatakis, 2014) and MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012), respectively, in the CIPRES Science Gateway 3.3 (Miller et al., 2010). To conduct ML and BI tree reconstructions, the best-fit nucleotide substitution models for cp and ITS trees were estimated in advance using jModeltest 2.1.10 (Darriba et al., 2012) with the Akaike information criterion (AIC). The best-fit nucleotide substitution models of the studied regions are available in Table 2. For ML tree reconstructions, 10 tree searches and 1000 rapid bootstrap (BS) procedures were run in RAxML 8.2.12 with the best-fit nucleotide substitution models. For BI tree reconstructions, two independent Markov chain Monte Carlo (MCMC) runs were performed in MrBayes 3.2.6 with the best-fit nucleotide substitution models. In each run, the algorithm began from a random tree, and four simultaneous chains were set at default temperature (Huelsenbeck & Ronquist, 2001). One tree was sampled every 1000th generation, and a total of 1×10^7 generations were performed in each run. The first 25% of trees were set as burn-in. To estimate the posterior probabilities (PP) on each branch of the consensus BI tree, the last 75% of trees were summed with a 50% majority rule applied. The ML and BI trees were then illustrated using FigTree 1.4.3 (Rambaut, 2014).

Shimodaira-Hasegawa (SH) (Shimodaira & Hasegawa, 1999) and Kishino-Hasegawa (KH) tests (Kishino & Hasegawa, 1989) implemented in PAUP* version 4.0a166 (Swofford, 2002) were conducted to test the topological incongruence between the ITS and cp trees. The combination of multiple loci from different genomic regions has been shown to provide better phylogenetic resolution (Burleigh & Mathews, 2004; Kupczok et al., 2010; Liu et al., 2017; Shi et al., 2017). To improve the discrimination of the *Daphniphyllum* phylogeny, combined data were used to reconstruct a species tree using both ML and BI algorithms with the criteria described above. Since all ITS clones from the same collection were clustered into a clade (Fig. 2), one ITS

Table 2. The statistics for maximum parsimony analyses, the best-fit nucleotide substitution models, and indels for DNA regions applied in this study.

Alignment (DNA region)	Number of ingroup sequences/ taxa	Number of outgroup sequences/ taxa	Length of alignment (bp + indel)	Number of parsimony-informative characters (all accessions / ingroup only)	Percentage of parsimony-informative characters (all accessions / ingroup only)	Number of informative indel characters	The best-fit nucleotide substitution model
cp (*psb*A-*trn*H spacer + *trn*L intron)	30 / 16	6 / 6	895 + 38	92 / 24	10.28% / 2.68%	23	GTR+G
ITS (ITS1+5.8S rDNA+ITS2)	55 / 20	6 / 6	707 + 87	235 / 54	33.24% / 7.64%	51	GTR+I

clone was randomly selected to represent the collection in the combined alignment. In addition, in order to understand the phylogenetic relationships among the main lineages in this family and to reduce the conflict signals from reticulate evolution, any taxa with possible hybrid origin(s), if any, were excluded from the combined tree analyses (Moody & Rieseberg, 2012; Triplett et al., 2014; Gong et al., 2017).

To test the classification hypotheses of *Daphniphyllum glaucescens* s.l., we employed topological conflict analyses, which were successfully applied in many earlier phylogenetic studies (Yang et al., 2012; Guo et al., 2017; Liu et al., 2018). For each alignment (cp, ITS, or ITS+cp), we first created a hypothesis tree (Ho) assuming all subspecies and varieties are in a monophyletic clade. Then, for each alignment, a constraint ML tree was generated with RAxML 8.2.12 as described in the previous paragraphs but using the Ho tree as a specific constraint, GTR+G as evolutionary model, and (-f g) as the optional parameters. Later, both SH and KH tests were conducted to test whether the constraint ML trees were significantly different from our real, unconstrained ML trees.

Results

In total, 45 *Daphniphyllum* accessions—representing 20 taxa (20/36 = 55.6%), including three taxa (3/4 = 75.0%) in section *Lunata*, eight taxa (8/10 = 80.0%) in section *Staminodia*, and nine taxa (9/22 = 40.9%) in section *Daphniphyllum* as proposed by Tang et al. (2012)—were analyzed in this study (Supplementary Table S1). Eleven *Daphniphyllum* accessions had ambiguous bases in their ITS sequences in our initial sequencing results, and the TA cloning was applied for these accessions. Two to five distinctive ribotypes were identified for these accessions (Supplementary Table S1, File S4). In sum, we produced 95 *Daphniphyllum* sequences newly generated in this study, six sequences (four ITS accessions, one *psb*A-*trn*H spacer accession, and one *trn*L intron accession) downloaded from GenBank, and six sequences obtained from the supermatrix of Folk et al. (2019). Detailed information of all studied *Daphniphyllum* sequences is provided in Supplementary Table S1. In total, 107 *Daphniphyllum* sequences were used. We also sequenced one additional outgroup and used 17 sequences from GenBank for six outgroup taxa in our analyses (Supplementary Table S1).

