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A second species of *Pseuduvaria* in China: the identity of the enigmatic species *Meiogyne kwangtungensis*

Qing-Long Wang[1], Hui Zhang[2], Yun-Yun Shao[3], Zhu-Nian Wang[1], Bine Xue[2]

1 *Tropical Crops Genetic Resources Institute, CATAS, Haikou 571101, Hainan, China* **2** *College of Horticulture and Landscape Architecture, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, Guangdong, China* **3** *Guangdong Provincial Key Laboratory of Digital Botanical Garden, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, Guangdong, China*

Corresponding author: Bine Xue (xuebine@zhku.edu.cn)

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Abstract

Meiogyne kwangtungensis is a rare species endemic to Hainan, China, known just from two fruiting collections made in the 1930s. Although it was published under the name *Meiogyne* in 1976, it was suggested that it might be better placed within *Pseuduvaria* or *Mitrephora*. For decades, this species was never collected again, thus its true generic affinity remained unresolved due to the lack of flowers. During a field exploration in Hainan, we re-discovered this species and collected a flowering specimen for the first time. The flower immediately confirmed its affinity with *Pseuduvaria*. Phylogenetic analyses of five chloroplast regions (*psbA-trnH*, *trnL-F*, *matK*, *rbcL*, and *atpB-rbcL*; ca. 4.2 kb, 70 accessions) also unambiguously placed *Meiogyne kwangtungensis* in the *Pseuduvaria* clade (PP = 1.00, ML BS = 99%). Morphologically, it is most similar to *P. multiovulata* which is endemic to Myanmar and Thailand, both with often-paired flowers, long pedicels and short peduncles, and often 1–2 monocarps. However, it differs in having smaller flowers with kidney-shaped glands on the inner petals, fewer stamens and carpels, smaller ovoid monocarps with an apicule and fewer seeds. On the basis of the combined molecular phylogenetic and morphological data, we propose a new combination, *Pseuduvaria kwangtungensis* (P.T.Li) Qing L.Wang & B.Xue. A full description including floral characters and a color plate are provided here for this species. A key to species in the genus *Pseuduvaria* in China is also provided.

Keywords

Annonaceae, *Meiogyne*, molecular phylogeny, morphology, *Pseuduvaria*

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Introduction

The genus *Meiogyne* Miq. is a medium-sized genus in tribe Miliuseae Hook.f. & Thomson of Annonaceae (Chatrou et al. 2012; Thomas et al. 2012; Xue et al. 2014; Guo et al. 2017). It consists of ca. 30 species of trees or shrubs, distributed in wet tropical lowland and lower montane rainforests across South-east Asia and the western Pacific (Thomas et al. 2012; Tan et al. 2014; Xue et al. 2014, 2017; Turner and Utteridge 2015; Johnson et al. 2019). Species in *Meiogyne* are characterized by sub-equal petals, inner petals with corrugated or verrucose base of the adaxial surface and innermost stamens with tongue-shaped apical prolongations (van Heusden 1994; Thomas et al. 2012; Xue et al. 2014).

Meiogyne kwangtungensis Li was published in 1976, based on two fruiting collections (*F. C. How 73305*, IBSC, A, IBK and *Z. Huang 33693*, IBSC) from Hainan, China, in 1935 and 1933 respectively (Li 1976; Tsiang and Li 1979). After that, it was not collected again. The long fruiting pedicle of this species (up to 50 mm in length) is unusual in *Meiogyne*, as most *Meiogyne* species have short flowering and fruiting pedicels (usually less than 30 mm in length, except *Meiogyne chiangraiensis* Chalermglin & M.F.Liu; Johnson et al. 2019). Rainer and Chatrou (2006) indicate that this species can also belong to *Pseuduvaria* or *Mitrephora*. Flower characters are essential for generic delimitation in Annonaceae, and the three genera, *Meiogyne*, *Pseuduvaria* and *Mitrephora* can be easily distinguished on that basis (van Hesuden 1992; Su and Saunders 2006). Therefore, flowers are required to confirm the correct generic placement of this species (Li and Gilbert 2011).

For the past few years, we have carried out several field explorations in Hainan to search for this species. The explorations finally resulted in new collections of *Meiogyne kwangtungensis*, including flowers and fruits. Based on the mature flowers, we are able to confirm that *Meiogyne kwangtungensis* should be placed in *Pseuduvaria*.

Pseuduvaria is a genus widely distributed in continental SE Asia and Malesia, extending from Indochina and the Philippines to New Guinea and NE Australia (Su and Saunders 2006). The only comprehensive taxonomic monograph recognizes 52 species in the genus (Su and Saunders 2006). Three new species and one new combination in Su et al. (2010) and one new species in Turner (2010) bring the total species recognized in *Pseuduvaria* to 57. The flowers of *Pseuduvaria* are often unisexual and it's unique in having inner petals apically connivent over the reproductive organs, forming a mitriform dome (Su and Saunders 2006; Su et al. 2008). Each inner petal is differentiated into a distinct blade and basal claw, which results in three lateral apertures between the petal claws, enabling access by floral visitors (Su and Saunders 2006). Moreover, the adaxial surface of the inner petals often bears one or two protruding glands (Su and Saunders 2006). In contrast, the flowers of *Meiogyne* are bisexual and the inner petals are spreading and corrugated at the base of the adaxial surface.

With the available flowering materials and silica-gel samples for DNA extraction, we clarify the generic placement of *Meiogyne kwangtungensis* based on morphological data and phylogenetic analysis in this study.

Materials and methods

Morphological studies

The morphological characters were examined based on the living plants and specimens kept in the HITBC, IBSC, IBK, and KUN herbaria. Comparisons were also made against published *Pseuduvaria* species in the monograph and recent papers (Su and Saunders 2006; Su et al. 2010; Turner 2010; Li and Gilbert 2011).

Phylogenetic studies

Total DNA of the silica-gel dried material of *Meiogyne kwangtungensis* (*Q. L. Wang 20200528002*, IBSC) was extracted using a modified CTAB method (Doyle and Doyle 1987). Five chloroplast regions (*psbA-trnH*, *trnL-F*, *matK*, *rbcL*, and *atpB-rbcL*) were newly generated. For detailed information on PCR amplification and primer sequences we refer to Su et al. (2008). 54 *Pseuduvaria* species from Su et al. (2010) were included in this study. *Monocarpia euneura* Miq. and 14 species in the tribe Miliuseae were selected as outgroups based on the phylogenetic framework reported in previous studies (Chatrou et al. 2012; Chaowasku et al. 2014; Guo et al. 2017; Xue et al. 2018, 2020a). Sequences were downloaded from the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The final data matrix comprised a total of 70 species of Annonaceae. The information on sequence alignment can be found in Xue et al. (2018).

Detailed information about the samples, localities and GenBank accession numbers are all listed in the Appendix 1.

Phylogenetic analyses were done using Bayesian Inference (BI) and maximum likelihood (ML) methods. The information on model selection of the sequence matrix constructed could refer to Xue et al. (2018). The best partition scheme suggested five partitions based on DNA region identity with GTR + I + Γ chosen for *matK* and *rbcL*; and GTR + Γ selected for *atpB-rbcL*, *psbA-trnH* and *trnL-F* regions. Detailed methods for tree reconstruction could refer to Xue et al. (2018) and Xue et al. (2020b).

Results

The morphological observation is illustrated in Figs 1, 2, and discussed in detail below.

The concatenated alignment of the 70-taxon dataset consisted of 4,261 aligned positions (*psbA-trnH*: 430 bp, *trnL-F*: 891 bp, *matK*: 810 bp, *rbcL*: 1,343 bp, and *atpB-rbcL*: 787 bp). The Bayesian analyses and ML resulted in similar topologies. The 50% majority-rule consensus tree resulting from the Bayesian analyses under five-partitioned model is shown as Fig. 3.

The backbone of the tribe Miliuseae is not well resolved as in previous studies. The sampled *Pseuduvaria* species form a well-supported clade (PP = 1; ML BS =

99%; Fig. 3). The three *Meiogyne* species, viz. *Meiogyne mindorensis* (Merr.) Heusden, *M. pannosa* (Dalzell) J. Sinclair, and *M. virgata* (Blume) Miq. form a well-supported clade (PP = 1; ML BS = 96%; Fig. 3). *Meiogyne kwangtungensis*, however, is not retrieved in the same clade as the three *Meiogyne* species sampled, but nested within *Pseuduvaria* clade, and closely related to *Pseuduvaria gardneri* Y. C. F. Su, Chaowasku & R. M. K. Saunders, *P. fragrans* Y. C. F. Su, Chaowasku & R. M. K. Saunders and *P. multiovulata* (C. E. C. Fischer) J. Sinclair (PP =1; ML BS = 91%; Fig. 3).

Discussion

With the new collections of the flowering specimens of *Meiogyne kwangtungensis*, the enigmatic identity of this species is resolved. The flowers of *Meiogyne kwangtungensis* are unisexual (Fig. 1D–G). Both female and male flowers are characterized by having longer inner petals that are apically connivent over the reproductive parts to form a mitriform dome (Fig. 1B, D, F). The inner petals are differentiated into distinct blades and claws, with the adaxial surface of the claw of the inner petal bearing two protruding glands (Fig. 1E). The stamen has a flat-topped connective extending over the thecae ('uvarioid' sesu Prantl 1891; Mols et al. 2004) (Fig. 1E, 2D). These characters are completely different from that of the *Meiogyne* species. In contrast, flowers of *Meiogyne* are bisexual; both whorls of petals are sub-equal and similar in shape; the inner petals are not connivent either. Therefore, the flower morphology of *Meiogyne kwangtungensis* is consistent with that of *Pseuduvaria*, which immediately confirmed its affinity with *Pseuduvaria*.

The molecular phylogeny further supported the placement of *Meiogyne kwangtungensis* in the genus *Pseuduvaria*. It falls into the same clade with *Pseuduvaria gardneri*, *P. fragrans* and *P. multiovulata* (PP =1.00; ML BS = 91%) (Fig. 3). Morphologically, *Meiogyne kwangtungensis* is most similar to *Pseuduvaria multiovulata* (C.E.C.Fischer) J.Sinclair in Thailand, both with 1–2 flowers per inflorescence, with long pedicels and short peduncles, and often with 1–2 monocarps (Su and Saunders 2006; Gardner et al. 2015). However, the two species differ in the size of the flowers, the shape of the inner petal glands, the number of stamens and carpels, the shape of the apex of the monocarps and the number of seeds per monocarp (Table 1). *Meiogyne kwangtungensis* has small flowers (outer petal ca. 7 mm long, inner petal ca. 8mm long) whereas *Pseuduvaria multiovulata* has larger flowers (outer petal 7.5–11 mm long, inner petal 9–18.5 mm long; Su and Saunders 2006). *Meiogyne kwangtungensis* has two kidney-shaped to ellipsoid glands on adaxial surface of the inner petals (Fig. 1E), whereas the inner petals glands of *Pseuduvaria multiovulata* are square (Su and Saunders 2006). *Meiogyne kwangtungensis* has 20–30 stamens in male flower and 3 carpels in female flower (Fig. 1E, G), whereas *Pseuduvaria multiovulata* has 110–115 stamens in male flower and ca. 11 carpels in female flower (Su and Saunders 2006). The monocarps of *Meiogyne kwangtungensis* have an apiculate apex, with 5–10 seeds per monocarp, whereas the monocarps of *Pseuduvaria multiovulata* do not have apicule, with ca. 17 seeds per monocarp (Su and Saunders 2006).

Table 1. Morphological comparison between *Pseuduvaria kwangtungensis* and *P. multiovulata*.

Characters	*P. kwangtungensis*	*P. multiovulata*
Length of the outer petals	ca. 7 mm long	7.5–11 mm long
Length of the inner petals	ca. 8 mm long	9–18.5 mm long
Shape of inner petal glands	kidney-shaped to ellipsoid	square
Number of stamens	20–30	110–115
Number of carpels	3	ca. 11
Shape of the apex of the monocarps	apiculate	do not have apicule
Number of seeds per monocarp	5–10	ca. 17

In China, only one *Pseuduvaria* species is recorded in Yunnan Province, i.e. *Pseuduvaria trimera* (Craib) Y.C.F.Su & R.M.K.Saunders (Li and Gilbert 2011) (Fig. 4). This species is relatively widely distributed, also occurring in Myanmar, Thailand and Vietnam. *Meiogyne kwangtungensis* and *Pseuduvaria trimera* could be easily differentiated from each other by the growth habit, the morphology of leaf, inflorescence, flower and fruit. *Pseuduvaria trimera* is a tree up to 20 m tall (Su and Saunders 2006; Li and Gilbert 2011), whereas *Meiogyne kwangtungensis* is a shrub to 4 m tall. The leaf laminas of *Pseuduvaria trimera* are subcoiaceous with 14–18 pairs of secondary veins (Fig. 4A), whereas leaf laminas of *Meiogyne kwangtungensis* are papery with ca. 10 pairs of secondary veins (Fig. 1A). The inflorescences of *Pseuduvaria trimera* are clustered on young branches with yellow flowers (Fig. 4B–G), whereas those of *Meiogyne kwangtungensis* are axillary with cream-colored or purple flowers (Fig. 1B–F). *Pseuduvaria trimera* is distinct in lacking any glands on the clawed inner petals (Fig. 4F), whereas *Meiogyne kwangtungensis* has a pair of glands on the adaxial surface of the inner petals (Fig. 1E). *Pseuduvaria trimera* has globose, stipitate monocarps with rugulose pericarps (Fig. 4H, I; Su and Saunders 2006; Li and Gilbert 2011), whereas *Meiogyne kwangtungensis* has ovoid, sessile monocarps with smooth pericarp (Fig. 1H).

Meiogyne kwangtungensis is different from all species in *Pseuduvaria*. Both the morphological and molecular phylogenetic data support the transfer of *Meiogyne kwangtungensis* to *Pseuduvaria*, thus a new nomenclatural combination is proposed here. Additionally, as the name *Meiogyne kwangtungensis* was published based on only two fruiting collections lacking flowers, we provide a detailed description of the flower morphology and an updated description for the fruit morphology. A key to the two *Pseuduvaria* species in China is also provided.

Taxonomic treatment

***Pseuduvaria kwangtungensis* (P.T.Li) Qing L.Wang & B.Xue, comb. nov.**
urn:lsid:ipni.org:names:77214765-1
Figs 1, 2

Chinese name. hai nan jin gou hua (海南金钩花)

Basionym. *Meiogyne kwangtungensis* P.T.Li, Acta Phytotax. Sin. 14(1): 104. 1976.

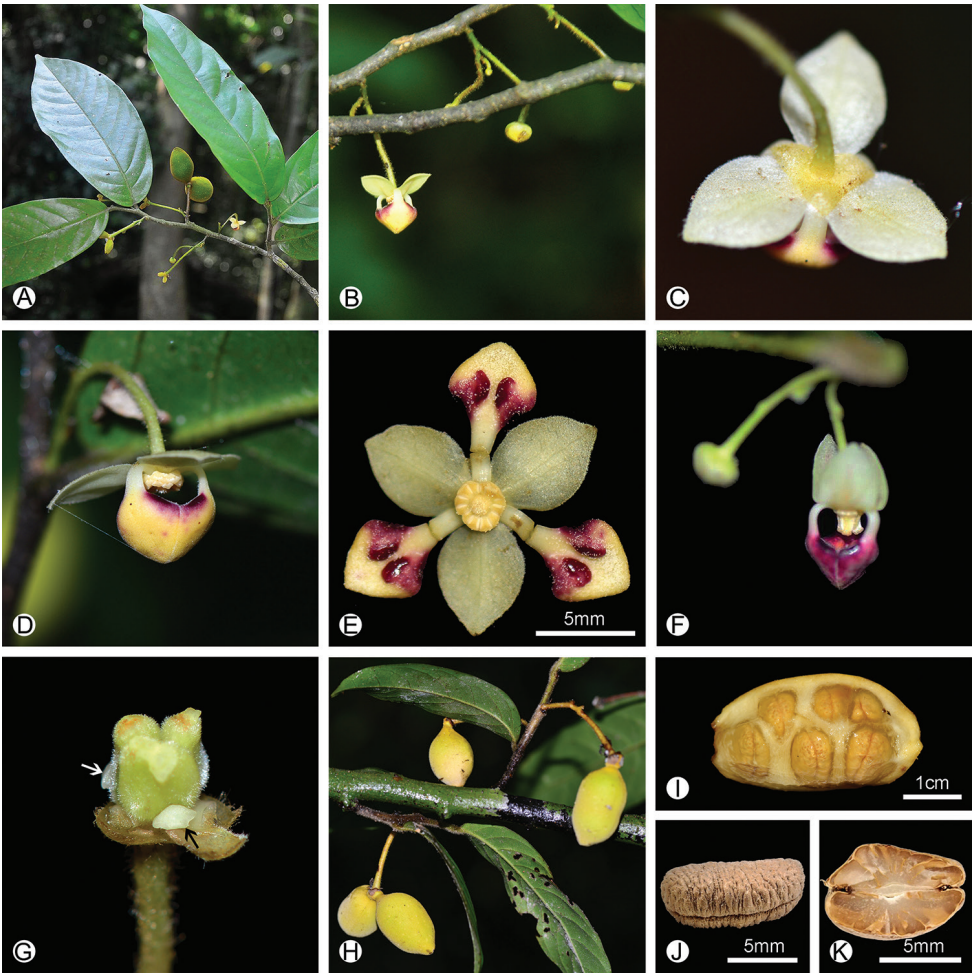

Figure 1. Morphology of *Pseuduvaria kwangtungensis*, comb. nov. **A** flowering branch **B** inflorescence **C** bottom view of a male flower **D** side view of a male flower **E** male flower, top view, inner petals manually separated to show adaxial inner petal surface with paired glands **F** a female flower **G** gynoecium of the female flower, showing three carpels and two staminodes (with black and white arrows) **H** fruits **I** inside of a monocarp, showing seeds in two series **J** Single dried seed, showing the grooved raphe **K** section of the seed, showing the spiniform endosperm rumination. Photos: Q. L. Wang (**A–I**); B. Xue (**J, K**).

Type. China. Hainan: Bao-ting Hisen, Xing-long, 25 Jul. 1935, *F. C. How 73305* (holotype: IBSC! [IBSC0003357]; isotypes, A [A00066602, photo!], IBK![IBK00190122], SN!).

Description. ***Shrubs*** to 3–4 m tall, d.b.h. ca. 5 cm. Monoecious. ***Branches*** black, densely villous when young, glabrescent (Fig. 1A). Petiole 2–3 mm, villous (Figs 1A, 2A); ***leaf blade*** oblong to elliptic, 6–18 × 2.5–5.5 cm, papery, adaxially glossy and glabrous except for pubescent midrib (Figs 1A, 2A), abaxially glaucous and villous (Figs 1A, 2B), midvein adaxially impressed, secondary veins ca. 10 on each side of midvein and

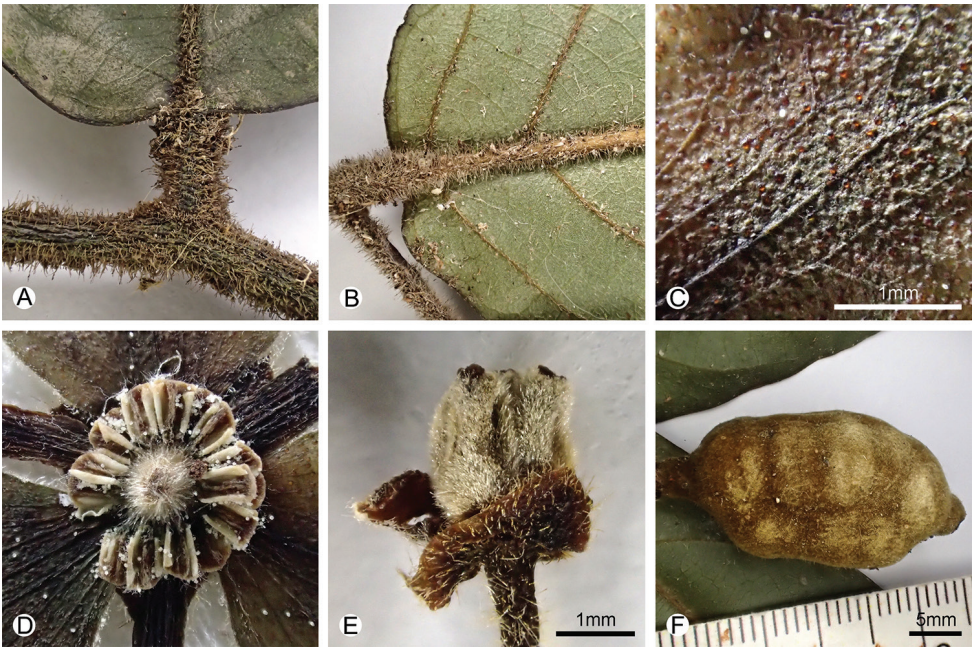

Figure 2. Specimen morphology of *Pseuduvaria kwangtungensis*, comb. nov. **A** adaxial view of the leaf base and the petiole **B** abaxial view of the leaf base and the petiole **C** close-up of the adaxial surface of the outer petal, showing the dense tiny golden glands **D** dried androecium, showing the morphology of the stamens **E** dried gynoecium, showing the hairy carpels **F** dried monocarp, showing the pubescent indumentum and the shallowly transversely constriction between seed. Photos: B. Xue.