We found that PCR was unsuccessful using herbarium samples in our study. Supplementary Table S7 shows all herbarium samples from which genomic DNA was extracted in this study. Only two *Daphniphyllum* samples were successfully amplified (*D. laurinum* (Benth.) Baill. collected by Ambri & Arifin #AA129 and *D. angustifolium* Hutch. collected by Yaodong Chen #2009, see Supplementary Table S1). Earlier studies revealed that DNA quality remains relatively good in the herbarium samples for some plant groups (e.g., Choi et al., 2015; Shipunov et al., 2020) but not for other plant groups (e.g., Do & Záveská Drábková, 2018; Paton et al., 2018). Our study shows that *Daphniphyllum* herbarium samples do not provide high quality usable DNA even after relatively short periods of time.

Phylogenetic trees were reconstructed based on 30 and 55 *Daphniphyllum* sequences and six outgroups in the cp and ITS alignments with lengths of 933 and 794 characters, respectively (Table 2). In total, 92 (10.28%) and 235 (33.24%) parsimony-informative base pairs and 23 and 51 indels were found in the cp and ITS analyses, respectively (Table 2).

Our ML and BI analyses based on ITS alignment were similar in topology but differed in branch support values. The ML and BI trees based on cp alignment were also similar to one another in topology. Therefore, only BI trees are shown here (Figs. 2, 3, Supplementary Fig. S8). The alignments and tree files are available in the supplementary data (Files S3, S4).

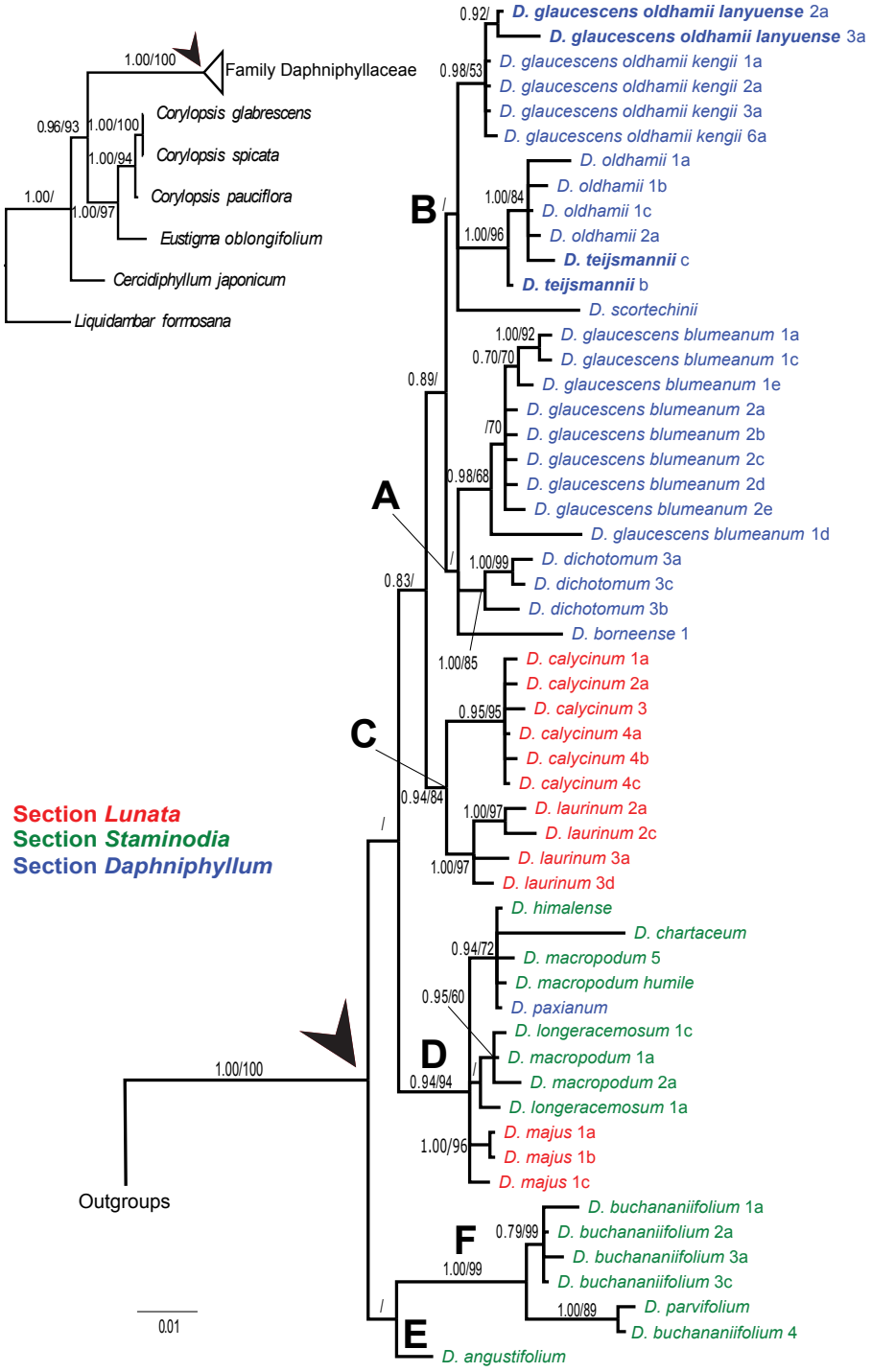

Figure 2. Bayesian inference (BI) of *Daphniphyllum* Blume based on ITS (ITS1, 5.8S rDNA, and ITS2) region. Fifty-five sequences, representing 55.6% of *Daphniphyllum* taxa (20 out of 36 taxa), and six outgroups were analyzed in a matrix with an alignment length of 707 bp and 87 indels. Numbers after taxa refer to Supplementary Table S1, and different clones of the same collection are labeled with letters. Numbers at nodes show Bayesian posterior probabilities/maximum likelihood bootstrap values only when posterior probability is > 0.70 or bootstrap value is > 50. The arrow shows the crown node of *Daphniphyllum*. Sectional assignments follow Tang et al. (2012) and are color-coded. Clades are indicated with capital A, B, C, D, E, and F. Putative hybrid taxa are highlighted in bold. Note that *D. glaucescens* Blume subsp. *oldhamii* (Hemsl.) T. C. Huang var. *lanyuense* T. C. Huang is elevated to *D.* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang in this study.