prominent on both surfaces, base rounded to sometimes shallowly cordate, apex acuminate (Fig. 1A). ***Inflorescences*** axillary, with up to 2–3 flowers, only one flower at anthesis per inflorescence (Fig. 1A, B). Flowering peduncles 3–10 mm long, ca. 1 mm in diameter, villous (Fig. 1A, B). Sympodial rachides inconspicuous (often less than 5 mm), internodes poorly developed with several bracts. Flowering pedicels 15–30 mm long, ca. 1 mm in diameter, densely villous with erect hairs (Fig. 1B, C). ***Sepals*** partially connate, triangular to ovate, ca. 2 mm long, ca. 2 mm wide, glabrous adaxially, densely puberulous with appressed hairs abaxially (Fig. 1C). ***Outer petals*** ca. 7 mm long, ca. 5 mm wide, thin, broadly circular, glabrous adaxially, puberulous with appressed hairs abaxially and on the edge, cream-colored, without claws, dried with dense tiny golden glands adaxially (Figs 1C–E, 2C). ***Inner petals*** ca. 8 mm long, 4 mm wide, rhombic, apex acute, base acute, ca. 1 mm thick, very densely puberulous with appressed hairs adaxially, densely puberulous with appressed hairs abaxially, cream-colored with purple tinge on adaxial surface of the blade in staminate flower (Fig. 1D, E), and purple in pistillate flower (Fig. 1F); basal claw ca. 3–4 mm long; glands paired on adaxial surface of inner petal, kidney-shaped to ellipsoid, surface smooth, raised (Fig. 1E); apical aperture absent. Flowers unisexual. ***Staminate flowers*** with androecium ca. 1 mm long, ca. 2 mm wide; stamens ca. 20–30 per flower, ca. 0.9 mm long, ca. 0.7 mm wide

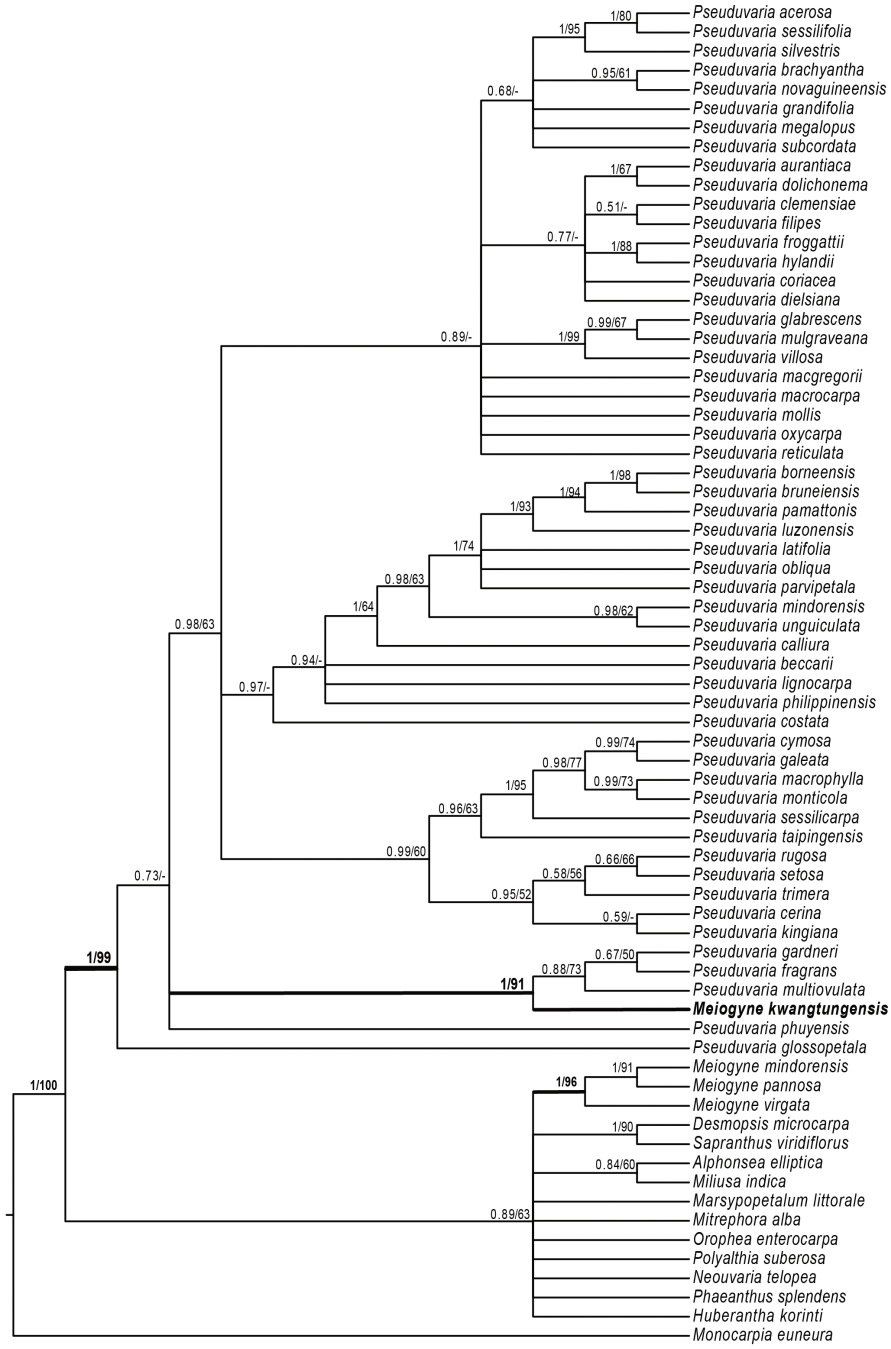

Figure 3. Bayesian 50% majority-rule consensus tree under partitioned models (cpDNA data: *atpB-rbcL*, *matK*, *psbA-trnH*, *rbcL*, and *trnL-F*; 70 taxa). Numbers at the nodes indicate Bayesian posterior probabilities and maximum likelihood bootstrap values (> 50%) in that order.

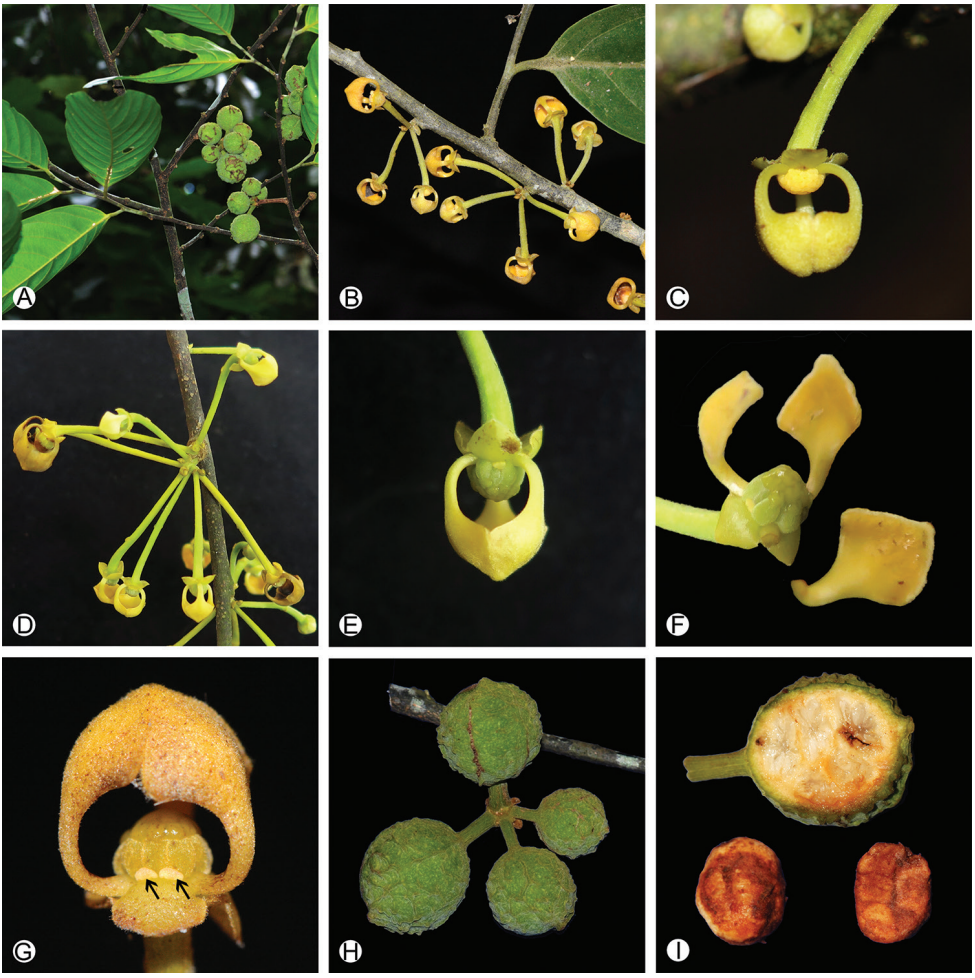

Figure 4. Morphology of *Pseuduvaria trimera* **A** fruiting branch **B** male inflorescence **C** side view of a male flower **D** female inflorescence **E** side view of a female flower **F** a female flower, showing the morphology of the inner petals and no glands on the inner petal **G** side view of the female flower, showing several carpels and two staminodes (with arrows) **H** fruits **I** section of one monocarp and seed morphology. Photos: Daniel Thomas (**A, H, I**); Yun-yun Shao (**B–G**).

(Fig. 2D). ***Pistillate flowers*** with gynoecium ca. 1.5 mm long, ca. 1.3 mm wide; carpels 3 per flower, ca. 1.2 mm long, ca. 0.5 mm wide (Figs 1F, G, 2E); ovules ca. 6–10 per carpel, bi-seriate; staminodes two (Fig. 1G). ***Fruiting*** peduncle 5–10 mm long, fruiting pedicel 20–50 mm (Fig. 1H). ***Monocarps*** 1–3, sessile or stipes to 3 mm long, ovoid, 20–37 cm long, 20–25 cm wide, very shallowly transversely constricted between seed when dry, densely tomentose, base rounded, apex apiculate (Figs 1H, 2F). ***Seeds*** 5–10 per monocarp, in 2 series, yellowish, semi-lenticular to ellipsoid, 12 to 14 mm long, 5–8 mm wide, 3–5 mm high, with rugose and pitted testa and a grooved raphe that is more or less straight (Fig. 1I, K), endosperm rumination spiniform (Fig. 1L).

Distribution and habitat. Known from several localities in Hainan province: Bai-cha Mountain in San-ya and Xing-long in Bao-ting, growing in rain forests, open woodland in valleys, at low elevations (ca. 600 m a.s.l).

Phenology. Flowering from March to June; fruiting from June to August.

Additional specimens examined. China. Hainan: San-ya, Bai-cha Mountain, 13 Aug. 1933, *Z. Huang 33693* (IBSC0078951, SN); Bao-ting Hisen, Qi-xian Mountain, on mountain slopes under forest, alt. 549 m, 25 Apr. 2020, *Q. L. Wang BT20200425001* (ATCH, IBSC); alt. 584 m, 28 May 2020, *Q. L. Wang BT20200528001*, *BT20200528002* (ATCH, IBSC).

Preliminary IUCN conservation status. CR D (IUCN 2012). *Pseuduvaria kwangtungensis* was assessed as CR D by Qin et al. (2017). Prior to this study, *P. kwangtungensis* was only represented in herbaria by two collections from Hainan, China, collected in 1933 and 1935 respectively. One of the authors, Dr. Qing-Long Wang, has undertaken extensive field surveys in Hainan, and only found this species again in two localities in Qi-xian Mountain in Bao-ting, with three and four mature individuals respectively. Although it's possible more individuals may be discovered with more extensive field surveys, we intended to maintain the CR category.

Key to *Pseuduvaria* in China

1 Shrub to 4 m tall, d.b.h. ca. 5 cm. Leaf laminas membranous, secondary veins ca. 10 pairs. Inflorescences axillary, with up to 2–3 flowers; flowers cream-colored or purple, glands paired on adaxial surface of inner petal; carpels 3 per flower, stamens 20–30 per flower. Fruits with 1–3 monocarps. Monocarps ovoid, apex apiculate, smooth; sessile or stipes to 3 mm long. Distributed in Hainan.....***P. kwangtungensis***

– Trees to ca. 20 m tall, d.b.h. ca. 42 cm. Leaf laminas subcoiaceous, secondary veins 14–18 pairs. Inflorescences clustered (3–6) on young branches, each with 1–2 flowers; flowers yellow or light green; inner petals lack gland; carpels 7–14 per flower, stamens 45–56 per flower. Fruits with 7–8 monocarps. Monocarps globose, apex slightly apiculate, rugulose; stipes 10–14 mm long. Distributed in Yunnan.....***P. trimera***

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Appendix I

Voucher information and GenBank accession numbers for samples used in this study (—, missing data; *, newly generated sequences). Voucher data are given for accessions for which DNA sequences were newly obtained, using the following format:

species, origin, voucher and Genbank accession numbers for *atpB-rbcL*, *matK*, *psbA-trnH*, *rbcL*, and *trnL-F*. For DNA sequences published in previous studies, voucher information is available from GenBank.

Alphonsea elliptica Hook. f. & Thomson, —, AY518807, JQ690402, —, AY319078; *Desmopsis microcarpa* R.E.Fr., —, AY518804, AY84146, AY319059, AY319173; *Huberantha korinti* (Dunal) Chaowasku, EU522345, EU522234, EU522124, EU522289, EU522179; *Marsypopetalum littorale* (Blume) B.Xue & R.M.K.Saunders, —, AY518835, JX544804, —, AY319140; *Meiogyne mindorensis* (Merr.) Heusden, —, JQ723776, —, JQ723863, JQ723916; *Meiogyne pannosa* (Dalzell) J.Sinclair, —, JQ723778, —, JQ723865, JQ723918; *Meiogyne virgata* (Blume) Miq., —, AY518798, JX544784, —, AY319094; *Miliusa indica* Lesch.ex A.DC., —, JQ723781, —, JQ723868, JQ723921; *Mitrephora alba* Ridl., —, AY518855, JQ889978, —, AY319106; *Monocarpia euneura* Miq., AY841381, AY518865, AY841477, —, AY319111; *Neo-uvaria telopea* Chaowasku, —, JX544751, JX544791, JX544755, JX544783; *Orophea enterocarpa* Maingay ex Hook.f. & Thomson, —, AY518815, JQ690417, —, AY319119; *Phaeanthus splendens* Miq., —, AY518864, JX544790, JX544754, AY319126; *Polyalthia suberosa* (Roxb.) Thwaites, AY841386, AY220439, AY841502, AY238956, AY319152; *Pseuduvaria acerosa* Y.C.F.Su & R.M.K.Saunders, EU522347, EU522236, EU522126, EU522291, EU522181; *Pseuduvaria aurantiaca* (Miq.) Merr., EU522348, EU522237, EU522127, EU522292, EU522182; *Pseuduvaria beccarii* (Scheff.) J.Sinclair, EU522349, EU522238, EU522128, EU522293, EU522183; *Pseuduvaria borneensis* Y.C.F.Su & R.M.K.Saunders, EU522350, EU522239, EU522129, EU522294, EU522184; *Pseuduvaria brachyantha* Y.C.F.Su & R.M.K.Saunders, EU522351, AY518837, EU522130, EU522295, AY319160; *Pseuduvaria bruneiensis* Y.C.F.Su & R.M.K.Saunders, EU522352, EU522241, EU522131, EU522296, EU522186; *Pseuduvaria calliura* Airy Shaw, EU522353, EU522242, EU522132, EU522297, EU522187; *Pseuduvaria cerina* J.Sinclair, EU522354, EU522243, EU522133, EU522298, EU522188; *Pseuduvaria clemensiae* Y.C.F.Su & R.M.K.Saunders, EU522355, EU522244, EU522134, EU522299, EU522189; *Pseuduvaria coriacea* Y.C.F.Su & R.M.K.Saunders, EU522356, AY518838, EU522135, EU522300, AY319161; *Pseuduvaria costata* (Scheff.) J.Sinclair, EU522357, EU522246, EU522136, EU522301, EU522191; *Pseuduvaria cymosa* (J.Sinclair) Y.C.F.Su & R.M.K.Saunders, EU522358, EU522247, EU522137, EU522302, EU522192; *Pseuduvaria dielsiana* (Lauterb.) J.Sinclair, EU522359, EU522248, EU522138, EU522303, EU522193; *Pseuduvaria dolichonema* (Diels) J.Sinclair, EU522360, EU522249, EU522139, EU522304, EU522194; *Pseuduvaria filipes* (Lauterb. & K.Schum.) J.Sinclair, EU522361, EU522250, EU522140, EU522305, EU522195; *Pseuduvaria fragrans* Y.C.F.Su, Chaowasku & R.M.K.Saunders, EU522397, EU522286, EU522176, EU522341, EU522231; *Pseuduvaria froggattii* (F.Muell.) Jessup, EU522362, EU522251, EU522141, EU522306, EU522196; *Pseuduvaria galeata* J.Sinclair, EU522363, EU522252, EU522142, EU522307, EU522197; *Pseudu-*

varia gardneri Y.C.F.Su, Chaowasku & R.M.K.Saunders, GQ174302, GQ174298, GQ174294, GQ174300, GQ174296; *Pseuduvaria glabrescens* (Jessup) Y.C.F.Su & R.M.K.Saunders, EU522364, EU522253, EU522143, EU522308, EU522198; *Pseuduvaria glossopetala* Y.C.F.Su & R.M.K.Saunders, GQ174303, GQ174299, GQ174295, GQ174301, GQ174297; *Pseuduvaria grandifolia* (Warb.) J.Sinclair, EU522365, EU522254, EU522144, EU522309, EU522199; *Pseuduvaria hylandii* Jessup, EU522366, EU522255, EU522145, EU522310, EU522200; *Pseuduvaria kingiana* Y.C.F.Su & R.M.K.Saunders, EU522367, EU522256, EU522146, EU522311, EU522201; *Pseuduvaria kwangtungensis* (P.T.Li) Qing L.Wang & B.Xue [= *Meiogyne kwangtungensis* Li], China, Hainan, *Q. L. Wang 20200528002* (IBSC), MW415929*, MW415930*, MW415931*, MW415932*, MW415933*; *Pseuduvaria latifolia* (Blume) Bakh.f., EU522368, EU522257, EU522147, EU522312, EU522202; *Pseuduvaria lignocarpa* J.Sinclair, EU522369, EU522258, EU522148, EU522313, EU522203; *Pseuduvaria luzonensis* (Merr.) Y.C.F.Su & R.M.K.Saunders, EU522370, EU522259, EU522149, EU522314, EU522204; *Pseuduvaria macgregorii* Merr., EU522371, EU522260, EU522150, EU522315, EU522205; *Pseuduvaria macrocarpa* (Burck) Y.C.F.Su & R.M.K.Saunders, EU522372, EU522261, EU522151, EU522316, EU522206; *Pseuduvaria macrophylla* (Oliv.) Merr, EU522373, EU522262, EU522152, EU522317, EU522207; *Pseuduvaria megalopus* (K.Schum.) Y.C.F.Su & Mols 16235, EU522374, EU522263, EU522153, EU522318, EU522208; *Pseuduvaria mindorensis* Y.C.F.Su & R.M.K.Saunders, EU522375, EU522264, EU522154, EU522319, EU522209; *Pseuduvaria mollis* (Warb.) J.Sinclair, EU522376, EU522265, EU522155, EU522320, EU522210; *Pseuduvaria monticola* J.Sinclair, EU522377, EU522266, EU522156, EU522321, EU522211; *Pseuduvaria mulgraveana* Jessup, EU522378, EU522267, EU522157, EU522322, EU522212; *Pseuduvaria multiovulata* (C.E.C.Fisch.) J.Sinclair, EU522379, EU522268, EU522158, EU522323, EU522213; *Pseuduvaria nova—guineensis* J.Sinclair, EU522380, EU522269, EU522159, EU522324, EU522214; *Pseuduvaria obliqua* Y.C.F.Su & R.M.K.Saunders, EU522381, EU522270, EU522160, EU522325, EU522215; *Pseuduvaria oxycarpa* (Boerl.ex Koord.) Y.C.F.Su & R.M.K.Saunders, EU522382, EU522271, EU522161, EU522326, EU522216; *Pseuduvaria pamattonis* (Miq.) Y.C.F.Su & R.M.K.Saunders, EU522383, AY518840, EU522162, EU522327, AY319163; *Pseuduvaria parvipetala* Y.C.F.Su & R.M.K.Saunders, EU522384, EU522273, EU522163, EU522328, EU522218; *Pseuduvaria philippinensis* Merr., EU522385, EU522274, EU522164, EU522329, EU522219; *Pseuduvaria phuyensis* (R.M.K.Saunders, Y.C.F.Su & Chalermglin) Y. C. F. Su & R. M. K. Saunders, EU522342, AY518841, EU522121, EU522287, AY319114; *Pseuduvaria reticulata* (Blume) Miq., EU522386, EU522275, EU522165, EU522330, EU522220; *Pseuduvaria rugosa* (Blume) Merr., EU522387, AY518839, EU522166, EU522331, AY319162; *Pseuduvaria sessilicarpa* (J.Sinclair) Y.C.F.Su & R.M.K.Saunders, EU522388, EU522277,

EU522167, EU522332, EU522222; *Pseuduvaria sessilifolia* J.Sinclair, EU522389, EU522278, EU522168, EU522333, EU522223; *Pseuduvaria setosa* (King) J.Sinclair, EU522390, EU522279, EU522169, EU522334, EU522224; *Pseuduvaria silvestris* (Diels) J.Sinclair, EU522391, EU522280, EU522170, EU522335, EU522225; *Pseuduvaria subcordata* Y.C.F.Su & R.M.K.Saunders, EU522392, EU522281, EU522171, EU522336, EU522226; *Pseuduvaria taipingensis* J.Sinclair, EU522393, EU522282, EU522172, EU522337, EU522227; *Pseuduvaria trimera* (Craib) Y.C.F.Su & R.M.K.Saunders, EU522394, EU522283, EU522173, EU522338, EU522228; *Pseuduvaria unguiculata* (Elmer) Y.C.F.Su & R.M.K.Saunders, EU522395, EU522284, EU522174, EU522339, EU522229; *Pseuduvaria villosa* Jessup, EU522396, EU522285, EU522175, EU522340, EU522230; *Sapranthus viridiflorus* G.E.Schatz, AY841391, AY743493, AY841515, JQ590194, AY319165.