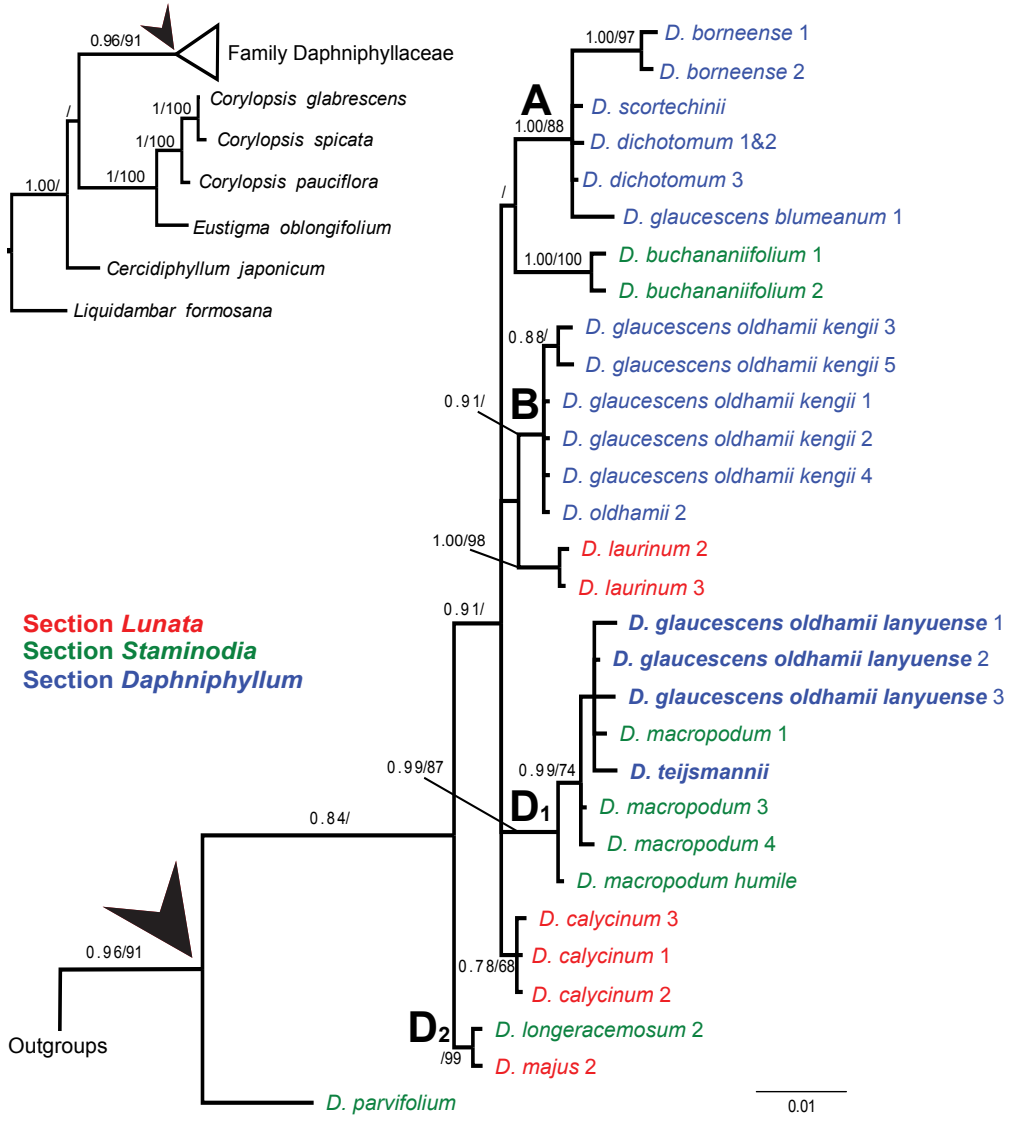

Figure 3. Bayesian inference (BI) of *Daphniphyllum* Blume based on two chloroplast regions (cp; *psb*A-*trn*H spacer and *trn*L intron regions). Thirty sequences, representing 44.4% of *Daphniphyllum* taxa (16 out of 36 taxa), and six outgroups were analyzed in a matrix with an alignment length of 895 bp and 38 indels. Numbers after taxa refer to Supplementary Table S1. Numbers at nodes show Bayesian posterior probabilities/maximum likelihood bootstrap values only when posterior probability is > 0.70 or bootstrap value is > 50. The arrow shows the crown node of *Daphniphyllum*. Sectional assignments follow Tang et al. (2012) and are color-coded. Clades are indicated as in Figure 2. Putative hybrid taxa are highlighted in bold. Note that *D. glaucescens* Blume subsp. *oldhamii* (Hemsl.) T. C. Huang var. *lanyuense* T. C. Huang is elevated to *D.* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang in this study.

Both SH and KH tests showed that the ITS tree (Fig. 2) and cp tree (Fig. 3) were incongruent ($P < 0.05$). Therefore, the tree based on combining cp and ITS regions, which might be informative, was provided as a supplemental file (Supplementary Figure S8) but not shown in the main text. *Daphniphyllum glaucescens* subsp. *oldhamii* (Hemsl.) T. C. Huang var. *lanyuense* T. C. Huang, *D. teijsmannii* Zoll. ex Teijsm. & Binn., and *D. macropodum* and its variety *D. macropodum* var. *humile* (Maxim. ex Franch. & Sav.) K. Rosenthal were clustered in the cp tree (Clade D_1; PP = 0.99; BS = 87; Fig. 3), but each of them grouped with different *Daphniphyllum* taxa in the ITS tree (Fig. 2). As shown in Figure 2, *D. teijsmannii* is grouped with *D. oldhamii*

Table 3. Tests of monophyly of *Daphniphyllum glaucescens* Blume s.l. with Shimodaira-Hasegawa (SH) tests and Kishino-Hasegawa (KH) tests. Asterisks indicate significant differences ($P < 0.05$) between unconstrained and constraint trees.

Alignment (DNA region)	–ln *L* difference	KH test	SH test
cp (*psb*A-*trn*H spacer + *trn*L intron)			
cp tree (unconstrained)	(best)		
Constraint ML tree	52.18592	0.0085*	0.0074*
ITS (ITS1+5.8S rDNA+ITS2)			
ITS tree (unconstrained)	(best)		
Constraint ML tree	56.21965	0.0005*	0.0010*

(Hemsl.) K. Rosenthal (PP = 1.00; BS = 96), *D. glaucescens* subsp. *oldhamii* var. *lanyuense* with *D. glaucescens* subsp. *oldhamii* var. *kengii* (Hurus.) T. C. Huang (PP = 0.98; BS = 53), and *D. macropodum* and *D. macropodum* var. *humile* with *D. longeracemosum* K. Rosenthal, *D. chartaceum* K. Rosenthal, *D. himalense* (Benth.) Müll. Arg., *D. majus* Müll. Arg., and *D. paxianum* K. Rosenthal (Clade D; PP = 0.94; BS = 94). All ribotypes of *D. glaucescens* subsp. *oldhamii* var. *lanyuense* allied with the ribotypes of *D. glaucescens* subsp. *oldhamii* var. *kengii*, and all ribotypes of *D. teijsmannii* allied with *D. oldhamii* (see Fig. 2, File S4). These topological conflicts likely imply the hybridization events in the evolutionary history, while the possibilities of other mechanisms, such as lineage sorting, introgression, gene exchange, or chloroplast capture cannot be ruled out (Soltis & Kuzoff, 1995; Liu et al., 2017; Wen et al., 2020).

The three proposed sections of *Daphniphyllum* were not recovered as monophyletic. Seven clades are revealed: A, B, C, D_1, D_2, E, and F (Figs. 2, 3). Clade A includes four taxa from section *Daphniphyllum*: *D. borneense* Stapf, *D. dichotomum* (T. C. Huang) T. C. Huang, *D. glaucescens* var. *blumeanum* (Baill. ex Müll. Arg.) J. J. Sm., and *D. scortechinii* Hook. f. Clade B comprises two taxa (*D. glaucescens* subsp. *oldhamii* var. *kengii* and *D. oldhamii*) from section *Daphniphyllum* as well. *Daphniphyllum calycinum* Benth. and *D. laurinum*, the type of section *Lunata*, are grouped in clade C, whereas *D. majus* is grouped with *D. longeracemosum* from section *Staminodia* in clade D_2. Clade D_1 contains one taxon from section *Daphniphyllum* (*D. paxianum*) and four from section *Staminodia* (*D. chartaceum*, *D. himalense*, *D. macropodum*, and *D. macropodum* var. *humile*). Members of clade F in the ITS tree (*D. buchananiifolium* and *D. parvifolium* Quisumb. & Merr.) were from section *Staminodia*, but they were not grouped in one clade on the cp tree. Clade E contained only *D. angustifolium*, which was assigned to section *Staminodia* in earlier studies.