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Phylogenetics of *Leptocereus* (Cactaceae) on Hispaniola: clarifying species limits in the *L. weingartianus* complex and a new species from the Sierra de Bahoruco

Lucas C. Majure[1], Yuley Encarnación[1,2], Teodoro Clase[3], Brígido Peguero[3], Kelly Ho[2], Duniel Barrios[4]

1 *University of Florida Herbarium (FLAS), Florida Museum of Natural History, Gainesville, FL 32611, USA* **2** *Department of Biology, University of Florida, Gainesville, Florida, USA* **3** *Departamento de Botánica, Jardín Botánico Nacional "Dr. Rafael M. Moscoso", Santo Domingo, Dominican Republic* **4** *Grupo de Ecología y Conservación, Jardín Botánico Nacional, Universidad de La Habana, Habana, Cuba*

Corresponding author: Lucas C. Majure (lmajure@floridamuseum.ufl.edu)

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Abstract

The Antillean genus *Leptocereus* represents an *in-situ* radiation among the Greater and Lesser Antilles of 19 currently recognized species. Extensive fieldwork carried out in the Dominican Republic over recent years has revealed that the species limits of *Leptocereus* of Hispaniola are more complex than previously thought. There are four currently recognized species that occur on the island, *L. demissus*, *L. paniculatus*, *L. undulosus* and *L. weingartianus*. We evaluate species limits in this group based on DNA sequence data and phylogenetic analysis, morphological characters and a survey of herbarium specimens from across Hispaniola. Based on our analyses, it is clear that at least five species occur on the island of Hispaniola, with the new species from Sierra de Bahoruco, *L. velozianus*, described here. We provide an identification key, distribution maps and photographic plates for all species on Hispaniola based on our own fieldwork and the study of herbarium specimens. The description of yet another species of *Leptocereus* on Hispaniola reiterates the importance of the poorly studied, but yet biodiverse, seasonally dry tropical forest in the Antilles.

Keywords

Biodiversity, Greater Antilles, plastome phylogeny, Seasonally Dry Tropical Forest

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Introduction

Seasonally dry tropical forests (SDTF) are one of the most threatened forest types on the planet with only around 10% of their original coverage still remaining intact in the Neotropics (Banda-R. et al. 2016) mostly owing to anthropogenic pressures, such as charcoal production and agriculture (Pennington et al. 2000, 2004). This forest type is extensive throughout the Greater Antilles; however, it has been poorly studied (Gentry 1982; Pennington et al. 2005) compared to other forest types on the islands or dry forest from other parts of the Neotropics. Recent work has revealed that these understudied forests have much undocumented biodiversity (e.g., Mejía and García 1997; Fryxell and Clase 2007; Areces-Mallea 2017, 2018; Majure et al. 2020; Martínez-Gordillo et al. 2020). Cactaceae are a conspicuous element of the diverse SDTF of the Greater Antilles, with roughly 94 taxa occurring in the region (Majure et al. unpubl. data). An estimated 35 species occur on the island of Hispaniola, including the Caribbean endemic *Leptocereus* (A. Berger) Britton & Rose. *Leptocereus* s.l. is a clade of 19 currently recognized species of trees and erect to sprawling shrubs, and represents an *in-situ* Antillean radiation that occurs in SDTF, primarily of the Greater Antilles, where it is most diverse, but also occurs in the Lesser Antilles on the island of Anegada (Barrios et al. 2020). Recent phylogenetic work by Barrios et al. (2020) showed that the two large tree species endemic to Cuba and Hispaniola, and traditionally circumscribed in the genus *Dendrocereus* (Britton and Rose 1920; Anderson 2001; Hunt et al. 2006), formed a monophyletic group [the *Dendrocereus* clade (D)] clearly nested within *Leptocereus*. Barrios et al. (2020) also resolved two other primary clades in *Leptocereus*, the Cuban (CU) clade consisting of species endemic to Cuba, and the Hispaniolan-Puerto Rican (EPR) clade consisting of taxa endemic to Hispaniola, Puerto Rico and outlying islands.

On Hispaniola, three species of *Leptocereus* (including *Dendrocereus*) were formerly recognized (*L. paniculatus* (Lam.) D. R. Hunt, *L. undulosus* (DC.) D. Barrios & Majure, and *L. weingartianus* E. Hartmann), and Areces-Mallea (2017) recently described a fourth species (*L. demissus* Areces) from southwestern Dominican Republic from the SDTF south of the Sierra de Bahoruco mountain range. Thus, currently, four species are recognized on Hispaniola.

Barrios et al. (2020) showed that the Hispaniolan endemic, *Leptocereus weingartianus*, was sister to a clade containing another Hispaniolan endemic, *L. paniculatus*, and a clade of two Puerto Rican species, *L. grantianus* Britton and *L. quadricostatus* Britton & Rose. *Leptocereus weingartianus* is widely distributed across Hispaniola and is morphologically heterogeneous, forming relatively large shrubs or small trees (Areces-Mallea 2017) with apical branches that are often sprawling among surrounding, dense vegetation of SDTF (Majure et al. pers. obsv.). The new species, *L. demissus*, described by Areces-Mallea (2017), is morphologically very similar to *L. weingartianus*, especially considering the sprawling growth form of ultimate stem segments (Majure et al. pers. obsv.), however, several reproductive characters appear to separate the two species morphologically (Areces-Mallea 2017). Recent fieldwork in the Sierra de Bahoruco has

revealed populations of an unidentified taxon of what morphologically appeared to be part of the *L. weingartianus* complex, however, which differed from typical *L. weingartianus* in stem and spine features. Therefore, it was clear given the morphological heterogeneity of *L. weingartianus*, and the wide distribution and phenetic similarity of putative close relatives, such as *L. demissus*, that phylogenetic analyses of these taxa on Hispaniola were greatly needed.

We wanted to determine whether the morphologically disparate populations from the Sierra de Bahoruco were indeed closely related to the *L. weingartianus* complex, and whether or not the morphologically similar *L. demissus* was distinct from *L. weingartianus* based on phylogenetic relationships. We sampled multiple populations of typical *L. weingartianus*, one population of the newly described *L. demissus* and several populations of the new morphotype from Sierra de Bahoruco, as well as taxa from all major clades of *Leptocereus* (sensu Barrios et al. 2020) and carried out a phylogenetic reconstruction based on nearly entire plastome sequencing from genome skimming. We also reviewed herbarium specimens from across the distribution of *Leptocereus* on Hispaniola, and we herein provide distribution maps, photographic plates, and an identification key to all species on Hispaniola, as well as a description of the new species uncovered during this work.

Materials and methods

All species of *Leptocereus* from Hispaniola, representing the EPR and D clades, as well as the new material from Sierra de Bahoruco, were sampled here for phylogeny reconstruction along with three taxa from the CU clade of *Leptocereus* and the Cuban *L. nudiflorus* (Britton & Rose) D.Barrios & S.Arias of the D clade. Likewise, *L. grantianus* and *L. quadricostatus* of the EPR clade were sampled. Outgroups included here were based on previous work by Barrios et al. (2020) and Majure et al. (unpubl. data) and included *Armatocereus*, *Calymmanthium*, *Cereus*, *Melocactus*, *Selenicereus*, and *Stenocereus* of Core Cactoideae (see Appendix 1).

Whole genomic DNAs of all taxa were extracted using a standard CTAB protocol with silica column cleaning (see Majure et al. 2019; Köhler et al. 2020). DNAs were resuspended in 300 ul of TE (Tris-EDTA) buffer (pH 8.0), and DNA quantity was analyzed on a Qubit 2.0 Fluorometer. Whole genomic DNAs were sent to Rapid Genomics LLC (http://rapid-genomics.com/home/; Gainesville, Florida, U.S.A.) for library preparation (including shearing) and sequencing via a genome skimming method, as in Majure et al. (2019). All taxa were sequenced on the Illumina HiSeq X platform using paired end reads (yielding 150 bp reads), and sixty samples were included per lane.

Raw reads of all taxa were imported in to Geneious (v. 11.1.5, Biomatters Ltd., Auckland, New Zeland) and reference-mapped using a previously, de-novo assembled partial plastome (including the large-single copy unit) of *Melocactus pedernalensis* M.M.Mejía & R.G.García (Majure, unpubl. data). Consensus sequences were then generated from reference-mapped plastomes, which were used for alignments.

Sequence alignment was carried out using the MAFFT (Katoh and Standley 2016) plugin in Geneious. We analyzed our 104,697 bp dataset (including indels) under maximum likelihood (ML) with the RAxML (Stamatakis 2014) plugin in Geneious using the rapid bootstrapping algorithm and undertaking 100 bootstrap pseudoreplicates.

Specimens (ca. 50) from multiple herbaria (JBSD, NY, S, US), as well as those generated from our own fieldwork (DES, FLAS, JBSD), were consulted for determining the morphological distinctiveness of the new material from the Sierra de Bahoruco, as compared to the phenetically similar taxa *L. weingartianus* and *L. demissus*. Those data also were used to determine the distribution of all species of *Leptocereus* across Hispaniola and to generate our identification key to the species (in part).

Results

Leptocereus s.l. was resolved as monophyletic in our phylogenetic analysis, and all three principal subclades recovered by Barrios et al. (2020) were recovered here as well (i.e., CU, D, and EPR subclades)-all three subclades were mostly well-supported (CU, bs = 100%; D, bs = 84%, EPR, bs = 100%). The CU subclade was sister to a well-supported clade (bs = 100%) composed of the D and EPR subclades. Multiple accessions of the two species composing the D clade, *L. undulosus* and *L. nudiflorus* formed clades and were resolved as sister taxa in our topology, further demonstrating their phylogenetic distinctiveness. Within the EPR clade, a clade composed of *L. demissus* and the new species *L. velozianus* was sister to a clade composed of two subclades, the first formed by all accessions of *L. weingartianus* and the second by *L. paniculatus*, *L. grantianus* and *L. quadricostatus*. Thus, our results show that *L. weingartianus* is not closely related to the new species *L. velozianus*, but rather more closely related to the rest of the EPR clade. Likewise, although *L. demissus* appears to be phenetically more similar to *L. weingartianus*, it was more closely related to the new species *L. velozianus*. In all cases where multiple accessions were used per species, those species formed well-supported clades (Fig. 1).

Leptocereus velozianus (Figs 2, 3), which is restricted to the northwestern part of the Sierra de Bahoruco (Fig. 4), is slightly phenetically similar to the Puerto Rican species *L. quadricostatus*, given the crenate rib margins and spiny pericarpels, although those two species are not close relatives (Fig. 1). *Leptocereus velozianus* is most phenetically similar to two other Hispaniolan species (*L. weingartianus* and *L. demissus* – Figs 5, 9), especially *L. weingartianus*, but differs from them based on the spine color (white or cream versus yellow to yellowish-red, although, younger spines in *L. velozianus* can be yellow-cream colored with darker tips, thus slightly overlapping with *L. weingartianus*), and the overall size of the joints (1.2–3.5 cm in diameter in the latter two species and 2.7–3.7 in *L. velozianus*). *Leptocereus velozianus* has conspicuously crenate margins, generally more so than either *L. weingartianus* or *L. demissus*, although this is a variable character (Areces-Mallea 2017). Although Areces-Mallea (2017) mentioned that *L. demissus* has straight rib margins between areoles and *L. weingartianus* is more

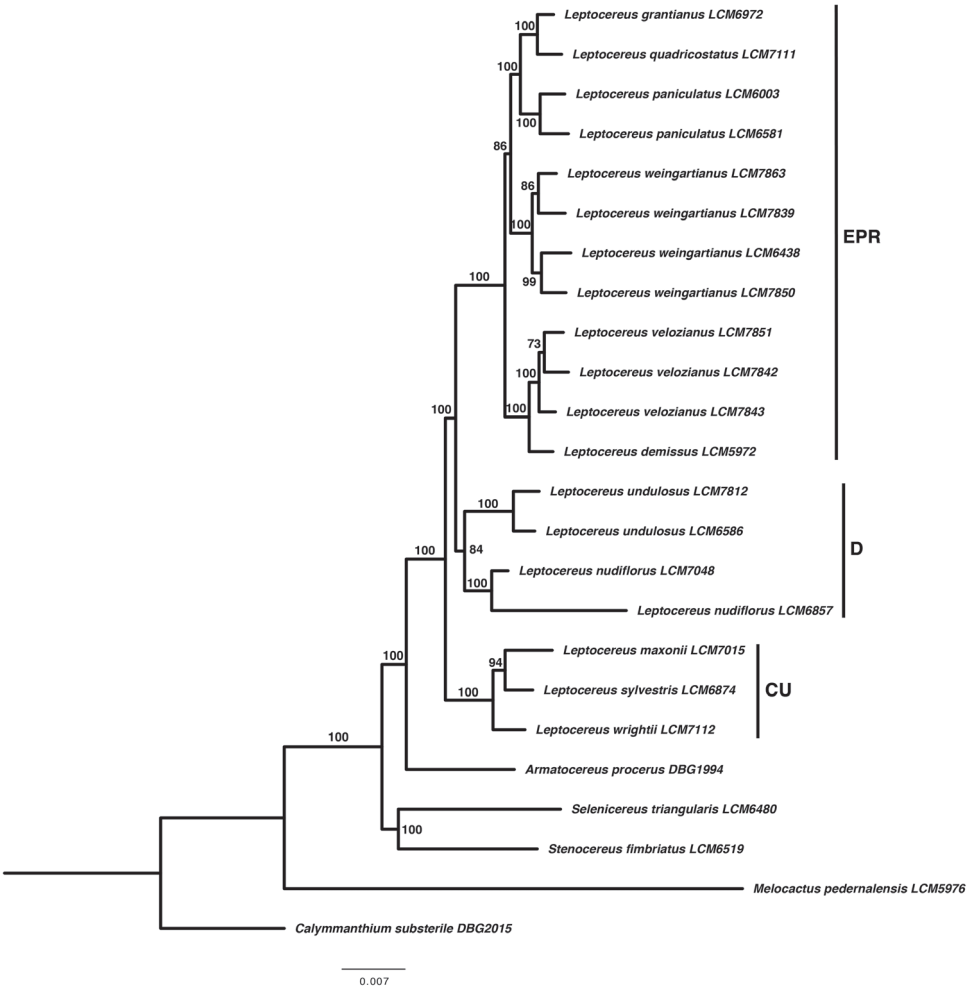

Figure 1. Phylogeny of *Leptocereus* with all major clades represented (sensu Barrios et al. 2020) and the new species *L. velozianus* included, resolved here as sister to *L. demissus*. Bootstrap values are given above the branches.

crenate, we found that character to be variable in both, with *L. weingartianus* also sometimes having nearly straight rib margins between areoles (Figs 5B, 9A). In general, *L. velozianus* can be separated from the other two species, *L. demissus* and *L. weingartianus*, by a suite of morphological characters, as well as its phylogenetic relationships to the other taxa (Fig. 1). *Leptocereus velozianus* does not share any major morphological features with either *L. paniculatus* or *L. undulosus* (Figs 6, 7).

As far as is known, *L. demissus* and *L. velozianus* are the most restricted species on Hispaniola, with *L. demissus* restricted to lower elevation dogtooth limestone of Parque Nacional Jaragua and the surrounding area south of the Sierra de Bahoruco, while *L. velozianus* is restricted to well-developed tropical dry forest along the north slopes

of the Sierra de Bahoruco (Fig. 4). *Leptocereus paniculatus*, *L. undulosus*, and *L. weingartianus* are much more widespread, with *L. weingartianus* occurring mostly on the north island from its eastern extremity near Cabo Engaño to its western extremity in Haiti on Gonave Island in elevations ranging from near sea level to 755 m in the Sierra Martín García, Dominican Republic (see Additional specimens examined). *Leptocereus paniculatus* is quite widespread across the island and is mostly found at lower elevations from -30 m on Isla Cabritos to around 400 m near Sierra Martín García, while *L. undulosus* is mostly restricted to areas near the coast in elevations ranging from near sea level to 245 m (Fig. 8, see also additional specimens examined).

Discussion

Our topology differs only slightly from Barrios et al. (2020) in that the CU clade was sister to the rest of *Leptocereus* here, whereas, in Barrios et al. (2020) the EPR clade was sister to the rest of *Leptocereus*. In both topologies, the D clade was nested within *Leptocereus*. Although it has been suggested that *L. undulosus* and *L. nudiflorus* may be conspecific (Anderson 2001), the multiple accessions sampled here clearly show them as genetically distinct entities, with multiple accessions of each forming clades, this likely being further driven by their reproductive isolation, being restricted to Hispaniola and Cuba, respectively. Morphological characters separating these two taxa are currently under investigation (D. Barrios unpubl. data).

Our phylogenetic results clearly demonstrate that the new species described here, *L. velozianus*, is genetically distinct from the more widespread *L. weingartianus*, and *L. demissus*, likewise, is more closely related to *L. velozianus* rather than the phenetically more similar *L. weingartianus*. Areces-Mallea (2017) mentioned that *L. demissus* is physically separated from *L. weingartianus* by the Sierra de Bahoruco. Our phylogenetic results and fieldwork support this idea, given that all *L. weingartianus* sampled were collected north of the Sierra de Bahoruco and form a well-supported clade, however, *L. weingartianus* does occur in the southern peninsula at nearly the same latitude as *L. demissus* south of Massif de la Hotte in Haiti based on the proposed neotype of *L. weingartianus* by Areces-Mallea (2017) from Cote le Fer. Interestingly, *L. weingartianus* grows alongside *L. velozianus* in the Sierra de Bahoruco; however, we have seen no evidence of hybridization between the two species. Ploidy, though, has not been examined in any species of *Leptocereus* from Hispaniola but is currently underway. *Leptocereus weingartianus* also occurs alongside *L. paniculatus* in populations near Jimaní and *L. demissus* occurs with *L. undulosus* in Parque Nacional Jaragua; however, we likewise have seen no putative hybrids among those species' pairs. We consider that phenology and perhaps pollinator differences could be driving the lack of hybridization in *Leptocereus* on Hispaniola (although we have very little information on specific pollinators of these species), and in other parts of their ranges, *L. weingartianus* appears to occupy different ecological niches than *L. paniculatus*. For example, in the Sierra Martín García, although both species occur there, *L. paniculatus* occurs at much

lower elevations than *L. weingartianus*, which begins to be found around 400 + meters (Majure et al. pers. obvs.). So, ecology also could be driving species divergence in this group of close relatives.

Below, we provide a description of the new species, *L. velozianus*, as well as a taxonomic treatment with identification key, distribution maps and photographic plates of the other four species of *Leptocereus* on Hispaniola. Updated descriptions of the other four species will be presented elsewhere (Encarnación, in prep.).

Taxonomic treatment

Key to the species of *Leptocereus* on Hispaniola

1 Large trees, floral buds and fruit spineless **2**
– Small trees, erect or sprawling shrubs, floral buds and fruit spiny (at least when immature) **3**
2 Mature stem segments mostly spineless, usually 3–4-ribbed, fruit broadly ovate to elliptic, smooth..... ***L. undulosus***
– Mature stem segments spiny, 4–5-ribbed, fruit elliptical, tuberculate..... .....***L. paniculatus***
3 Stems 1.2–3.5 cm in diameter, ribs crenate or straight, young spines yellow to yellowish-red **4**
– Stems 2.7–3.7 cm in diameter, ribs strongly crenate, young spines white to cream.....***L. velozianus***
4 Small trees or erect shrubs (ultimate stem segments often sprawling in age), stems mostly 4–5 ribbed, hypanthium oblong ..... ***L. weingartianus***
– Sprawling shrubs, stems 5–7 ribbed, hypanthium obconic.....***L. demissus***

1. *Leptocereus velozianus* Clase, Y.Encarn., Peguero & Majure, sp. nov.
urn:lsid:ipni.org:names:77214766-1
Figs 2–4

Type. República Dominicana. Prov. Independencia. Sierra de Bahoruco, Parque Nacional Sierra de Bahoruco, Puerto Escondido, Rabo de Gato, 433 m, 14 mayo 2019, *Majure 7843* (Holotype: JBSD!, Isotype: FLAS!).

Diagnosis. Differing from both *L. weingartianus* and *L. demissus* by the white young spines (vs. yellowish spines) and larger stem diameter (up to 3.7 cm in diameter in *L. velozianus*). Differing from *L. demissus* by the erect, primary trunk rather than the sprawling growth form, and oblong hypanthium in *L. velozianus* rather than obconic hypanthium as in *L. demissus*.

Description. Erect shrubs or small trees 2–4 m tall, ultimate stem segments often sprawling among associate vegetation in smaller individuals, or merely pendent on larger individuals, 13–40+ cm long, 2.7–3.7 cm wide, ribs 4–5 per stem, 12–17 mm

deep, rib margins strongly crenate, 18–28 mm between areoles, spines white (or yellowish around the developing base), aging gray, tips brownish-red to black, 13–15 per areole, erect, central spine 16–22 mm long, longest radial spines 18–30 mm long, shortest pair of reflexed radial spines at base of areole, 3–6 mm long, a tuft of brown, crisped trichomes filling areoles; flower, including hypanthium, ca. 7.6 cm long, spiny, outer tepals green, inner tepals greenish-white, apices obtuse, anthers white, 1.3–1.5 mm long, style ca. 3 cm long, stigma included in the corolla, lobes 8, pale green, apparently at the level of the ring of anthers, nectar chamber about 8 mm wide, immature fruit, 5–7.5 × 2.6–3.4 cm (excluding dried perianth), lustrous green, spiny, areoles ca. 28–36, with 8–13 spines, those 3–12 mm long, strongly brownish-red and white banded, the tips dark brownish-red to black, seeds 3.3–3.7 × 2.3–2.5 mm, dull black (only appearing shiny when not cleaned) with colliculate-pitted surface.

Etymology. The specific epithet, "*velozianus*" is given honoring the Dominican botanist Alberto Veloz, who is the Head and Curator of the Herbarium JBSD of the "Dr. Rafael M. Moscoso" National Botanical Garden of Dominican Republic. For 27 years, Veloz has dedicated his life to the study of the Hispaniolan flora and has conducted extensive fieldwork across the island, with many collections from the Sierra de Bahoruco, where this new species was found. Together with other botanists he has collected over 10,000 specimens and has published several papers on the flora in national and international journals. His publications have included different approaches, such as floristics, taxonomy, ecology and conservation. Veloz has also contributed to the formation of young botanists by involving students as part of the staff in the herbarium JBSD and through fieldwork.

Distribution and habitat. *Leptocereus velozanius* grows in well-formed, seasonally dry tropical forest over limestone from around 158–433 m in elevation occurring with the following associate species: *Bursera simaruba* Sarg., *Celtis ehrenbergiana* (Klotzsch.) Liebm., *Coccoloba diversifolia* Jacq., *Consolea microcarpa* (K.Schum.) E.F.Anderson, *Cordia globosa* Kunth, *Cylindropuntia caribaea* (Britton & Rose) F.M.Knuth, *Eugenia rhombea* (O.Berg.) Krug & Urb., *Gouania lupuloides* Urb., *Guaiacum sanctum* L., *Helicteres semitriloba* Bert. ex DC., *Hybanthus havanensis* Jacq., *Krugiodendron ferreum* Urb., *Leptocereus weingartianus* (E.Hartmann) Britton & Rose, *Melochia tomentosa* L., *Opuntia repens* Bello, *Pisonia aculeata* L., *Phyllostylon rhamnoides* (J.Poiss.) Taub., *Prosopis juliflora* (Sw.) DC., *Senna angustisiliqua* (Lam.) H.S.Irwin & Barneby, *Senegalia skleroxyla* (Tussac) Seigler & Ebinger, *Tournefortia stenophylla* Urb., *Zanthoxylum nashii* P.Wilson, *Ziziphus rignonii* Delponte. Currently, the species is only known from the northwestern slope of the Sierra de Bahoruco, southwest of the town of Jimaní (Fig. 4). Given the extent of tropical dry forest in and around that area, it is very likely that more localities of this species will be found.

Phenology. *Leptocereus velozianus* has been collected in flower and immature fruit during May and with mature fruit in June, July and November. Thus, it appears likely that *L. velozianus* may flower over the early to mid-summer months with fruit ripening later in the year.

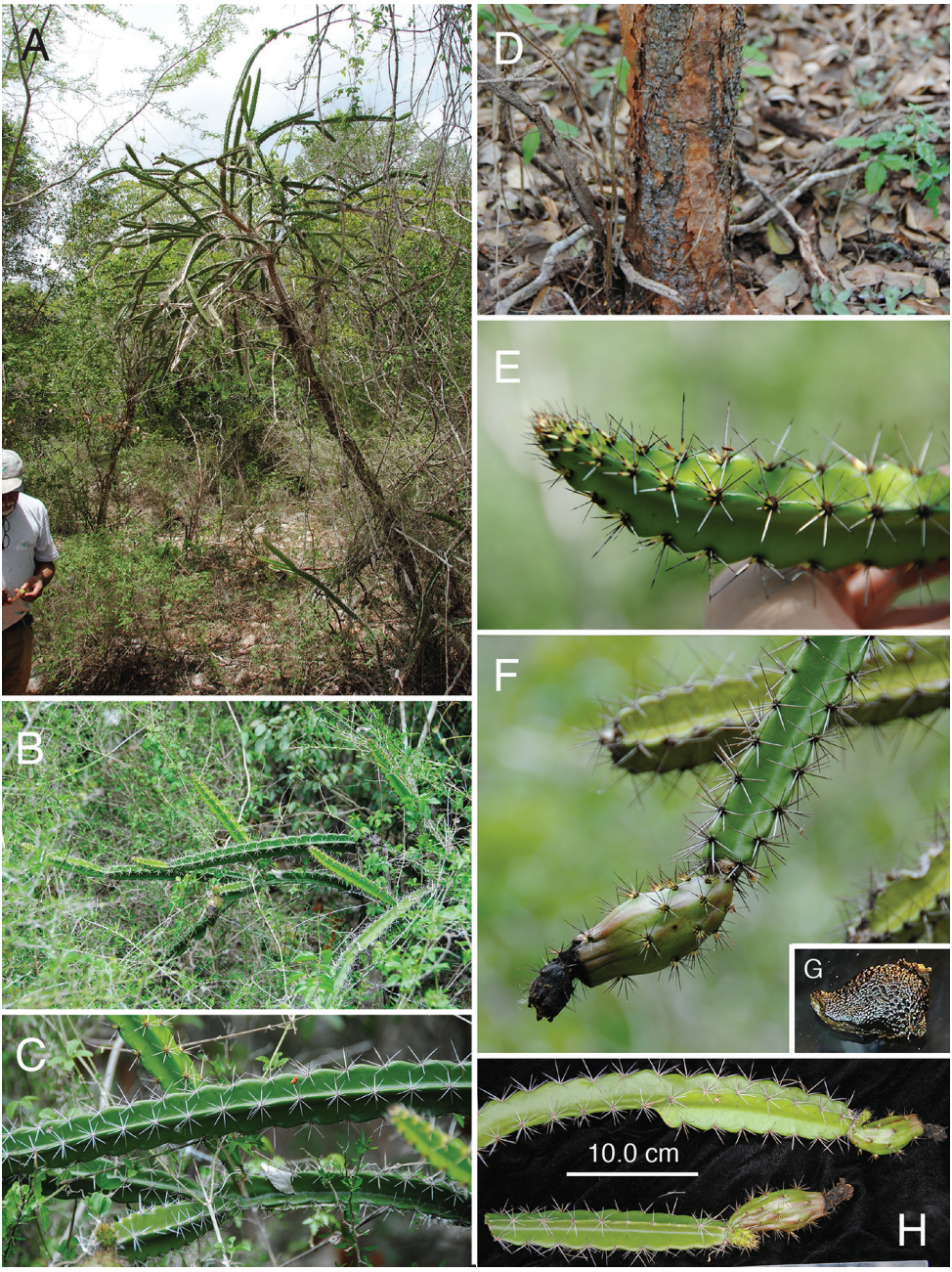

Figure 2. Photographic plate of *L. velozianus* **A** small tree habit of *L. velozianus* alongside T. Clase for scale **B, C** arching branches and spine color **D** trunk and bark **E** growing stem showing white spines as they mature **F** spiny, immature fruit **G** colliculate-pitted seed, and **H** stems with immature fruit, showing crenate rib margins. **A, D, F, G** from *Majure 7851* **B, C, H** from *Majure 7843*, and **E** from *Majure 7842*. Photos taken by L.C. Majure.

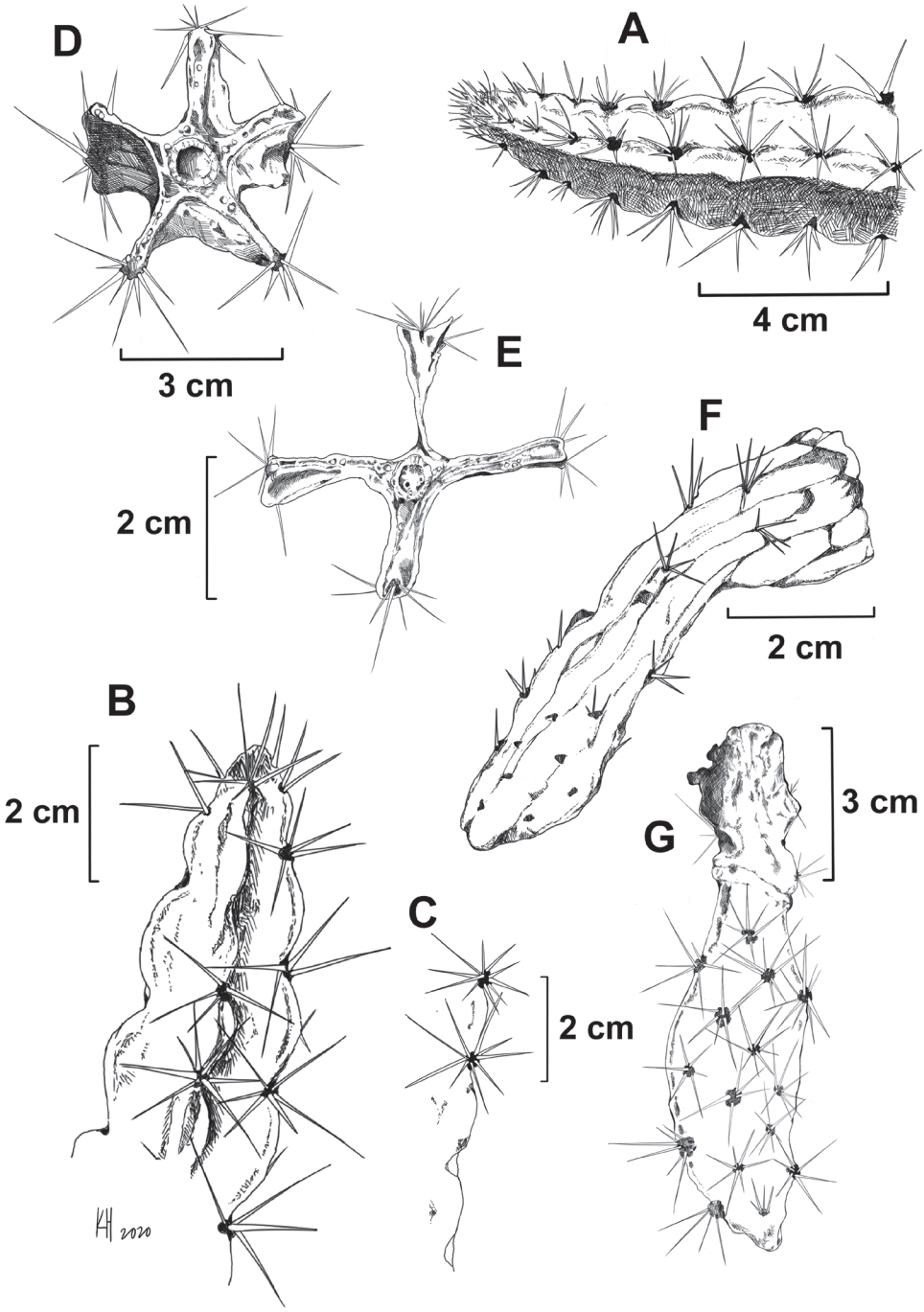

Figure 3. Illustration of *L. velozianus* **A–C** stems showing crenate margins **D, E** cross sections showing 4-5 ribbed stems, also showing large crystals in the parenchyma **F** day old flower showing rounded external tepal apices and spiny pericarpel, and **G** immature fruit showing spiny pericarpel and remnant floral tube. **A** from *Majure 7842*, and **B–F** from *Majure 7843*.

Conservation status. Formal evaluation of conservation status will be undertaken by Encarnación (in prep.) for *L. velozianus* based on further field work and demographic study. However, based on the currently known limited distribution of the species, the few numbers of individuals that have been observed, as well as anthropogenic activity near populations of the species, we consider that this species could be Near Threatened based on IUCN criteria. Further fieldwork will be essential for providing a comprehensive assessment of the conservation status of *L. velozianus*. Fortunately, the larger population known for this species is within the Sierra de Bahoruco National Park, so it is mostly protected from large-scale anthropogenic disturbances. Furthermore, the SDTF in that area is very well developed (i.e., non-fragmented) and should provide extra protection for the species.

Additional specimens examined. Dominican Republic. Prov. Independencia. Sierra de Bahoruco, municipio Duvergé, comunidad Puerto Escondido, lugar denominado Rabo de Gato, yendo hacia Cañada de Pedro Bello, 26 jun 2013, *Clase et al. 8004* (JBSD). Sierra de Bahoruco, municipio Duvergé, Rabo de Gato, yendo hacía la cañada La Cuaba, Pedro Bello, 23 nov 2017, *Clase et al. 9931* (JBSD). Sierra de Bahoruco, municipio Duvergé, Puerto Escondido, lugar denominado Rabo de Gato, yendo hacía la Cañada de Pedro Bello, 27 jul 2017, *Clase et al. 10202* (JBSD). Sierra de Bahoruco, municipio Jimaní, yendo desde El Limón, lugar denominado Guzmán, lado Sur de la carretera, 27 jul 2017, *Clase et al. 10205* (JBSD). Sierra de Bahoruco, justo al Este de Jimaní en la carretera 46 (entre Jimaní y El Limón) en el lugar denominado Guzmán; 158 m, 14 mayo 2019, *Majure 7842* (FLAS, JBSD). Parque Nacional Sierra de Bahoruco, Puerto Escondido, Rabo de Gato; 433 m, 14 mayo 2019, *Majure 7851* (FLAS, JBSD).

2. *Leptocereus demissus* Areces Cactus Succ. J. (Los Angeles) 89: 118. 2017.
Figs 4, 5

Type. Dominican Republic. Prov. Pedernales. On limestone terraces of maritime origin along both sides of the road to Pedernales, approx. 10 km southeast of Cabo Rojo, in dry forests dominated by large *Dendrocereus undulosus* trees, 22 Dec. 1998, *Areces 6812* (Holotype: JBSD-n.v.; Isotypes: NY, HAJB, HNT-n.v.).

Notes. Although type material of *L. demissus* is reported to be at JBSD, HAJB, HNT, and NY (Areces-Mallea 2017), we have been unable to locate types at any of these institutions for comparative analysis for this work. Thus, we have based our knowledge on the morphological traits of this species using those characters given by Areces-Mallea (2017), geography, and the one collection from near Oviedo made by us (*Majure 5972*-see specimens examined; Fig. 5).

Additional specimens examined. Dominican Republic. Prov. Pedernales. Oviedo, Sierra de Bahoruco, Municipio Oviedo, lugar denominado Fondo Paradí, Parque Nacional Jaragua, 86 m, 3 feb 2016, *Majure 5972* (DES, JBSD). Cabo Rojo, próx. al punto No. 7 de la Concesión, [no date], *Veloz et al. 945* (JBSD). A 18 km de Oviedo en la carretera hacia Pedernales, 1 mayo 1998, *Villardebó s.n.* (JBSD).

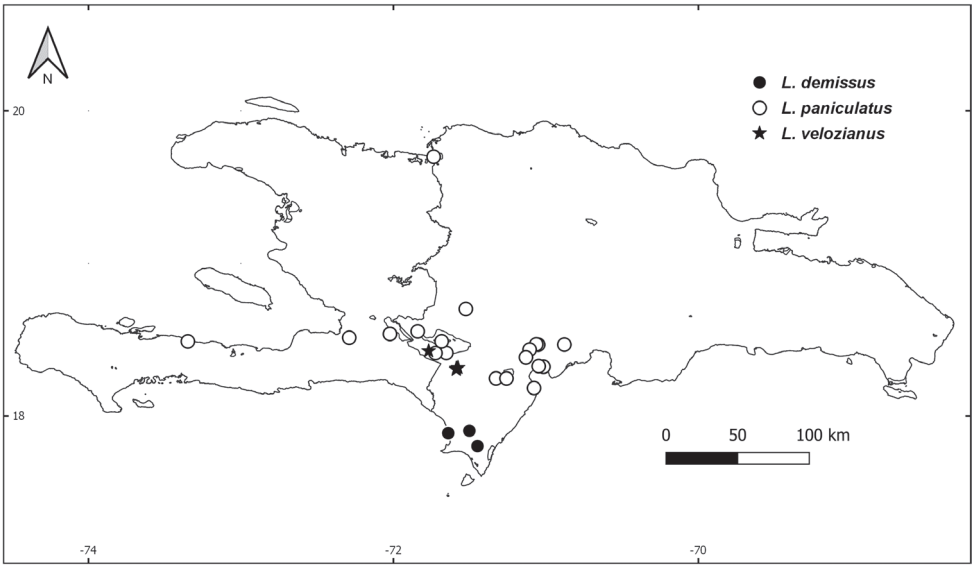

Figure 4. Distribution map of *L. demissus* (black circles), *L. paniculatus* (open circles), and *L. velozianus* (black stars) on Hispaniola.

Figure 5. Photographic plate of *L. demissus* **A** spiny fruit, and **B** slightly crenate stem of *L. demissus* (from *Majure 5972*). Photos by L.C. Majure.

3. *Leptocereus paniculatus* (Lam.) D. R. Hunt *Bradleya* 9: 89. 1991.

Figs 4, 6

Cactus paniculatus Lam. Encyl. [J. Lamarck & al.] 1: 540. 1785. *Cereus paniculatus* (Lam.) DC. Prodr. [A. P. de Candolle] 3: 466. 1828. *Neoabbottia paniculata* (Lam.) Britton & Rose Smithsonian Misc. Collect. 72: 3. 1921.

Type. Presumably from near Cul de Sac, Haiti, where Plumier collected numerous species. Lectotype (designated by Mottram 2002), plate t.21 in Plumier, which was cited by Lamarck (1785) and thus formed part of the protologue. This includes an illustration of a fragment of a stem segment, flower and fruit.

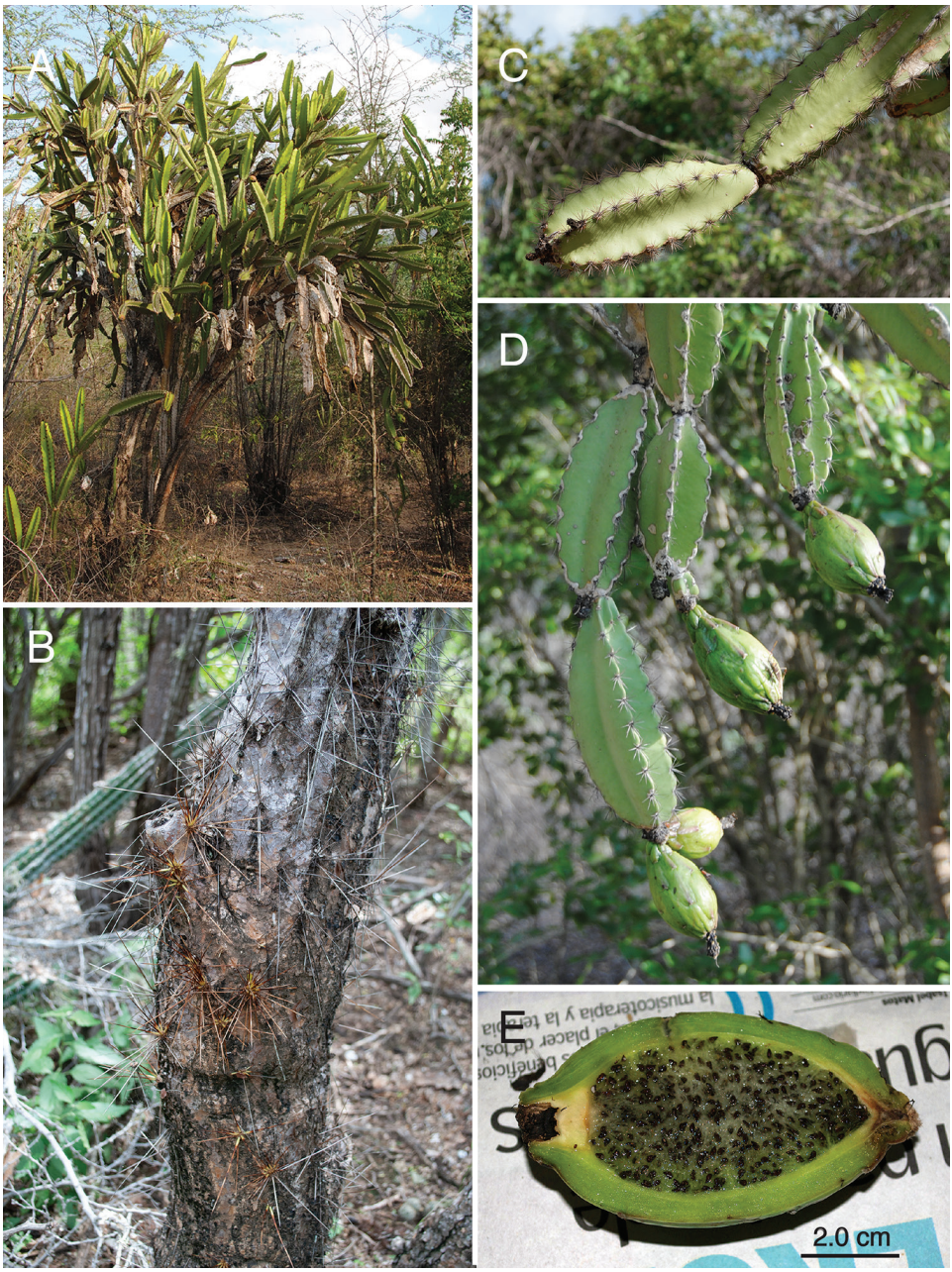

Figure 6. Photographic plate of *L. paniculatus* **A** habit **B** spiny trunk **C** apical, spiny, four-angled stems **D** fruiting stems and tuberculate fruit, and **E** longitudinal section of mature fruit. **A, D** from *Majure 7817* **B, C, E** from *Majure 6581*. Photos by L.C. Majure.