For the topological conflict analyses, we included all sampled subspecies and varieties under *Daphniphyllum glaucescens* s.l. (Huang, 1965, 1966, 1992, 1993) in our hypothesis trees (Ho)—which are *D. borneense*, *D. dichotomum*, *D. glaucescens* var. *blumeanum*, *D. oldhamii*, *D. glaucescens* subsp. *oldhamii* var. *kengii*, *D. glaucescens* subsp. *oldhamii* var. *lanyuense*, *D. paxianum*, and *D. teijsmannii* (Table 1). The Ho tree files and constraint ML tree files are available in the supplementary data (Files S5, S6). Our results suggest that all constraint ML trees are significantly different from our ML trees (Table 3). In other words, our analyses reject all Ho trees that assume the monophyly of *D. glaucescens* s.l.

Discussion

The scope of sections and evolutionary relationships among taxa

Earlier studies divided *Daphniphyllum* taxa into three sections based on morphological data (Huang, 1965, 1966; Tang et al., 2012), whereas our phylogenetic results suggest that none of the three sections are monophyletic (Figs. 2, 3). The inconsistencies between molecular and morphological data also occur in many other plant groups (Holyoak & Pedersen, 2007; Bateman et al., 2018; Schuster et al., 2018). These incongruences in *Daphniphyllum* likely infer some hybridization, lineage sorting, introgression, gene exchange, or chloroplast capture events (Soreng & Davis, 2000; Liu et al., 2020; Wen et al., 2020)

The three of four sampled species in section *Lunata* were not monophyletic. Our cp tree (Fig. 3) was not able to infer the relationships among *Daphniphyllum calycinum* and *D. laurinum* because of low resolution at the basal branches, but these two taxa are grouped in clade C in the ITS tree (Fig. 2). *Daphniphyllum majus* is clustered with members from section *Staminodia* in clade D (Fig. 2) or D_2 (Fig. 3).

The results that *Daphniphyllum majus* is closest to members in section *Staminodia* are not too surprising to us because of the habitats and leaf morphology of this species. While all of *D. majus*, *D. calycinum*, and

D. laurinum have lunate (new-moon-shaped) stamens in the staminode flowers and persistent calyx in fruit (Huang, 1965, 1966; Tang et al., 2012), *D. majus* grows in habitats remarkably different from *D. calycinum* and *D. laurinum*. *Daphniphyllum majus* appears in inland, high-altitude forests (500–1500 m), while both *D. calycinum* and *D. laurinum* occur in coastal, low-altitude forests (60–750 m and 0–1500 m, respectively) (Baillon, 1858; Ming & Kubitzki, 2008). The leaf morphology and geographic distribution of *D. majus* are more similar to *D. chartaceum*, a member of section *Staminodia* (Zhang & Lu, 1989; Tang et al., 2009), which is also placed in clade D (Fig. 2, Supplementary Fig. S8). Moreover, while stigma form is an important trait to characterize sections in *Daphniphyllum* (Huang, 1965, 1966; Tang et al., 2012), the stigma form of *D. majus* remains unclear (Table 1). Above all, here, we suggest removing *D. majus* from section *Lunata* based on the evolutionary position, leaf morphology, and habitats of *D. majus*. However, the sectional assignment for *D. majus* is unclear due to the non-monophyly of sections in *Daphniphyllum* (see the following discussions).

Section *Lunata* would be a monophyletic section only if *Daphniphyllum majus* were removed from this section and *D. peltatum* Yan Liu & T. Meng (not sampled in this study) were grouped with *D. calycinum* and *D. laurinum*.

Moreover, the nine sampled taxa in section *Daphniphyllum* (Table 1) are grouped into three different clades: clades A, B, and D (Figs. 2, 3). Except for two putative hybrid taxa—*D. glaucescens* subsp. *oldhamii* var. *lanyuense* and *D. teijsmannii* (discussed in the following subsections)—and one taxon in clade D, all other taxa are in clades A and B. Thus, section *Daphniphyllum* was not monophyletic, although only 40.9% (= 9/22) of taxa in this section were sampled (Table 1).

Interestingly, our analyses infer not only morphological similarity but also geographical patterns in section *Daphniphyllum*. East Asian *Daphniphyllum* are clustered in clade B as expected based on their morphology and geographical distributions, and Southeast Asian species are grouped in clade A (Fig. 2) (Hooker, 1890; Hayata, 1904; Rosenthal, 1919; Hurusawa, 1942a, 1942b; Huang, 1965, 1966, 1993; Tang et al., 2012).

Species of section *Staminodia* are divided into three main clades, namely D, E, and F (Figs. 2, 3). Clades E and F contain only members from section *Staminodia* (Fig. 2). Other sampled taxa are placed in clade D (or D_1 and D_2) with taxa from sections *Daphniphyllum* and *Lunata*. Considering sampled members in section *Staminodia* only, our results are consistent with earlier systematic studies. *Daphniphyllum angustifolium* and *D. parvifolium* (clade E), *D. buchananiifolium* (clade F), and *D. macropodum* (clade D) have three distinct pistil morphologies (Table 1) (Tang et al., 2012) and leaf anatomies (Tang et al., 2009). *Daphniphyllum macropodum* var. *humile*, *D. himalense*, and *D. longeracemosum* are morphologically similar with *D. macropodum* in their pistillate flower, fruit, and leaf anatomies (Rosenthal, 1919; Huang, 1966; Ming & Kubitzki, 2008; Tang et al., 2009, 2012) (see Table 1). Our results infer that current section *Staminodia* likely contains three (clades D, E, and F) or more sections after considering *D. majus* and *D. paxianum*.