Notes. *Leptocereus paniculatus* is consistently recovered as closely related to the *L. quadricostatus*/*L. grantianus* clade (Fig. 1; see also Barrios et al. 2020), although the species is much more robust and treelike compared to the other two species. It is the

only species on Hispaniola that has spineless, tuberculate fruits and forms large populations at lower elevations in seasonally dry tropical forest across the island (Figs 4, 6).

Additional specimens examined. Dominican Republic. **Prov. Azua.** Llanura de Azua, 4 km al oeste del poblado de Tábara Abajo, en la carretera hacía Barahona, 400 m, 2 sept 1994, *García et al. 5630* (JBSD). **Provs. Azua-Barahona.** Este de Quita Coraza en el camino a Azua, 25 mayo 1982, *Zanoni & Mejía 20797* (JBSD). **Provs. Baoruco-Barahona.** A 3 km al este del poblado de Quita Coraza, 500 m, 10 dic 1994, *Perdomo & Villafaña 18* (JBSD). **Prov. Barahona**. 1.5 km al sur del poblado Fondo Negro, siguiendo un camino en dirección suroeste, 100 m, 15 oct 1994, *Camejo et al. 31* (JBSD). Barahona, 1913, *Fuertes 13.19* (NY, on 3 sheets). Sierra Martín García, approx 1.5 km al S del Higuito, 120 m, 16 dic 1995, *García et al. 6119* (JBSD). Sierra Martín García, ca 0.6 km al noreste del Cruce Vicente Noble, al costado de un arroyo seco justo al este de la Carretera 44., 108 m, 3 nov 2016, *Majure 6581* (DES, JBSD). Along road between Canoa and Puerto Alejandro, 5–20 m, 17 Oct 1982, *Proctor 39106* (JBSD). **Prov. Independencia.** Municipio Jimaní, distrito municipal Boca de Cachón, parte arriba del balneario, lugar denominado la Loma, 4 m, 6 jun 2009, *Clase et al. 5638* (JBSD). Sierra de Neiba, Ángel Félix, 400 m, 24–26 mar 1975, *Liogier et al. 22786* (JBSD). Entre Duvergé y Cabral, 3 ene 1977, *Liogier 26214* (JBSD). Independencia, Sierra de Bahoruco, al lado (sur) del Lago Enriquillo, al oeste ca. 15 km de Duvergé en la carretera 46, 5 feb 2016, *Majure 6003* (DES, FLAS, JBSD). Justo al sur de la Laguna Rincón ca. 2.5 km al oeste de La Lista y 3.4 km al este de Las Salinas a lo largo de la Carretera 46, 104 m, 13 mayo 2019, *Majure 7817* (FLAS, JBSD). Sierra de Bahoruco, ca. 1.5 km al este de El Limón en la Carretera 46 al sur del Lago Enriquillo, 43 m, 13 mayo 2020, *Majure 7840* (FLAS, JBSD). Isla Cabritos en el Lago Enriquillo, próximo al Campamento de Parques Nacionales, -30 m, 14 oct 1981, *Mejía & Pimentel 17261* (JBSD). **Prov. Montecristi.** Pepillo Salcedo (Manzanillo), al este del pueblo, en la zona donde se desarrollará el proyecto Puerto Cristal, 5 m, 5 nov 2002, *García & Gómez 7512* (JBSD). **Haiti.** [**Dept. de Nippes**]. Massif de la Hotte, western group, Anse a Veau, quaternary limestone west of town, 2 Jan 1936, *Ekman H5393* (NY). **Dept. de l'Ouest.** Vicinity of Etang Saumatre, 4–12 Apr 1920, *Leonard 3500a-b* (NY, US). Ibid, *Leonard 5344* (NY, US). Vicinity of Petionville, 350 m, 15–28 Jun 1920, *Leonard 5326* (NY).

4. *Leptocereus undulosus* (DC.) D. Barrios & Majure, Plant Syst. Evol. 306: 12. 2020.

Figs 7, 8

Cereus undulosus DC., Prod. Syst. Nat. 3: 467. 1828. *Dendrocereus undulosus* (DC.) Britton & Rose, J. New York Bot. Gard. 26(310): 220. 1925. *Acanthocereus undulosus* (DC.) Croizat, Caldasia 2: 137. 1943.

Lectotype. (designated by Barrios et al. 2020): illustration in Plumier, Plantarum americanarum fasc. 8. 187. t. 194. 1758. **Epitype.** (designated by Barrios et al. 2020): Haiti, Jean Rabel Rd., vicinity of Cabaret, Baie des Moustiques, plain west of Cabaret, 12–18 Jan 1929, *E.C. Leonard & G.M. Leonard 12085* (NY 03305544!).

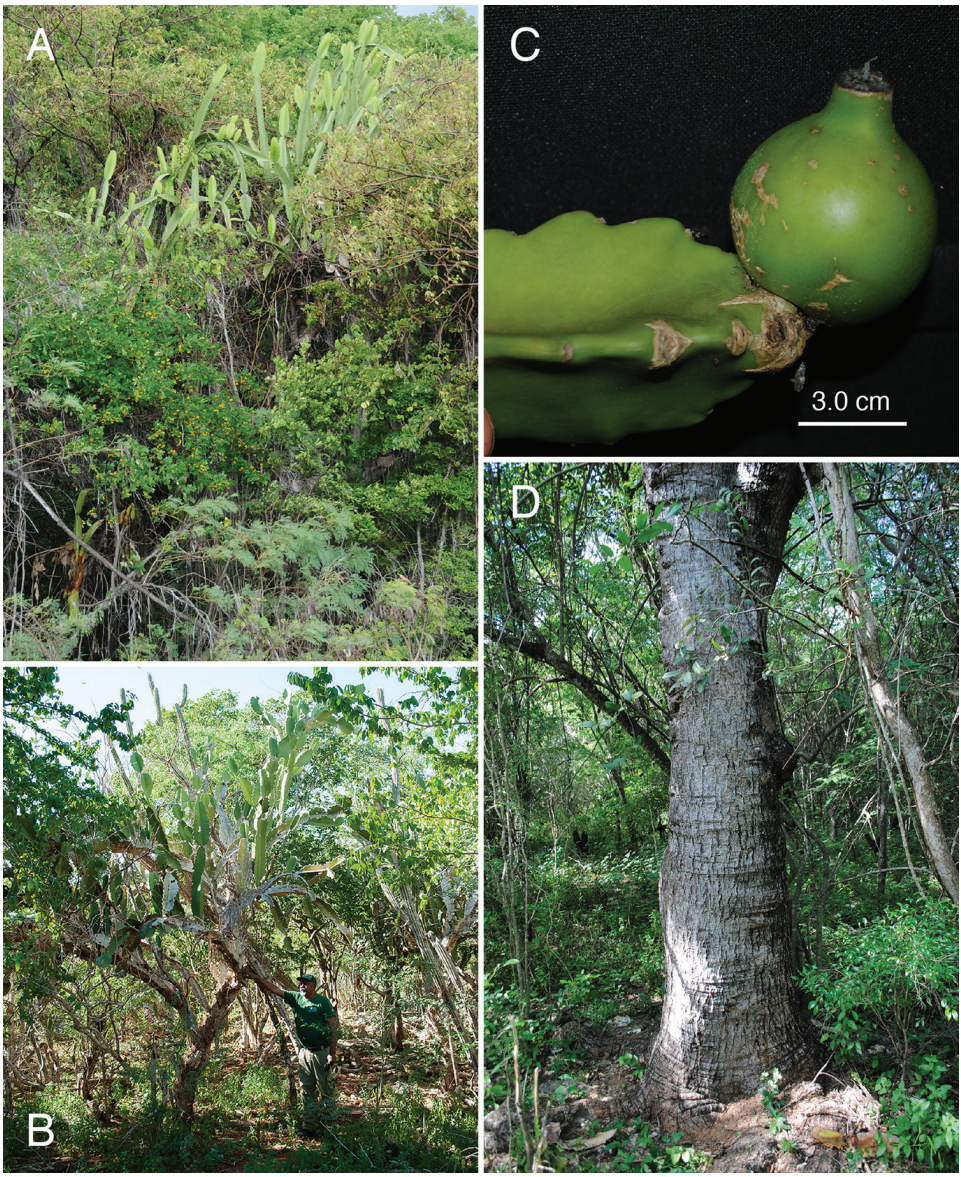

Figure 7. Photographic plate of *L. undulosus* **A** large tree of *L. undulosus* erupting from surrounding tropical dry forest **B** small individual of *L. undulosus* with T. Clase for reference **C** fruit and three-angled stem **D** large, woody trunk (ca. >64 cm dbh). **A** from *Majure 7812* **B, D** from *Majure. 6586* and **C** from *Majure 5974.* Photos by L.C. Majure.

Notes. Although several authors have suggested that *L. undulosus* may be conspecific with *L. nudiflorus* of Cuba (Anderson 2001, Hunt et al. 2006), Barrios et al. (2020) showed that the two taxa were sister to one another and likewise were genetically divergent; however, they only sampled one accession of *L. undulosus*. We reaffirm

that relationship here with one more sample of *L. undulosus* included in our phylogenetic analysis from a population near Barahona, east of the previous accession used in Barrios et al. (2020) from Parque Nacional Jaragua. Both samples form a strongly supported clade, sister to *L. nudiflorus.* More detailed morphological studies are necessary across the populations of *L. undulosus* on Hispaniola. Considering the distance between populations of the two (Cuba vs. Hispaniola), it is very likely that they are reproductively isolated. Thus, there seems to be no reason to consider these two taxa as conspecific. We anticipate that further morphological and genetic study will provide further support for the recognition of these two species.

Although not represented in our distribution map of *L. undulosus*, as there are no collections from that site, Île de la Tortue likely represents one of the larger populations of the species on Hispaniola (Peguero pers. obsv.). This reiterates the extensive fieldwork that still needs to be carried out to fully document populations of species of cacti with herbarium specimens from across the island. Cacti are often uncommonly collected because of the difficulty in preparing specimens, and thus, are generally poorly represented in herbaria (Majure et al. 2017).

Additional specimens examined. Dominican Republic. **Prov. La Altagracia.** 500 m al sur del cruce hacia Bayahibe en la carretera al Parque Nacional del Este, 60 m, 22 mayo 1986, *García & Pimentel 1051* (JBSD). **Prov. Barahona.** Sierra de Bahoruco, a lo largo de Carretera 44, ca. 0.8 km al norte de Playa Azul, 13 mayo 2019, *Majure 7812* (FLAS, JBSD). **Prov. Pedernales.** Entre Pedernales y Oviedo, 150 m, 24–27 jun 1975, *Liogier & Liogier 23339* (JBSD). Oviedo, Paraje Tres Charcos, Parque Nacional Jaragua, área de sisal, 138 m, 3 feb 2016, *Majure 5974* (DES, FLAS, JBSD). Oviedo, Parque Nacional Jaragua, al suroeste de Manuel Goya y Carretera 44, Lugar denominado "Los Sisales", cerca 14.6 km al oeste de Oviedo, 245 m, 16 nov 2016, *Majure 6586* (DES, JBSD). En la carretera hacia Las Mercedes, desde Pedernales a 2.5 km del cruce, 1 mayo 1998, *Villardebó s.n.* (JBSD). **Haiti. Dept. de l'Ouest.** Massif de la Selle, group Morne des Commissaires, Anses-a-Pitre, limestone cliff at Río Pedernales, on the road to Banane, 25 Oct 1926, *Ekman H6730* (NY). Place de la Paix, Port au Prince, no date, *Buch?* (NY). **Dept. de Nord-Ouest.** Coastal terrace between Mole St. Nicholas and Jean Rabel, 19 Jan 1995, *Areces 6790* (S). Between Port-des-Paix and Moustique, 1924, *Buch s.n.* (NY).

5. *Leptocereus weingartianus* (E.Hartmann) Britton & Rose Cactaceae 2: 77. 1920.
Figs 8, 9

Cereus weingartianus E. Hartmann Monatsschr. Kakteenk. 14: 155. 1904.

Type. Haiti. Lectotype (designated by Barrios and Majure, in review). Photo of type material of *L. weingartianus* in Hartmann (1904).

Notes. There has been some confusion around the type of *L. weingartianus* – Areces-Mallea (2017) designated a neotype of *L. weingartianus* considering that the original

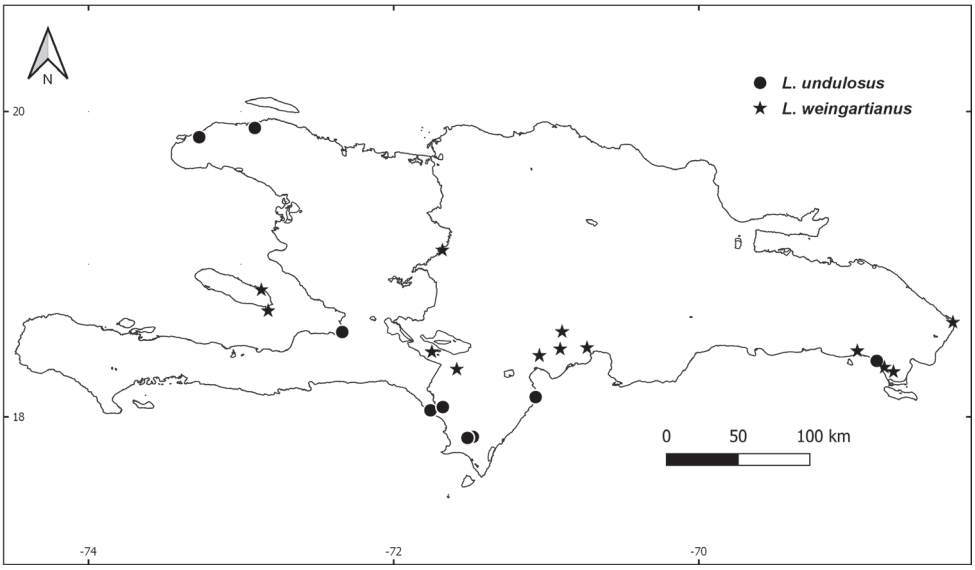

Figure 8. Distribution map of *L. undulosus* (black circles) and *L. weingartianus* (black stars) on Hispaniola.

type specimen is likely not extant, however, the photo of the type material in the protologue (Hartmann 1904) represents original material (Fig. 9C) and thus should serve as the type for this species. Thus, the neotype designation by Areces-Mallea (2017) from Cote le Fer, Haiti, is superfluous. Although supposedly deposited in JBSD and NY, we have been unable to find those specimens. Likewise, we have been unable to locate the other specimens of *L. weingartianus* (*Areces 5973*, *6438*, *6814*, *6815*, *6875*) cited by Areces-Mallea (2017) and supposedly at NY, so those are not cited in our specimens examined here.

Additional specimens examined. Dominican Republic. **Prov. La Altagracia.** Bayahíbe, La Romana, 1–5 m, 21 Feb 1976, *Liogier & Liogier 24907* (JBSD, NY). Cabo Engaño, on coastal road, 14 May 1980, *Mejía & Zanoni 6299* (JBSD). Parque Nacional del Este, Sector Guaraguao, 21 ene 1986, *Salazar et al. 317* (JBSD). Parque Nacional del Este, en el camino hacía La Cueva de José María, Guaraguao, 1–30 m, 28 abr 2001, *Veloz & Cedeño 2648* (JBSD). **Prov. Azua.** Municipio Sabana Yegua, después del cruce del 15, yendo hacía San Juan, aprox. 600 m antes del poblado de Las Guanábanas, 241 m, 30 jul 2011, *Clase et al. 6824* (JBSD). Sierra Martín García, Sept 1976, *Liogier 25277* (JBSD). Azua, Mar 1913, *Rose et al. 3941* (NY, US). **Prov. Barahona.** Barahona, Sierra Martín García, ca. 0.6 km al noreste del Cruce de Vicente Noble, al noreste ca. 11.2 km de la Carretera 44, a lo largo del un arroyo seco, 685 m, 3 nov 2016, *Majure 6438* (DES, JBSD). Sierra Martín García, ca. 0.6 km al noreste del Cruce de Vicente Noble, al noreste ca. 11.2 km de la Carretera 44, a lo largo del un arroyo seco, 755 m, 3 nov 2016, *Majure 6464* (DES, JBSD). **Prov. Elías Piña.** Cerro San Francisco (lado del sur), afuera del poblado de Bánica al sur de Pedro Santana, 490 m, 14 mayo 2019, *Majure 7863* (FLAS, JBSD). **Prov. Independencia.**

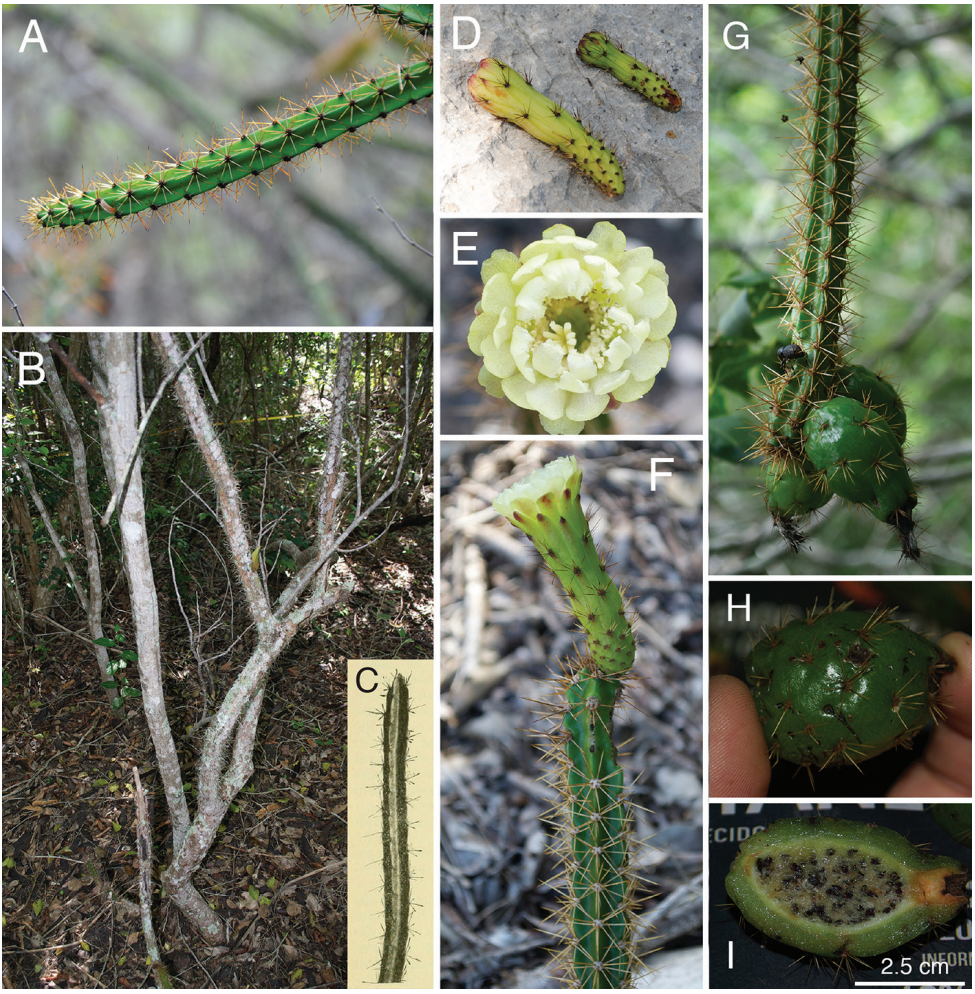

Figure 9. Photographic plate of *L. weingartianus* **A** young developing stem showing yellowish spines **B** trunk of adult individual showing erect growth form **C** type of *L. weingartianus* from Hartmann (1904) **D** floral buds showing spiny pericarpels **E** opened flower **F** side view of open flower and associated stem showing yellowish spines **G** stem with crenate margins and spiny fruit **H** shiny, green, spiny fruit with yellowish spines, and **I** longitudinal section of fruit showing mature seeds. **A** taken from *Majure 7839* **B, E, F, H, I** from *Majure 6464* **D** from *Majure 7863* and **G** from *Majure 6438*. Photos by L.C. Majure.

Sierra de Bahoruco, ca. 1.5 km al este de El Limón en la Carretera 46 al sur del Lago Enriquillo, 43 m, 13 mayo 2019, *Majure 7839* (FLAS, JBSD). Parque Nacional Sierra de Bahoruco, Puerto Escondido, Rabo de Gato, 433 m, 13 mayo 2019, *Majure 7850* (FLAS, JBSD). **Haiti. [Ouest].** Vicinity of Anse Galette, Gonave Island, 3–14 Mar 1920, *Leonard 3123* (NY, US). Petite Gonave Island, 9–10 Jul 1920, *Leonard 5256* (NY, US).

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Appendix 1

Species sampled in our phylogenetic analysis and GenBank accession numbers. Collector and collector number are given, or Desert Botanical Garden (DBG) accession number, as well as herbarium repository in parentheses. Herbarium acronyms follow Thiers (2017).

Armatocereus procerus DBG1994 (DES-SAMN16988911); ***Calymmanthium substerile*** DBG 2015 (DES-SAMN16988912); ***Leptocereus demissus*** *Majure 5972* (DES, JBSD-SAMN16988913); ***Leptocereus grantianus*** *Majure 6972* (FLAS-SAMN16988914); ***Leptocereus maxonii*** *Majure 7015* (DES, FLAS, HAJB-SAMN16988915); ***Leptocereus nudiflorus*** *Majure 6857* (DES, FLAS-SAMN16988916), *Majure 7048* (DES, HAJB-SAMN16988917); ***Leptocereus paniculatus*** *Majure 6003* (DES, FLAS, JBSD-SAMN16988918), *Majure 6581* (DES, JBSD-SAMN16988919); ***Leptocereus quadricostatus*** *Majure 7111* (FLAS-SAMN16988920); ***Leptocereus sylvestris*** *Majure 6874* (DES-SAMN16988921); ***Leptocereus undulosus*** *Majure 6586* (DES, JBSD-SAMN16988922), *Majure 7812* (FLAS, JBSD-SAMN16988923); ***Leptocereus velozianus*** *Majure 7842*, *7843*, *7851* (FLAS, JBSD-SAMN16988924–SAMN16988926); ***Leptocereus weingartianus*** *Majure 6438* (DES, JBSD-SAMN16988930), *Majure 7839*, *7850*, *7863* (FLAS, JBSD-SAMN16988927–SAMN16988929); ***Leptocereus wrightii*** *Majure 7112* (DES, FLAS-SAMN16988931); ***Melocactus pedernalensis*** *Majure 5976* (DES, JBSD-SAMN16988932); ***Selenicereus triangularis*** *Majure 6480* (DES, JBSD-SAMN16988933); ***Stenocereus fimbriatus*** *Majure 6519* (DES, JBSD-SAMN16988934).

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RESEARCH ARTICLE

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Two new hybrids of the genus *Diplazium* (Athyriaceae) from Japan

Kiyotaka Hori[1], Hironobu Kanemitsu[2]

1 *The Kochi Prefectural Makino Botanical Garden 4200-6 Godaisan, Kochi 781-8125, Japan* **2** *IDEA Consultants, Inc., 1-5-12 Higashihama, Higashi-ku, Fukuoka City, Fukuoka 812-0055, Japan*

Corresponding author: Kiyotaka Hori (khori@makino.or.jp)

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Abstract

In this study, we describe the ferns *Diplazium* ×*kanayamaense* hyb. nov. and *D.* ×*tsukushiense* hyb. nov. and further compare them to parental species *D. chinense*, *D. deciduum* and *D. fauriei* in terms of morphological characteristics, plastids and nuclear DNA markers. These new hybrids have been determined to be endemic to western Japan. The International Union for Conservation of Nature and Natural Resources status was evaluated for *D.* × *kanayamaense* as endangered (EN) and *D.* ×*tsukushiense* as critically endangered (CR).

Keywords

Athyriaceae, *Diplazium*, Japan, new hybrid

Introduction

One of the important characters of Japanese fern flora is the richness of hybrids. Japanese pteridologists have been recognising morphological variations of Japanese ferns and they recognised many hybrids confuse identification of Japanese ferns (Tagawa 1959; Nakaike 1992; Ebihara 2017). However, some Japanese hybrid ferns still are not described in Latin or English languages and they do not have scientific names. Especially, Athyriaceae has still many undescribed hybrids (Ebihara 2017) and there is still not enough evidence to support a combination of their hypothesised parents.

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Diplazium has been identified as the largest genus of Athyriaceae (PPG I 2016). It reportedly has 300–400 species (Wei et al. 2013; Liu et al. 2018), of which 26 species, eight varieties and 25 hybrids are recorded in Japan. Of these, 15 hybrids still do not have scientific names (Ebihara 2017). Hori and Murakami (2019) reported reticulate evolution of apogamous and sexual species in the *D. hachijoense* complex and found four undescribed apogamous species, based on plastid and nuclear markers. However, describing these ferns has been difficult as DNA phylogenies suggest that several undetected species are present in this complex. Otherwise, providing a description for these hybrids is easy when parents are endemic to Japan. In this study, we focused on *D.* ×*toriianum* Sa.Kurata, *D. mettenianum* complex (Ohta and Takamiya 1999) and *D. chinense* (Baker) C.Chr.

Kurata (1960) has described *D.* ×*toriianum* [*D. mettenianum* (Miq.) C.Chr.× *D. squamigerum* (Mett.) Matsum.] in terms of morphological characteristics. Subsequently, Ohta and Takamiya (1999) have recognised that the Japanese *D. mettenianum* complex contains *D. deciduum* N.Ohta & M.Takamiya sp. nov., *D. fauriei* Christ, *D. griffithii* T.Moore, *D. hayatamae* N.Ohta & M.Takamiya and *D. mettenianum sensu stricto*, based on cytology. Parents of *D.* ×*toriianum* are likely to be *D. deciduum* and *D. mettenianum* (Ebihara 2017).

Meanwhile, Tsutsui (1988) found one undescribed fern from Kyushu, Japan. He considered the fern to be a hybrid of *D. chinense* and *D. fauriei*, based on its morphological characteristics; this claim was supported by Ebihara (2017). Further, Kanemitsu (2019) has also found one undescribed fern nearby that might be a hybrid of *D. chinense* and *D. deciduum*. However, both Tsutsui (1988) and Kanemitsu (2019) did not provide scientific names with appropriate descriptions. Thus, this study identifies two new hybrids, *D.* ×*kanayamaense* (*D. chinense* × *D. deciduum*) and *D.* ×*tsukushiense* (*D. chinense* × *D. fauriei*), with descriptions based on morphological characteristics, plastids and nuclear DNA markers.

Materials and methods

Plant materials and DNA extraction

Total DNA for molecular analyses was extracted from silica-dried leaves using cetyltrimethylammonium bromide, as previously described (Doyle and Doyle 1990).

Diplazium chinense, *D. squamigerum*, *D.* ×*kanayamaense*, *D.* × *tsukushiense*, *D.* ×*toriianum*, members of the *D. mettenianum* complex (*D. deciduum*, *D. fauriei*, *D. hayatamae*, *D. mettenianum* and *D. griffithii*) and several additional species of *Diplazium* (*D. amamianum*, *D. donianum*, *D. esculentum*, *D. nipponicum*, *D. takii* and *D. wichurae*) were examined using molecular DNA analysis. We used four species of the genus *Deparia* as an outgroup (*De. japonica*, *De. lancea*, *De. unifurcata* and *De. viridifrons*). Voucher information for all samples is provided in Appendix 1. All voucher specimens are deposited in the herbarium of the Kagoshima University Museum (**KAG**), Tokyo

Metropolitan University (**MAK**), the Kochi Prefectural Makino Botanical Garden (**MBK**) or the National Museum of Nature and Science (**TNS**).

Additionally, we considered specimens from the Collection Database and Materials of TNS (http://db.kahaku.go.jp/webmuseum/), PE (http://pe.ibcas.ac.cn/en/), TAIF (http://taif.tfri.gov.tw/search.php), JSTOR Global Plants (https://plants.jstor.org/) and the Global Biodiversity Information Facility (https://www.gbif.org) database.

For conservation assessment, area of occupancy (AOO) and extent of occurrence (EOO) were estimated using GeoCAT (Bachman et al. 2011); default settings for grid size were also applied.

Plastid and nuclear DNA sequencing

We sequenced plastid *trnL-F* and nuclear *AK1* gene following methods from Hori and Murakami (2019), but with modified conditions for polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis of the nuclear *AK1* gene. Electrophoresis of *AK1* PCR products used 50% MDE gels (Lonza, Basel, Switzerland) containing 2% glycerol at 15 °C for 16 h at 300 V or 5% glycerol at 15 °C for 20 h at 300 V, followed by silver staining. To sequence the bands separated on the SSCP gels, the polyacrylamide gel was dried after silver staining by sandwiching the gel between Kent paper and a cellophane sheet on an acrylic backplate at 55 °C for 4 h. To extract the DNA, a piece of the DNA band was peeled from the dried gel by using a cutter knife and was incubated in 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) at 4 °C overnight. The supernatant solution was used as a template for further PCR amplification with the same primer set employed for the original PCR amplification. Sequence information obtained from voucher materials is provided in Table 1 and the Appendix 1.

Molecular analysis

The data set of plastid *trnL-F* phylogeny reflects what we directly sequenced from all the materials. In the dataset of nuclear *AK1* phylogeny, we used all the alleles which we separately picked up from PCR-SSCP gels. Sequences were aligned using MUSCLE (Edgar 2004) and assessed with Bayesian Inference analysis using MrBayes 3.2.6 (Ronquist et al. 2012) and Maximum Parsimony (MP) analysis with MEGA X software (Kumar et al. 2018). Indels were treated as missing characters in the analysis. The best-fit model (*trnL-F*: GTR+G model; *AK1*: HKY+G model) of sequence evolution for DNA regions was selected using jModelTest 2.1.10 (Darriba et al. 2012). Four Markov Chain Monte Carlo loops were run simultaneously and sampled every 100 of 1 million simulations. Tracer 1.7.1 (Rambaut et al. 2018) was used in examining posterior distributions of all parameters and associated statistics, including estimated sample sizes. The first 2500 sample trees from each run were discarded as burn-in periods. An MP tree was obtained using a subtree pruning-regrafting algorithm (Swafford et al. 1996) at search level 1, where initial trees were obtained by random addition of sequences (10 replicates). Confidence levels for monophyletic groups were estimated with 1,000 MP bootstrap pseudo-replicates.

Table 1. Haplotypes from *trnL-F* and allele *AK1* of *D.* × *kanayamaense*, *D.* × *tsukushiense* and related species. Any alleles of nuclear gene *AK1* that were identified by sequencing are in boldface. Otherwise, the alleles of nuclear gene *AK1* were deduced from comparisons of band positions in SSCP gels.

Voucher	Species/hybrid	*trnL-F*	*AK1*	Locality
H.Kanemitsu3746	*D.* ×*kanayamaense*	1	**A1A2CK**	Fukuoka Prefecture, Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu2883		1	A1A2CK	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu2884		1	A1A2CK	
H.Kanemitsu2906		1	A1A2CK	
H.Kanemitsu3755	*D.* ×*tsukushiense*	1	**A1A2DI**	Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei
H.Kanemitsu3756		1	A1A2DI	
H.Kanemitsu3757		1	A1A2DI	
H.Kanemitsu3750	*D.* ×*toriianum*	7	**CHKL1**	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu3751		7	CHKL1	
H.Kanemitsu3752		7	CHKL1	
K.Hori3023	*D. chinense*	1	**A1A2**	
H.Kanemitsu3760		1	A1A2	Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei
H.Kanemitsu3761		1	A1A2	
H.Kanemitsu3773	*D. deciduum*	4	**CHK**	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu3774		4	CK	
H.Kanemitsu3775		4	CHK	
H.Kanemitsu3892		4	CHK	
H.Kanemitsu3893		4	CHK	
H.Kanemitsu3905		4	CHK	
H.Kanemitsu3914		4	**CK**	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Nishiyama
H.Kanemitsu3951		4	CK	Saga Pref., Saga City, Fuji-Cho
H.Kanemitsu3758	*D. fauriei*	5	**DJ**	Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei
H.Kanemitsu3759		5	DJ	
H.Kanemitsu3992		5	DJ	
H.Kanemitsu3881		5	**DI**	Fukuoka Pref., Iizuka City, Naijukyo
H.Kanemitsu3989	*D. squamigerum*	6	**L2**	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu3990	*D. squamigerum*	6	L2	
H.Kanemitsu3991		7	L2	
K.Hori2336		4	**B**	Mie Pref., Minamimuro gun, Kiho-Cho, Takaoka
H.Kanemitsu3753		4	B	Fukuoka Pref., Fukuoka City, Sawara-Ku, Mt. Kanayama
H.Kanemitsu3754		4	B	
K.Hori3159	*D. hayatamae*	2	**BF**	Kagoshima Pref., Kumage gun, Yakushima-Cho, Kusugawa
K.Hori3160	*D. griffithii*	3	**EG**	

Results

Plastid and nuclear DNA phylogenetic trees

We sequenced 709–736 bp fragments of the *trnL-F* intergenic spacer from different specimens. The aligned *trnL-F* matrix was 753 bp, of which 140 bp (18%) were parsimony-informative. We then sequenced 280–520 bp of the *AK1* intron for each specimen, yielding a 574 bp aligned matrix, of which 78 bp (13%) were parsimony-informative. The accessions of DNA sequences were listed in Appendix 2.

The 50% majority consensus trees resulting from Bayesian Markov Chain Monte Carlo Bayesian (B/MCMC) analysis of plastid *trnL-F* and nuclear *AK1* gene are shown in Figures 1, 2, respectively.

Diplazium chinense, *D.* ×*kanayamaense* and *D.* ×*tsukushiense* in *trnL-F* phylogeny displayed haplotype 1. *Diplazium* ×*toriianum* exhibited the same haplotype 7 as

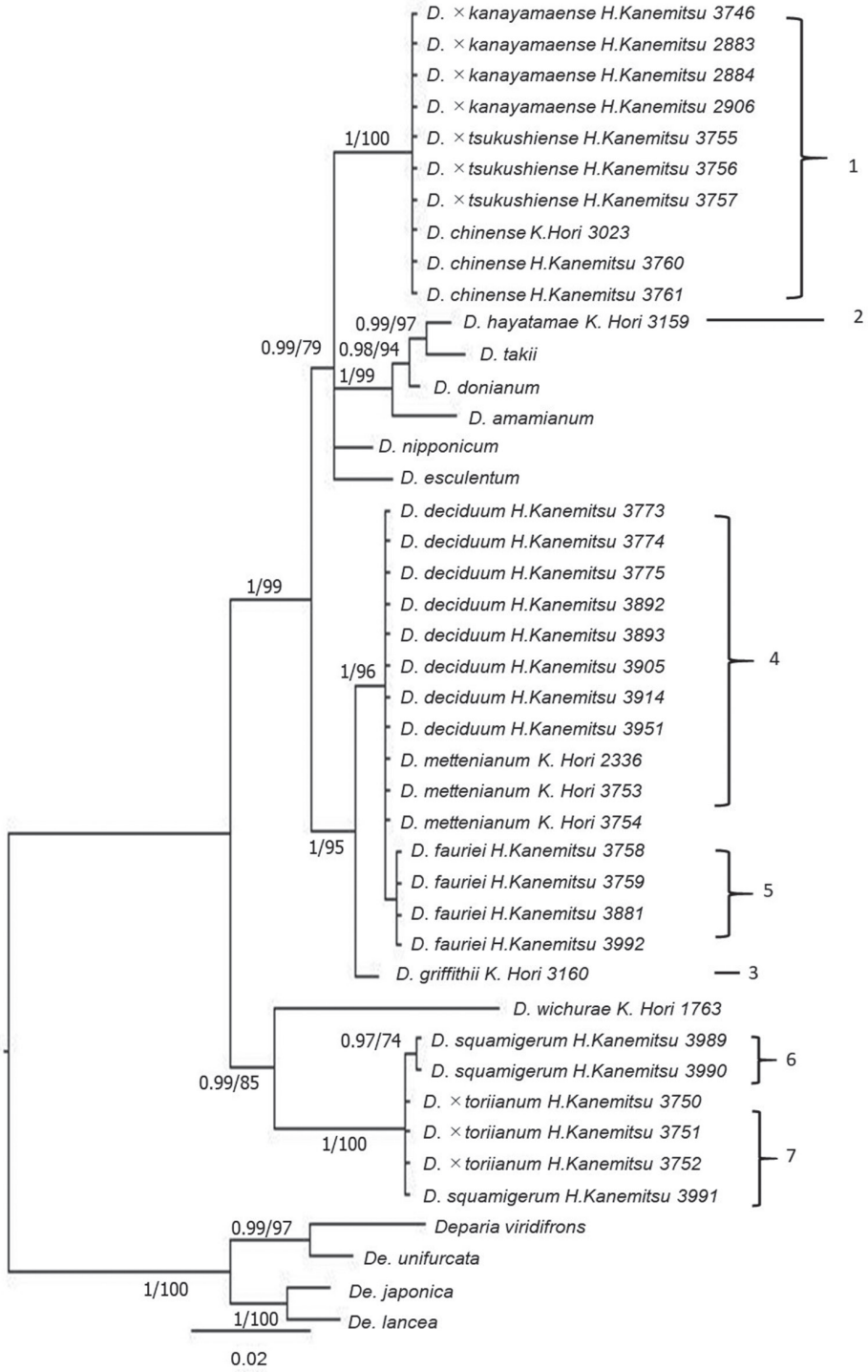

Figure 1. A 50% majority consensus tree resulting from Bayesian Markov Chain Monte Carlo Bayesian (B/MCMC) analysis of plastid *trnL-F* with BI PP (> 0.90) and MP BP (> 70) node support values.

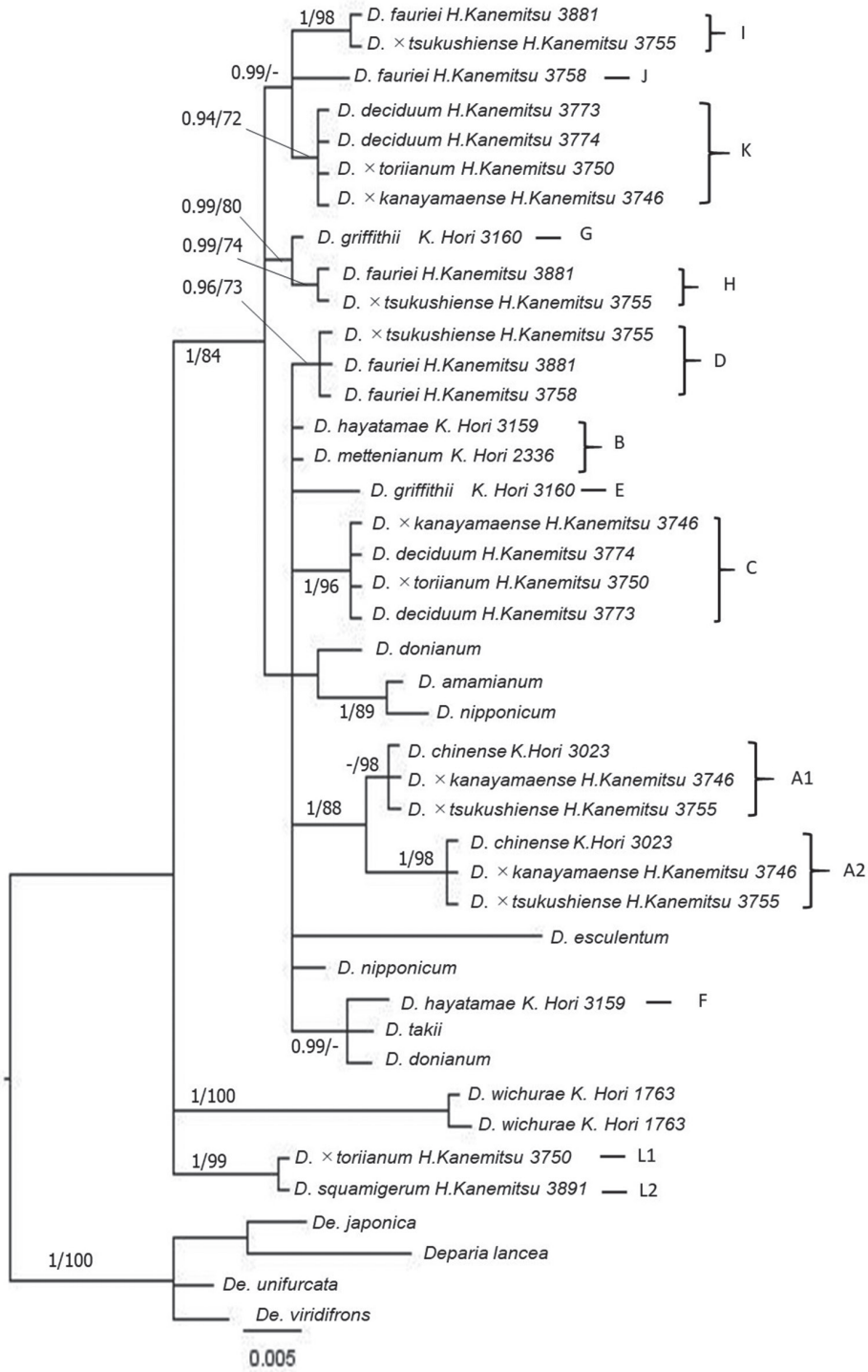

Figure 2. A 50% majority consensus tree resulting from Bayesian Markov Chain Monte Carlo Bayesian (B/MCMC) analysis of nuclear gene *AK1* with BI PP (> 0.90) and MP BP (> 70) node support values.

D. squamigerum, whereas *D. deciduum* and *D. mettenianum* in the *D. mettenianum* complex showed haplotype 4. Other species in the *D. mettenianum* complex were distinguished in *trnL-F* phylogeny: *D. fauriei*, 5; *D. hayatamae*, 2; and *D. griffithii*, 3. Haplotype 2 of *D. hayatamae* belonged to a different clade than the other species of the complex. *Diplazium hayatamae* has been determined to be closely related to *D. amamianum*, *D. donianum* and *D. takii*.