In summary, our molecular data do not support current infrageneric classification schemes of *Daphniphyllum* that are based on morphology. Thorough species sampling and additional molecular data will be required to explicate the evolutionary mechanisms of molecular-morphological inconsistencies and for further taxonomic revisions.

TEST THE CLASSIFICATION HYPOTHESIS OF *DAPHNIPHYLLUM GLAUCESCENS* S.L.

The taxonomic histories of the subspecies and varieties under *Daphniphyllum glaucescens* are extremely complex. For example, Müller Argoviensis (1869) recognized *D. glaucescens* and *D. teijsmannii*. Rosenthal (1919) elevated *D. glaucescens* var. *oldhamii* as *D. oldhamii* and treated *D. roxburghii* Baill. including *D. teijsmannii*. Later, Hurusawa (1942b) regarded *D. teijsmannii* as containing *D. oldhamii*. Then, Huang (1965, 1966) treated *D. oldhamii* and *D. teijsmannii* as the subspecies of *D. glaucescens*: *D. glaucescens* subsp. *oldhamii* and *D. glaucescens* subsp. *teijsmannii* (Zoll. ex Teijsm. & Binn.) T. C. Huang. The former subspecies was then treated as *D. oldhamii* by Ming and Kubitzki (2008) and later studies, and the latter subspecies was then accepted as *D. teijsmannii* by Noshiro (1999) and following articles.

Here, we provide the first analyses to test the classification hypothesis of *Daphniphyllum glaucescens* s.l. embraced by Huang's earlier studies (Huang, 1965, 1966, 1992, 1993). Our phylogenetic results show that synonyms of, or taxa included in, *D. glaucescens* s.l.—including *D. borneense*, *D. dichotomum*, *D. glaucescens* var. *blumeanum*, *D. oldhamii*, *D. glaucescens* subsp. *oldhamii* var. *kengii*, *D. glaucescens* subsp. *oldhamii* var. *lanyuense*, *D. paxianum*, and *D. teijsmannii*—are distributed over several clades rather than in a clade comprised of *D. glaucescens* (Figs. 2, 3). Moreover, our topological conflict analyses reject the broad classification of *D. glaucescens* s.l. (Table 3). Our results strongly suggest that *D. glaucescens* s.l. is not an evolutionary clade. The classifications in later studies (Ming, 1980; Huang, 1996, 1997; Ming & Kubitzki, 2008; Tang et al., 2009, 2012) better represent the diversity of *D. glaucescens* and its allies.

Table 4. Comparisons among *Daphniphyllum* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang, *D. teijsmannii* Zoll. ex Teijsm. & Binn., and their putative parental species. Morphological and distributional information is obtained from earlier studies (Huang, 1992, 1993; Noshiro, 1999; Ming & Kubitzki, 2008; Tang et al., 2009, 2012).

Species	*D. oldhamii*	*D. teijsmannii*	*D. macropodum*	*D.* ×*lanyuense*	*D. glaucescens* subsp. *oldhamii* var. *kengii*
Evolutionary relationships	putative paternal taxon of *D. teijsmannii*	natural hybrid	putative maternal taxon of *D.* ×*lanyuense* and *D. teijsmannii*	natural hybrid	putative paternal taxon of *D.* ×*lanyuense*
Epidermal cell on abaxial leaf surface	papillae	epapillae	epapillae	papillae	epapillae
Leaf apex	acute to shortly acuminate	acute to shortly acuminate	acute	round or cuspidate	shortly acuminate
Stigma	oblong	oblong	reniform	reniform	oblong
Calyx	parted	parted	asepalous or free	parted	parted
Surface of endocarp	slightly tuberculate	slightly tuberculate	slightly tuberculate	tuberculate	smooth or slightly tuberculate
Drupe size	6.5–14 mm long, 4.5–7 mm diam.	9–11 mm long, 5–6.5 mm diam.	9–13 mm long, 6–7 mm diam.	8–13 mm long, 9.5–10 mm diam.	7–11 mm long, 4.5–7 mm diam.
Geographical distribution	Southeast China; Taiwan	Japan, Korea	China, Japan, Korea, Taiwan	Lanyu Island	Taiwan
Elevation	30–1300 m	5–650 m	60–2100 m	20–500 m	100–2400 m

PUTATIVE HYBRIDS AND THEIR PUTATIVE PARENTAL TAXA

The topological conflicts between maternal (chloroplast) and biparental (nuclear tree) inherited genomes likely infer hybridization events in the studied plant groups (e.g., Palmer et al., 1988; Chiang et al., 2013; Wen et al., 2020) while other mechanisms might also contribute to the conflicts. In our study, the incongruent signals between our cp (Fig. 3) and ITS (Fig. 2) trees suggest that *Daphniphyllum glaucescens* subsp. *oldhamii* var. *lanyuense* and *D. teijsmannii* probably have hybrid origins. The former putative natural hybrid likely arose from *D. macropodum* (maternal parent) and *D. glaucescens* subsp. *oldhamii* var. *kengii* (paternal parent). The latter putative natural hybrid (*D. teijsmannii*) probably evolved from *D. macropodum* (maternal parent) and *D. oldhamii* (paternal parent). As with many natural hybrids in other plant groups (Schwarzbach et al., 2001; Peng et al., 2010; Liu et al., 2019), both putative natural hybrids show a mix of morphological traits of their parental taxa (Table 4). Both putative natural hybrids have limited distributions while their parental taxa are widespread (Table 4). The distribution patterns might be due to the shorter evolutionary histories and/or the lower fitness of the putative natural hybrids compared to their parental species (Song et al., 2004; Rieseberg & Willis, 2007; Arnold & Martin, 2010).

Topological conflicts between chloroplast and nuclear trees could also be caused by hybridization, lineage sorting, introgression, gene exchange, and chloroplast capture (Soltis & Kuzoff, 1995; Liu et al., 2017; Wen et al., 2020). Population-level data or additional nuclear regions will be required to provide better insight into the origins of *Daphniphyllum glaucescens* subsp. *oldhamii* var. *lanyuense* and *D. teijsmannii* (e.g., Suarez-Gonzalez et al., 2018; Kleinkopf et al., 2019; Nobis et al., 2019).

Daphniphyllum glaucescens subsp. *oldhamii* var. *lanyuense* has a unique evolutionary history (putative natural hybrid, inferred in the present study), distinguishable morphology (Huang, 1993; Tang et al., 2012), and a restricted geographical range (Huang, 1993) (Table 4). Here, we propose to raise *D. glaucescens* subsp. *oldhamii* var. *lanyuense* as a nothospecies, *D.* ×*lanyuense* (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang. The taxonomic treatment is provided below.

Taxonomic Treatment

Daphniphyllum ×lanyuense (T. C. Huang) M. S. Tang, S. H. Liu & Yuen P. Yang, stat. nov. Basionym: *Daphniphyllum glaucescens* Blume subsp. *oldhamii* (Hemsl.) T. C. Huang var. *lanyuense* T. C.

Huang, Taiwania 37(2): 134. 1992. TYPE: Taiwan. Taitung: Lanyu Island, 27 Aug. 1969, *T. C. Huang 5132* (holotype, TAI!; isotype, TAI!).

Shrubs or small trees, 1.5–8 m tall. Branchlets stout, black or dark brown, almost rugose; lenticels elliptic. Leaf scars acutely triangular or circular. Leaves elliptic, obovate, or oblanceolate, 6–12.5 × 2.5–5.5 cm; petioles stout, 2–4.5 cm; blade symmetrical, sometimes dentate near the apex; shiny, olive green or brown adaxially; papillose, glaucous or brown abaxially; base acute to cuneate; margins slightly revolute; apex mostly obtuse, convex, round, or cuspidate; lateral veins 6 to 10 pairs. Male inflorescences erect, 2–4 cm. Male flowers: pedicels 2.5–4 mm; calyx 4- to 6-parted, shorter than the filaments; stamens 6 to 10, anthers oblong, 0.8–2.0 mm, connective broadly acute at apex, cordate at base; filaments equal to or slightly shorter than anthers. Female inflorescences ascent, 1.4–3.5 cm. Female flowers: pedicels 2.5–6 mm; calyx 4- to 6-parted, caducous, ca. 0.6 mm; staminodes absent; ovary ovoid or globose, ca. 1.6 mm; stigmas reniform. Infructescences with axes to 0.8–4.0 cm. Fruit stalks 5–15 mm, slightly thickened close to the base of fruits. Drupes ellipsoid, 8–13.5 × 6–9.5 mm, noticeably tuberculate.

Distribution and habitat. *Daphniphyllum* ×*lanyuense* is endemic to Lanyu Island (Orchid Island), Taitung County, Taiwan, and occurs on open hilltops, roadsides, or forest edges.

Note. On the basis of the comparative morphology of pistillate flowers (Tang et al., 2012) and phylogenetic analyses inferred in this study, we conclude that *Daphniphyllum* ×*lanyuense* is a putative natural hybrid between *D. macropodum* (maternal parent) (Fig. 3) and *D. glaucescens* subsp. *oldhamii* var. *kengii* (paternal parent) (Fig. 2) and should be recognized as a species.

Paratypes. TAIWAN. **Taitung:** Lanyu, 16 Feb. 1970, *C. E. Chang 6143* (TAI); 14 Aug. 1977, *Y. C. Jeng 1639* (TAI); 26 June 1985, *Y. L. Jong 941* (TAI).