Diplazium chinense had two alleles (A1 and A2) in the same clade in the *AK1* phylogeny. Two clones of *D. deciduum* exhibited two paraphyletic out of three allelic types (CK or CHK). Two clones of *D. fauriei* also exhibited two paraphyletic out of two allelic types (DI or DJ), whereas *D.* ×*kanayamaense* displayed the same allele of *D. chinense* (A1A2) and one allelic type of *D. deciduum* (CK) completely. *Diplazium* ×*tsukushiense* displayed the same alleles of *D. chinense* (A1 and A2) and one allelic type of *D. fauriei* (DJ). *Diplazium* ×*toriianum* had the allele L1 which is closely related to allele L2 of *D. squamigerum* and the three alleles of one allelic type *D. deciduum* (CHK). Furthermore, other species of the *D. mettenianum* complex exhibited different alleles: *D. mettenianum*, B; *D. hayatamae*, BF; and *D. griffithii*, EG.

If the hybrids partly (or incompletely) shared the nuclear DNA allele of parents (in such a case, the hybrid had only nuclear allele A1A2C, A1A2K, A1A2H, A1A2I etc.), we need to assume the relationships between unknown species and present hybrids. In *D. chinense*, there was only one allelic type. There were different allelic combinations in *Diplazium fauriei* (DJ or DI) and *D. deciduum* (CK or CHK). However, the allele of hybrids (*D.* ×*kanayamaense*: A1A2CK; *D.* ×*tsukushiense*: A1A2DI) matched either allelic combination of two individuals of *D. deciduum* (*H. Kanemitsu 3914*, *3951*, CK) and one individual of *D. fauriei* (*H. Kanemitsu 3881*, DI) completely. This means we can simply interpret the origin of hybrids to be *D. chinense*, *D. deciduum* and *D. fauriei*. In addition, there are no morphological differences between different allelic types of *D. deciduum* and *D. fauriei*.

Haplotypes of *trnL-F* suggest that one maternal parent of the two new hybrids was *D. chinense*. Alleles in species in the *D. mettenianum* complex were variable and no species of the complex composed a monophyletic group. This might mean members of the *D. mettenianum* complex are allopolyploid or they have incomplete lineage sorting. However, allelic constituents of hybrids suggest that *D.* ×*kanayamaense* originated as a hybrid of *D. chinense* and *D. deciduum* and that *D.* ×*tsukushiense* originated as a hybrid of *D. chinense* and *D. fauriei*. These combinations are concordant with intermediate morphological characteristics between likely parents.

Taxonomic treatment

***Diplazium* ×*kanayamaense* K. Hori & H. Kanemitsu, hyb. nov.**
Figure 3

Diagnosis. *Diplazium* ×*kanayamaense* has been determined to be similar to *D.* ×*toriianum* in having 1-pinnate pinnatifid pinnae curved to an apex. However, lobes of *D.* ×*toriianum* are obtuse at the apex and scales are more entire on the margin. In contrast,

lobes of *D.* ×*kanayamaense* are acute at the apex and scales show small projections on their margins.

Type. Japan. Kyushu: Fukuoka Prefecture, Fukuoka City, Sawara-ku, Mt. Kanayama, 33°28'35.89"N, 130°19'23.57"E, alt. 700 m, semi-evergreen forest near streams containing *Carpinus laxiflora* (Siebold et Zucc.) Blume, *Neolitsea sericea* (Blume) Koidz., *Quercus acuta* Thunb and *Stewartia pseudocamellia* Maxim., on soil, 4 Jul 2020, *H. Kanemitsu 3746* (holotype: KAG 151589).

Description. *Terrestrial summergreen fern*. *Rhizomes:* creeping, non-branched, black, 10–15 × 0.5–0.8 cm in diam., closely set with roots and persistent, densely clothed with old stipe bases, glabrous; *fronds:* 2–5 per rhizome; *stipes:* purplish-green, 8–11 × 0.2–0.3 cm in diam., glabrous in middle to upper sections, sparsely clothed with dark brown scales (2.0–4.0 × 0.5–1.0 mm, with small projection on margin) of basal sections, lanceolate; *blades:* fresh green on adaxial surface, 1-pinnate pinnatifid, 1-pinnate at the apex, 15–26.5 × 8–23 cm, ovate; *rachises:* purplish-green, glabrous; *pinnae:* 9–10 pairs, ascending, lanceolate, alternate or opposite, petiolated (2–4 mm long), serrate to lobed, curved from base to apex, acute at the apex, sessile near the apex of blades, widely spaced, lowest pair slightly reduced or the same as second, second lowest pair usually largest, 15–17 cm × 1.5–3 cm; *pinnules*: alternate, 9–10 pairs on the basal sections of the blade, reduced distally, ovate to lanceolate, entirely to shallowly serrated, acute at apex in basal part of blade, obtuse at the apex in the middle section of blades, vein-free, single or double, close to or reaching to the margin, 5–7 pairs in the middle lobe; *the most basiscopic pinnules on the lowest pinnae*: occasionally absent, clearly short, independent from the costa, 2–10 mm × 1.5–4.0 mm; *sori:* long linear- or J-shaped, 1.0–3.0 mm long, on the middle of veinlets, 4–10 pairs per ultimate segment, persistent; *indusia:* cloudy white or brown, same shape as sori, entire, persistent; *spores:* absent or irregular-shaped, abortive.

Etymology. The name derives from Mt. Kanayama, Sawara-ku, Fukuoka City, Fukuoka Prefecture, west Japan, where *Diplazium* ×*kanayamaense* was initially found.

Specimens examined. Japan. Kyushu: Fukuoka Prefecture, Fukuoka City, Sawara-ku, Mt. Kanayama, 33°28'35.89"N, 130°19'23.57"E, alt. 700 m, semi-evergreen forest near streams containing *Carpinus laxiflora* (Siebold et Zucc.) Blume, *Neolitsea sericea* (Blume) Koidz., *Quercus acuta* Thunb and *Stewartia pseudocamellia* Maxim., on soil, 15 Jul 2018, *H. Kanemitsu 2883* (TNS1307641), *H. Kanemitsu 2884* (TNS1307641), *H. Kanemitsu 2906* (TNS1307645).

Distribution and ecology. *Diplazium* ×*kanayamaense* has been identified to be from Kyushu, western Japan (Figures 3, 5). The species is observed on soil under semi-evergreen forest near streams. This hybrid is endemic to Japan and exists in a population of approximately 124 individuals with juveniles, although parents, *D. chinense* and *D. deciduum*, were both absent near its side.

Conservation status. *IUCN Red List Category.* Based on estimates from GeoCAT, the EOO of *D.* ×*kanayamaense* was 0.002 km^2. AOO of *D.* ×*kanayamaense* was 4.0 km^2. There were only approximately 124 individuals in the type locality and the population size is not decreasing. According to IUCN (2012) criteria, this hybrid is endangered (EN). A formal evaluation of endangerment can be summarised by the following IUCN hierarchical alphanumeric coding system of criteria: EN D.

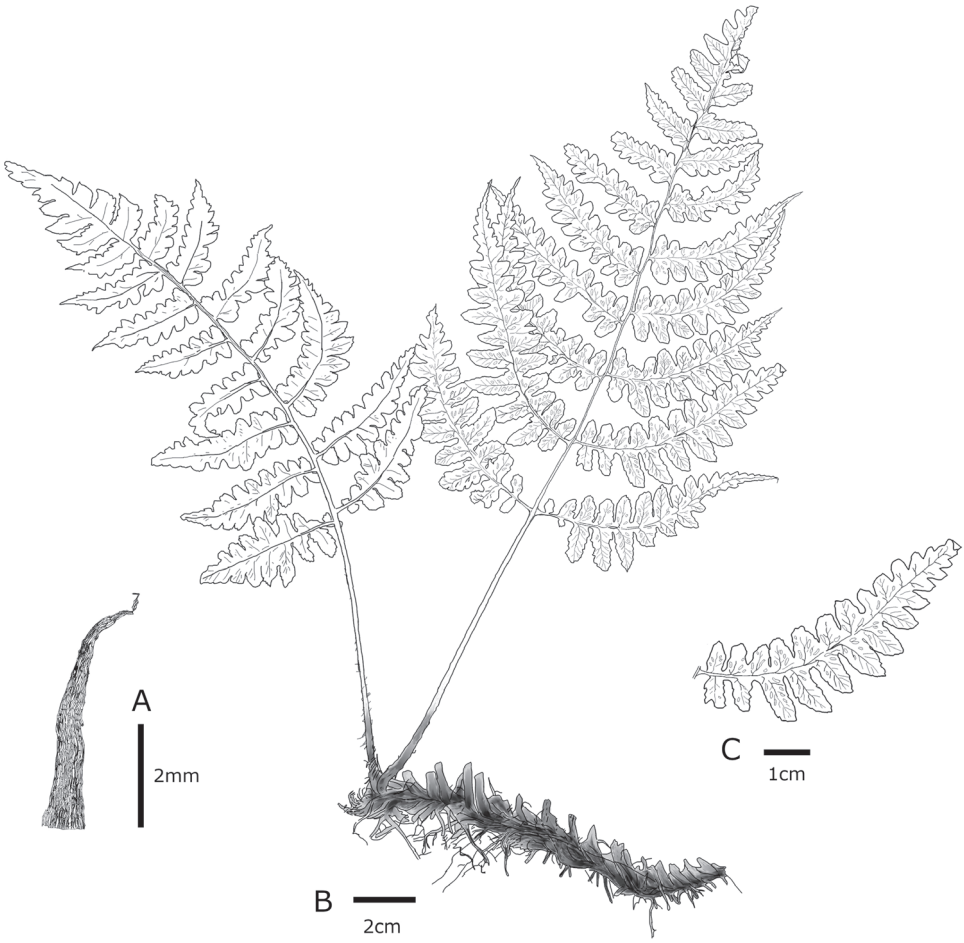

Figure 3. *Diplazium* ×*kanayamaense* K.Hori & H.Kanemitsu **A** lower stipe scale **B** habit **C** detail of abaxial pinnule **A–C** from the holotype (KAG151589 (illustration by K. Hori).

***Diplazium* ×*tsukushiense* K.Hori & H.Kanemitsu, hyb. nov.**
Figure 4

Diagnosis. *D.* ×*tsukushiense* is likened to *D. fauriei* with fronds 1-pinnate at the apex. However, lower pinnae of *D. fauriei* are not lobed, finely serrated on the margin. In contrast, lower pinnae of *D.* ×*tsukushiense* are lobed deeply and 1-pinnate pinnatifid.

Type. Japan. Kyushu: Fukuoka Prefecture, Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei, 33°26'22.87"N, 130°25'36.76"E, alt. 266 m, planted coniferous forest containing *Cryptomeria japonica* (Thunb. ex L.f.) D.Don, on soil, 4 Jul 2020, *H. Kanemitsu 3755* (holotype: KAG 151590),

Description. *Terrestrial semi-evergreen fern*. *Rhizomes:* creeping, occasionally two-branched, black, 7–30 cm × 0.8–1.3 cm in diam., closely set with roots and persistent, densely clothed with old stipe bases, glabrous; *fronds:* 2–5 per rhizome; *stipes:* purplish-

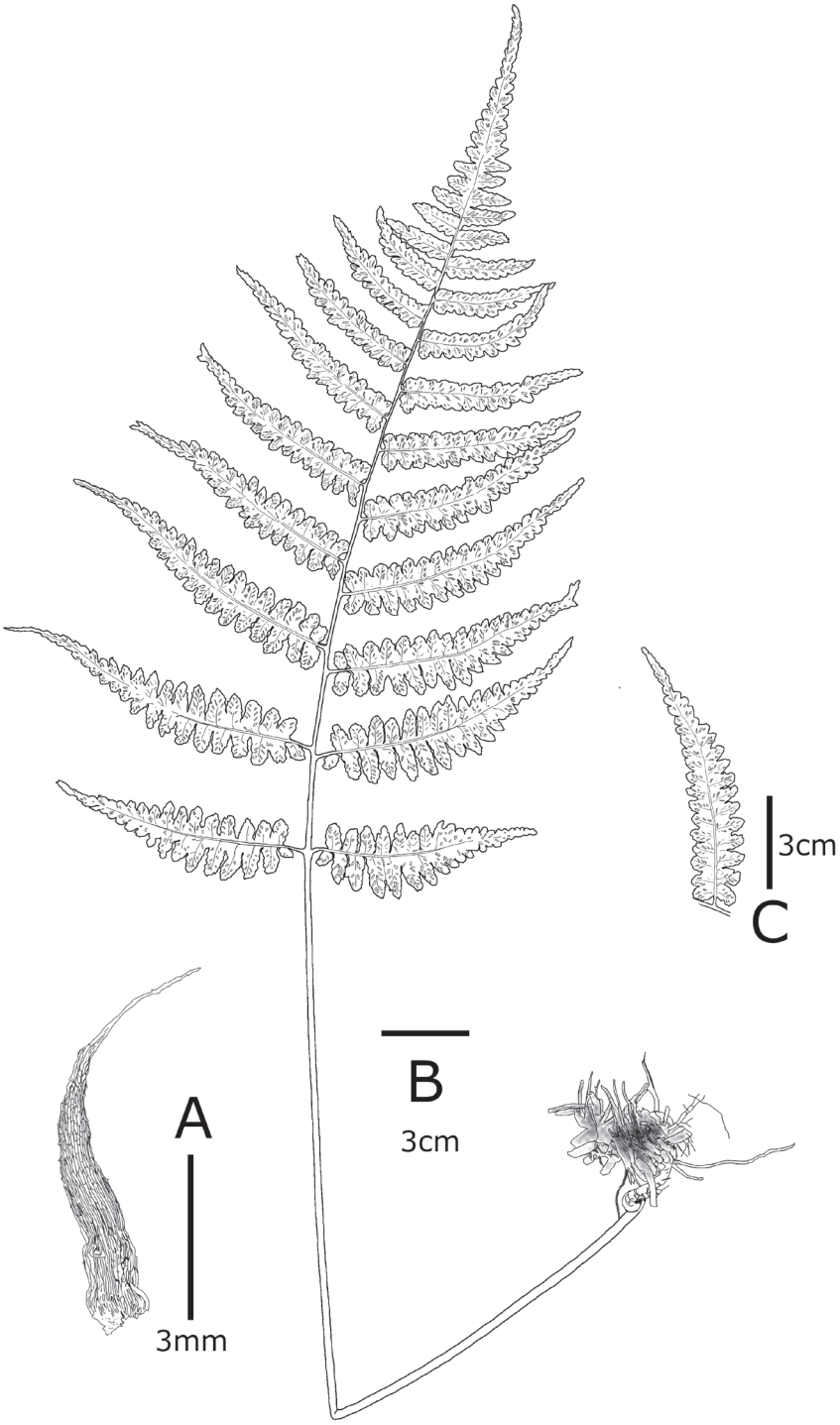

Figure 4. *Diplazium* ×*tsukushiense* K.Hori & H.Kanemitsu **A** lower stipe scale **B** habit **C** detail of abaxial pinnule **A–C** from the holotype (KAG151590) (illustration by K. Hori).

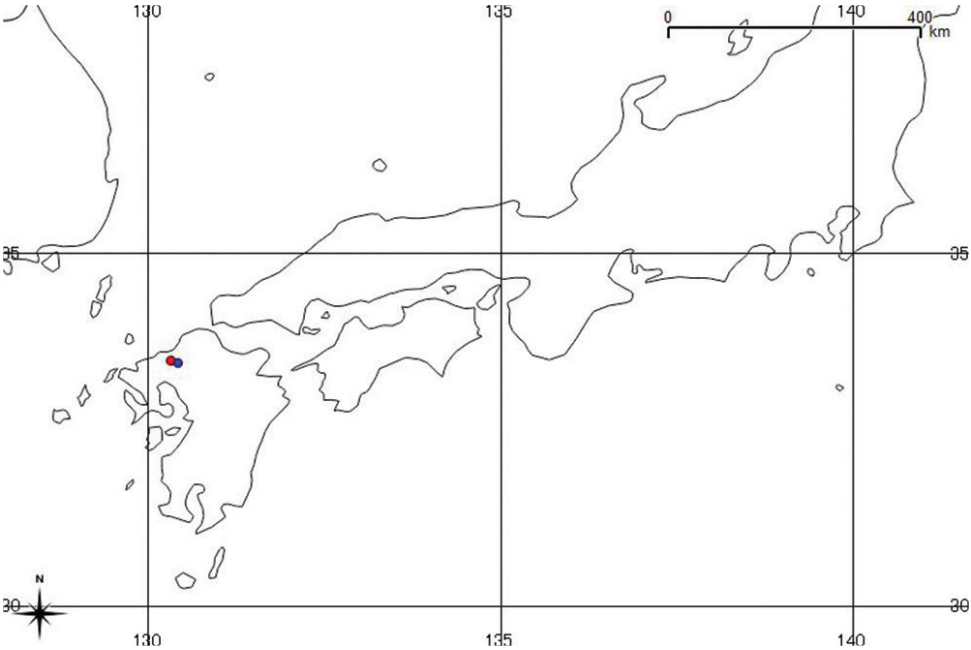

Figure 5. Map showing the known distribution in Japan of *D.* ×*kanayamaense* = Red circle and *D.* ×*tsukushiense* = Blue circle.

green, 20–30 cm × 0.2–0.3 cm in diam., glabrous in the middle to upper sections, sparsely clothed with dark brown scales (3.0–6.0 mm × 1.0–1.5 mm, with small projection on margin) on basal sections, lanceolate; *blades:* dark green on adaxial surface, 1-pinnate at the apex, 1-pinnate pinnatifid at the base and middle, 30.0–43.5 cm × 20.0–26.0 cm, narrowly ovate; *rachises:* purplish-green, glabrous; *pinnae:* 10–15 pairs, ascending, straight, lanceolate, alternate, petiolated (2–11 mm long), serrate to lobed, acute at the apex, sessile near the apex of blades, widely spaced, lowest pair of pinnae slightly reduced, second lowest pair usually largest, 10–16 cm × 2.0–5.0 cm; *pinnules*: alternate, 10–15 pairs on the basal sections of the blade, reduced distally, ovate to lanceolate, entirely to shallowly serrated, acute at apex in basal part of blade, rather acute at apex in middle part of blade, vein-free, single or double, close to or reaching to the margin, 5–7 pairs in the middle lobe; *the most basiscopic pinnules on the lowest pinnae*: occasionally absent, slightly short, rather independent from the costa, 3–7 mm × 3–4 mm; *sori:* long linear- or J-shaped, 1.0–5.0 mm long on the middle of veinlets, 4–10 pairs per ultimate segment, persistent; *indusia:* cloudy white or brown, same shape as sori, entire, persistent; *spores:* absent or irregular-shaped, abortive.

Etymology. The name derives from Tsukushiyabakei, Ooaza-Gokayama, Nakagawa City, Fukuoka Prefecture, west Japan, where *Diplazium* ×*tsukushiense* was initially found.

Specimens examined. Japan. Kyushu: Fukuoka Prefecture, Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei, 33°26'22.87"N, 130°25'36.76"E, alt. 266 m, planted coniferous forest containing *Cryptomeria japonica* (Thunb. ex L.f.) D.Don,

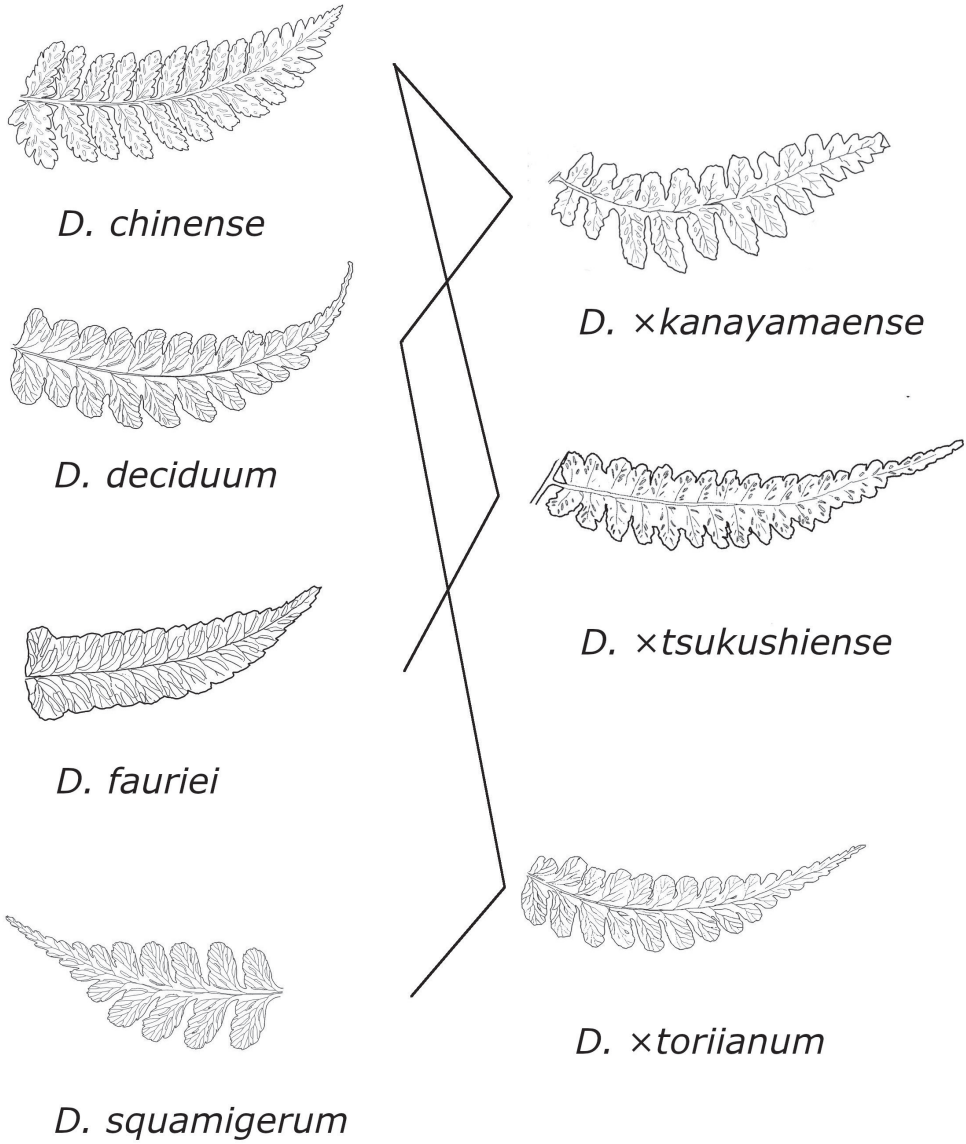

Figure 6. Abaxial surface of pinnae of *D. chinense*, *D. deciduum*, *D. fauriei*, *D. squamigerum*, *D.* ×*kanayamaense*, *D.* ×*tsukushiense* and *D.* ×*toriianum* with relationships of hybrids (illustration by K. Hori).

on soil, 4 Jul 2020, *H. Kanemitsu 3756*, *H. Kanemitsu 3757*, *loc.cit.*, 16 Jul 2018, *H. Kanemitsu 2930* (TNS1307651), *loc.cit.*, 12 Dec 1976, *S. Tsutsui 13341-3* (TNS341595), *loc.cit.*, 8 Jul 1978, *S. Tsutsui 15807* (TNS424776), *loc.cit.*, 6 Nov 1999, *Y. Inoue Y-37* (TNS1159163), *loc.cit.*, 11 Aug 1986, coll. by *T. Yamanaka* (TNS1135951), *loc.cit.*, 11 Aug 1986, coll. by *T. Yamanaka* (TNS1135952), *loc.cit.*, 11 Aug 1986, coll. by *T. Yamanaka* (TNS1135953), *loc.cit.*, 11 Aug 1986, coll. by *T. Yamanaka* (TNS1135954).