Specimens examined. TAIWAN. **Taitung:** Lanyu, *C. C. Liao 1205*, *1215* (HAST); *C. C. Wang et al. s.n.* (TNM); *C. E. Chang 3074* (PPI), *6143* (TAI), *14953* (HAST), *16822* (HAST, PPI), *19668* (PPI); *C. H. Tsou 509* (HAST); *C. H. Ou et al. s.n.* (TNM); *C.-I Peng 10704*, *12698* (HAST); *C. K. Yang 295* (TNM); *C. M. Wang 3562* (TNM); *C. S. Tung s.n.* (TNM); *C. Y. Kuo 437* (TAIF); *G. S. Wang & C. N. Koh 169*, *267* (TNM); *J. C. Wang 5579* (HAST); *L. C. Shih s.n.* (SYSU); *M. C. Ho & H. C. Huang 114*, *147* (NTUF); *M. F. Lao 215* (TAIF); *Mamoru 9910* (TAI); *P. F. Lu 1063*, *6269*, *8459* (HAST, TAIF); *M. S. Tang T800*, *T801*, *T803*, *T805*, *T806*, *T810* (SYSU); *Q. L. Ye 5784* (PPI); *S. F. Huang 2670* (TAI); *S. H. Liu et al. 901* (TAIF); *S. T. Chiu & H. F. Yen s.n.* (TNM); *S. T. Chiu & J. N. Chen 4158* (HAST, TAIF, TNM); *S. W. Chung 6545*, *6591* (HAST, TAIF); *S. Y. Lu 17604* (TAIF), *18499* (TNM), *s.n.* (TAIF, TNM); *T. C. Huang 5132*, *s.n.* (TAI); *T. Y. Yang & C. H. Chu 8004* (TNM); *T. Y. Yang et al. 3876*, *6630*, *6631* (TNM), *T. Y. A. Yang 1501* (HAST, TNM); *T. Y. A. Yang & C. N. Wang 10117* (HAST); *T. Y. A. Yang & C. C. Yen 10632* (HAST); *T. Y. A. Yang & C. H. Chuang 10310* (HAST); *T. Y. A. Yang & C. Y. Liu 14651* (HAST); *T. Y. A. Yang et al. 6630*, *6631* (HAST), *8247*, *8248* (TNM), *8482*, *8483* (HAST, TAIF, TNM), *8601*, *9891*, *9892*, *9921*, *10022*, *11861*, *12123*, *13037*, *13356*, *13716*, *13757*, *15166* (TNM); *W. L. Chiou 10119* (HAST, TAIF); *W. L. Wagner 6709* (HAST); *W. P. Leu 1395* (HAST); *Y. C. Lu 1596* (HAST, TAIF, TNM); *Y. C. Lu & C. C. Liao 1596* (TAIF, TNM); *Y. C. Lu et al. 920* (TAIF); *Y. P. Yang s.n.* (HAST, TAIF).

Conclusions

Our study provides new insight into *Daphniphyllum* sections and species-level systematics based on both nuclear and chloroplast regions by sampling half (55.6%) of the taxa in the genus. Our results did not support the monophyly of any of the three proposed sections that have previously been based on morphological classification schemes in *Daphniphyllum*. Moreover, our analyses reveal the evolutionary relationships among *Daphniphyllum*, support the classification hypotheses of *D. glaucescens* s. str. that its varieties and some synonymous taxa should be treated separately, and recognize two, or more, putative natural hybrids. In addition, we elevated *D.* ×*lanyuense* to nothospecies status. Phylogenies with inclusive sampling and higher resolutions will be required for further revision of the sectional and species delimitation.

Supplementary Data

Supplementary data are available online (<https://annals.mobot.org/>) and consist of the following. Supplementary Table S1 presents sampling information and GenBank accession numbers of the studied *Daphniphyllum* plants and outgroups. Supplementary Table S2 includes the primers and amplification protocols applied in this study. File S3 contains the cp (*psb*A-*trn*H spacer + *trn*L intron) alignments and trees; File S4, the ITS alignments and trees; File S5, the Ho tree and constraint ML tree for the topological conflict analyses using cp alignment; and File S6, the Ho tree and constraint ML tree for the topological conflict analyses using ITS alignment. Supplementary Table S7 is a list of the *Daphniphyllum* herbarium samples. Finally, Supplementary Figure S8 presents Bayesian inference (BI) of *Daphniphyllum* based on the ITS+cp combined alignment with a length of 1602 bp and 127 indels. Note that putative natural hybrid taxa are removed. Numbers after taxa refer to Supplementary Table S1. Numbers at nodes show Bayesian posterior probabilities/ML bootstrap values only when posterior probability is > 0.70 or bootstrap value is > 50. The arrow shows the

crown node of *Daphniphyllum*. Clades are indicated as Figure 2.

Authors' Contributions

Y. P. Y. and M. S. T. initiated the study; M. S. T. collected plant samples from China, Japan, Malaysia, Philippines, Singapore, Taiwan, and West Java; M. S. T. took color photographs of studied samples from the wild; M. S. T. and C. C. T. extracted DNA and conducted the molecular wet lab work; C. C. T., Y. P. Y., and S. H. L. contributed analysis tools; S. H. L. and M. S. T. performed phylogenetic analyses and prepared figures and tables; S. H. L. prepared and archived the sequence data to NCBI; S. H. L., M. S. T., and C. R. S. wrote and revised the manuscript; all authors read and approved the final manuscript.

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