Distribution and ecology. *D. ×tsukushiense* has been determined to be from Kyushu, western Japan (Figures 4, 5). Observed to grow on soil under coniferous forest containing *Cryptomeria japonica* (Thunb. ex L.f.) D.Don near streams. This hybrid is endemic to Japan. In the type locality, the population is only approximately 10 individuals. Parents *D. chinense* and *D. fauriei* are observed in the same locality.

Conservation status. *IUCN Red List Category.* Based on estimates from GeoCAT, the EOO of *D. ×tsukushiense* is 0.001 km^2. The known AOO of *D. ×tsukushiense* is 4.0 km^2. Only 10 individuals are found in the type locality and population size is decreasing because of illegal waste dumping in forests. Therefore, this hybrid should be considered critically endangered (CR), as per the IUCN (2012) criteria. A formal evaluation of endangerment is summarised by the following IUCN hierarchical alphanumeric coding: CR B1ab (i, iv, v)+B2ab (i, iv,v)+C1+C2 a (i, ii) b+D.

Discussion

The parents of *D. ×kanayamaense* and *D. ×tsukushiense* have been determined to be *D. chinense*, *D. deciduum* and *D. fauriei*. These three species are rather common in western and southern Japan. Therefore, hybridisation amongst these three species is natural to occur more frequently. However, the distribution of *D. ×kanayamaense* and *D. ×tsukushiense* was very narrow in the northern part of Kyushu. We suppose mixed large populations of parents and environmental conditions supported the establishment of hybridisation in the northern part of Kyushu.

We also found that there were differences between the distribution of hybrids and parents. In the type locality of *D. ×tsukushiense*, the allelic composition of *D. fauriei* did not match *D. ×tsukushiense*. We surveyed a wide area around type localities, but eventually, we found the parental individual (allelic type, DI) of *D. fauriei* in a location 30 km away from the type locality of *D. ×tsukushiense*. The difference in the distribution of parents and hybrids suggested hybridisation can decrease or cause the extinction of populations of parents.

This study could not estimate the ploidy level of these hybrids because of the difficulty of cultivation. However, for parents of these hybrids, previous cytological studies were well studied by using enough individuals, including type locality and around areas of hybrids (Ohta and Takamiya 1999; Takamiya et al. 2000). Previous cytological studies reported ploidy levels and reproductive modes of parents as follows: *D. chinense*, diploid sexual (Mitui 1968) or tetraploid sexual (Takamiya et al. 2000); *D. deciduum*, hexaploid sexual (Ohta and Takamiya 1999; Takamiya 2006); and *D. fauriei*, tetraploid sexual or hexaploid sexual (Ohta and Takamiya 1999; Takamiya 2006). In addition, Takamiya (2006) reported *D. ×tsukushiense* (*D. chinense* × *D. fauriei*) as a tetraploid sterile. Therefore, hexaploid *D. fariei* had no relationship with *D. ×tsukushiense*. Our materials can be also tetraploid sterile because we collected samples from the same place as Takamiya (2006). We assumed that the ploidy level of *D. ×kanayamaense* can be pentaploid sterile, based on ploidy levels of *D. chinense* (tetraploid) and *D. deciduum* (hexaploid). We do not expect the existence of diploid *D. chinense* because Takamiya et al. (2000) showed

Table 2. Morphological comparison amongst *D.* ×*kanayamaense*, *D.* ×*tsukushiense* and related species.

Characteristics	Summergreen/ evergreen	Shape of blade	Serration of blade at the base	Apex of pinnules in the basal part of blades	Size of blades (L: long, W: wide)
D. chinense	summergreen	deltoid	2-pinnate pinnatifid	acute	40.0–50.0 cm (L) 30.0–40.0 cm(W)
D. deciduum	summergreen	ovate	1-pinnate pinnatifid	obtuse	30.2–38.0 cm (L) 20.5–26.0 cm(W)
D. fauriei	evergreen	lanceolate	1-pinnate	acute	20.0–30.0 cm (L) 7.0–12.0 cm(W)
D. squamigerum	summergreen	ovate	1-pinnate pinnatifid	obtuse	30.0–40.0 cm (L) 25.0–35.0 cm(W)
D. × *kanayamaense*	summergreen	ovate	1-pinnate pinnatifid	acute	15.0–26.5cm(L) 8.0–23.0 cm(W)
D. × *tsukushiense*	semi-evergreen	narrowly ovate	1-pinnate pinnatifid	acute	30.0–43.5 cm (L) 20.0–26.0 cm (W)
D. × *toriianum*	summergreen	broadly ovate or ovate	1-pinnate pinnatifid	obtuse	21.3–22.3 cm (L) 17.5–20.5 cm (W)

enough cytological data of tetraploid *D. chinense*, which were derived from the populations that were sampled across the distribution range of *D. chinense* in Japan. We show the relationships of *D.* ×*kanayamaense*, *D.* ×*tsukushiense* and its relatives in Figure 6.

The respective plant size of *D.* ×*kanayamaense* and *D.* ×*tsukushiense* shows different characteristics. *Diplazium* ×*kanayamaense* is smaller than its parents *D. chinense* and *D. deciduum*, but *D.* ×*tsukushiense* is intermediate between *D. chinense* and *D. fauriei* (Table 2). In *D.* ×*kanayamaense* and *D.* ×*tsukushiense*, roots and rhizome both seem to be too weak to survive and difficult to cultivate, especially as most individuals of *D.* ×*kanayamaense* are juvenile, which are 10 cm tall or less. Therefore, environmental stability is important to maintain individual fern hybrids. The locality of *D.* ×*kanayamaense* has remained unchanged for years, whereas the locality of *D.* ×*tsukushiense* seemed to be altered due to illegal dumping activities. Thus, we expect that the discovery of these two new hybrids can assist the conservation efforts for Japanese fern flora.

Acknowledgements

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Appendix 1

Voucher specimens for DNA analysis in this study. **Data are in the order: Species name – locality voucher (Herbarium); haplotype of plastid *trnL-F*; allele of nuclear *AK1*.**

***Diplazium* ×*kanayamaense* K.Hori & H.Kanemitsu**– JAPAN. Fukuoka Pref., Fukuoka City, Sawara-ku, Mt. Kanayama, 4 Jul 2020, *H. Kanemitsu 3746* (KAG); 1; A1A2CK. ibid., 15 Jul 2018, *H. Kanemitsu 2883*, *2884*, *2906* (KAG); 1; A1A2CK.

***D.* ×*tsukushiense* K.Hori & H.Kanemitsu**– JAPAN. Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei, 4 Jul 2020, *H. Kanemitsu 3755*, *3756*, *3757* (KAG); 1; A1A2DI.

***D.* ×*toriianum* Sa.Kurata**– JAPAN. Fukuoka Pref., Fukuoka City, Sawara-ku, Mt. Kanayama, 4 Jul 2020, *H. Kanemitsu 3750*, *3751*, *3752* (KAG); 7; CHKL1.

***D. chinense* (Baker) C.Chr.**– JAPAN. Kochi Pref., Agawa County, Niyodogawa-cho, Iwayagawa, 16 June 2018, *Hori 3023* (MBK, Hori and Murakami 2019); 1; A1A2. Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei, 4 Jul 2020, *H. Kanemitsu 3760*, *3761* (KAG); 1; A1A2.

***D. deciduum* N.Ohta & M.Takamiya**– JAPAN. Fukuoka Pref., Fukuoka City, Sawara-ku, Mt. Kanayama, 11 Jul 2020, *H. Kanemitsu 3773* (KAG); 4; CHK. ibid., *H. Kanemitsu 3774* (KAG); 4; CK. ibid., *H. Kanemitsu 3775* (KAG); 4; CHK. ibid., 29 Aug 2020, *H. Kanemitsu 3892*, *3893* (KAG); 4; CHK. ibid., 6 Sep 2020, *H. Kanemitsu 3905* (KAG); 4; CHK. ibid., Mt. Nishiyama, 13 Sep 2020, *H. Kanemitsu 3914* (KAG); 4; CK. ibid., Saga Pref., Saga City, Fuji-cho, 22 Sep 2020, *H. Kanemitsu 3951* (KAG); 4; CK.

***D. fauriei* Christ**– JAPAN. Fukuoka Pref., Nakagawa City, Ooaza-Gokayama, Tsukushiyabakei, 4 Jul 2020, *H. Kanemitsu 3758*, *3759*, *3992* (KAG); 5; DJ. ibid., Iizuka City, Naijukyo, 22 Aug 2020, *H. Kanemitsu 3881* (KAG); 5; DI.

***D. squamigerum* (Mett.) Matsum.**– JAPAN. Fukuoka Pref., Fukuoka City, Sawara-ku, Mt. Kanayama, 4 Jul 2020, *H. Kanemitsu 3989*, *3990* (KAG); 6; L2. ibid., *H. Kanemitsu 3991* (KAG); 7; L2.

***D. mettenianum* (Miq.) C.Chr.**– JAPAN. Mie pref., Minamimuro gun, Kiho-cho, Takaoka, 6 Jul 2016, *Hori 2336* (MAK, Hori and Murakami 2019); 4; B. Fukuoka Pref., Fukuoka City, Sawara-ku, Mt. Kanayama, 4 Jul 2020, *H. Kanemitsu 3753*, *3754* (KAG); 4; B.

D. hayatamae **N.Ohta & M.Takamiya**– JAPAN. Kagoshima Pref., Kumage gun, Yakushima-cho, Kusugawa, 23 Jan 2019, *Hori 3159* (MBK); 2; BF.

D. griffithii **T.Moore**– JAPAN. Kagoshima Pref., Kumage gun, Yakushima-cho, Kusugawa, 23 Jan 2019, *Hori 3160* (MBK); 3; EG.

D. amamianum **Tagawa**– JAPAN. Kagoshima Pref., Amami City, Naze, Honchyatouge, 250 m alt., 7 May 2017, *K. Hatake 615* (MBK, Hori and Murakami 2019).

D. donianum **(Mett.) Tardieu var.** ***donianum***– JAPAN. Kagoshima Pref., Amami City, Sumiyou-machi, Nishinakama, *K. Hori 3228* (MBK).

D. esculentum **(Retz.) Sw.**– JAPAN. Kagoshima Pref., Isa City, Oguchisogi, *H1109* (MBK, Hori and Murakami 2019).

D. nipponicum **Tagawa**– JAPAN. Mie Pref., Minamimuro County, Kiho-cho, 70 m alt., 135°59'29.5", 33°45'55.2", 6 July 2016, *K. Hori 2339* (MAK, Hori and Murakami 2019).

D. takii **Sa.Kurata**– JAPAN. Fukuoka Pref., Kasuya County, Hisayama-machi, 140 m alt., 130°32'2.27", 33°40'44.18", 26 May 2018, *K. Hori 2924* (MBK, Hori and Murakami 2019).

D. wichurae **(Mett.) Diels** – JAPAN. Kanagawa Pref., Zushi City, Jinnmuji, 60 m alt., 139°36'18.19", 35°18'14.71", 14 Apr 2015, *K. Hori 1763* (MAK, Hori and Murakami 2019).

Deparia viridifrons **(Makino) M.Kato**– JAPAN. Kochi Pref., Takaoka County, Ochi Town, Mt. Yokogura, 30 May 2018, *K. Hori 2971* (MBK, Hori and Murakami 2019).

De. unifurcata **(Baker) M.Kato–** JAPAN. Kochi Pref., Agawa County, Niyodogawa-cho, Iwayagawa, 16 June 2018, *K. Hori 3021* (MBK, Hori and Murakami 2019).

De. japonica **(Thunb.) M.Kato**– JAPAN. Kyoto Pref., Sakyo-ku, Kibune, 300 m alt., 135°45'50.79", 35°7'30.85", July 14 2018, *K. Hori 3031* (MBK, Hori and Murakami 2019).

De. lancea **(Thunb.) Fraser-Jenk.**– JAPAN. Kochi Pref., Takaoka County, Ochi Town, Mt. Yokogura, 25 June 2020, *K. Hori 3378* (MAK).

Appendix 2

DNA data accession numbers of the obtained nucleotide sequences used for the construction of molecular phylogenetic trees in this study.

< *trnL-F* >

1, LC468193; 2, LC592258; 3, LC592259; 4, LC592260; 5, LC592261; 6, LC592262; 7, LC592263; *Diplazium donianum*, LC592264; *Deparia lancea*, LC592265.

<*AK1*>

A1, LC468179; A2, LC468182; B, LC468178; C, LC592244; D, LC592245; E, LC592246; F, LC592247; G, LC592248; H, LC592249; I, LC592250; J, LC592251; K, LC592252; L1, LC592253; L2, LC592254; *Diplazium donianum*, LC592255, LC592256; *Deparia lancea*, LC592257.

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Flueggea acicularis (Phyllanthaceae), a narrow endemic species rediscovered in central China

Songzhi Xu[1], Qiliang Gan[2], Lianzhong Fu[3], Mingxi Jiang[4], Zhenyu Li[3]

1 *School of Life Science, Nantong University, 226019, Nantong, China* **2** *Zhuxi Qiliang Biological Institute, Zhuxi 442300, Hubei, China* **3** *State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 100093, Beijing, China* **4** *Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Institute of Botany, Chinese Academy of Science, Wuhan 430074, China*

Corresponding author: Zhenyu Li (lizy@ibcas.ac.cn)

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Abstract

Flueggea acicularis (Phyllanthaceae) is endemic to the karst region of central China. Male specimens of this species were first collected in 1908. In 1989, female plants of *F. acicularis* were found for the first time, but misidentified as a new species. Throughout this period the male plants of *F. acicularis* were mismatched with female plants of other species, and male plants had not been collected since 1908. Then, in March, 2009, the authors rediscovered a wild population of *F. acicularis* consisting of both male and female plants in Wuxi county, Chongqing municipality, China. Based on field investigation and examination of specimens, we matched the correct female and male plants of this species for the first time since its initial publication a century ago. A complete and accurate morphological description, distribution, habitat and phenology of this species are also provided. Furthermore, the conservation status of *F. acicularis* is assessed as "Near Threatened" (NT) according to the IUCN Red List criteria.

Keywords

Flueggea acicularis, morphology, rediscovery, taxonomy, Three Gorges Area

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Introduction

Flueggea (Phyllanthaceae) consists of 16 species, widespread in tropical to warm temperate regions, along with 3 Eurasian narrowly distributed taxa that have been interpreted as relictual. An unusually obligate pollination mutualism (pollination and seed parasite) exists between Epicephala moth and Phyllanthaceae trees (Kawakita 2010, Hu and Li 2011). Hu et al. (2011) also reported that *Epicephala relictella* fed on the seeds of *F. suffruticosa*, but was not pollinating its host. Some secondary products (alkaloids, diterpenoids) that are of medicinal use have been extracted from *Flueggea* (Hu et al. 2011; Wang et al. 2010).There are four *Flueggea* species in China, and *Flueggea acicularis* is endemic to the karst region of central China. *F. acicularis* is extremely similar to *F. tinctoria* in morphology (Webster 1984). However, the geographical disjunction between the Chinese *F. acicularis* and *F. tinctoria* in the Iberian Peninsula is one of the most remarkable in the Euphorbiaceae, which might represent the relicts of the flora of Tethys in Tertiary period. But the taxonomic status of *F. acicularis* is somewhat in doubt.

The English botanist E.H. Wilson collected a male shrub assigned to Euphorbiaceae s.l. in the Three Gorges Area of central China in March, 1908. Hutchinson (1916) misidentified these specimens as *Flueggea leucopyra* Willd., a species producing berries. Croizat (1940) published a new species *Securinega acicularis* Croizat based on the three specimens of Wilson. Airy Shaw (1971) accepted this new species and described the morphology of fruits as "a shallowly 3-lobed, depressed-globose, dehiscent capsule, 6–7mm in diam.", but no specimens documenting the fruit were cited. Webster (1984) used a broad generic concept in the taxonomic revision of *Flueggea* and combined *Securinega acicularis* Croizat into *Flueggea acicularis* (Croizat) Webster, but indicated that pistillate flowers and the fruit were not seen. Li (1994) and Li and Gilbert (2008) described this species with "Female flowers: pedicels ca. 3mm; sepals 5,……Berry globose, 6–7 mm in diam, 3-locular". Thus, up to this point, the taxonomic status of *F. acicularis* and the morphological description of the staminate plant have not been doubted in academia, but the morphological characters of pistillate flowers and capsules have been confused throughout this period.

After a careful examination of the specimens stored under the name of *Flueggea acicularis* in main herbaria of China, we found that 25 sheets representing ten gatherings collected from the Jinshajiang valley flanking northwestern Yunnan province and southwestern Sichuan province had been misidentified. The whole plants of these specimens are entirely glabrous and bear flowers with 5 sepals, clearly differing from *F. acicularis* which has hirtellous young branchlets and has 6 sepals in a flower. These misidentified specimens belong to *Flueggea virosa* (Roxb. ex Willd.) Voigt, *F. suffruticosa* (Pall.) Baill. and *F. leucopyra* Willd. Besides, a most recent study of Huang et al. (2020) claimed that diterpenoids have been extracted from the aerial parts of *F. acicularis* collected in Yunnan. Given that the locality of collection is in Yunnan, it seems that the materials used in their study should be *F. leucopyra*, instead of *F. acicularis*.

In August, 1989, three botanists (Mingxi Jiang, Zongqiang Xie and Jinsheng He) collected specimens of the female plants of *F. acicularis* for the first time, in Luyou-

dong, Wushan county, during the vegetation investigation in the Three Gorges Area. But the specimens were not identified, because of their lack of flowers and fruits. In May, 1990, Zongqiang Xie et al. collected specimens of *F. acicularis* with capsules in Bawuxia located in the midstream of Daning river. However, since most floras in China still confused *F. acicularis* with *F. leucopyra* (Fu, 1979), or considered *F. acicularis* to have berries (Li 1994), the real female specimens of *F. acicularis* with capsules were misidentified as a new species under a nomen nudum "*Securinega wuxiensis*" (Chen et al. 1994). Thus, the male plants of *F. acicularis* remained paired with female plants of other species, and the true female plants of *F. acicularis* were considered as another species.

In April, 2009, during a field trip to Wuxi county, Chongqing municipality, the authors accidentally discovered a population consisting of shrubs of Phyllanthaceae in a remote area called Jingzhuba. The place is located in the limestone canyon of the headwaters of the Daning river in the northeast of Wuxi county. We collected specimens of both male and female plants containing staminate and pistillate flowers and capsules from the same population. After a careful morphological investigation, we confirmed that this species is *F. acicularis*. Based on an intensive study of the male and female plants from the same population, the male plant of *F. acicularis* has finally been matched with the correct female plant of the same species.

Materials

Specimens were collected and photographs were taken in the field of Badong county, Hubei, Wushan county, and Wuxi county, Chongqing municipality, Central China in 1989, 1990, 1997 and 2009. Specimens from the main herbaria of China (PE, KUN, IBK, IBSC) and some digital specimen databases (CVH, A, MO, US, K, GH, JSTOR Global Plants) were checked. The morphology of the species was observed and measured based on living plants, dry specimens and preserved materials. All morphological characters were measured with dissecting microscopes and were described using the terminology presented in Harris and Harris (1994).

Taxonomy

***Flueggea acicularis* (Croizat) Webster in Allertonia 3(4): 304. 1984.**

Securinega acicularis Croizat in Journ. Arn. Arb. 21(4):491. 1940.
Flueggea leucopyra auct. *non* Willd.: Hutch. in Sargent, Pl. Wilson. 2:520. 1916.

Types. China: Hubei (Hupeh) province, Badong (Patung Hsien) county, bush 2–6 feet, cliffs and rocky places, alt. 30–304 m, 24 March 1908, E. H. Wilson 3336 (**holotype**, A, A00048778; **isotypes**, A, GH, K, MO, US); l.c.3335 (paratypes, A, US, K);

Figure 1. *Flueggea acicularis* (Croizat) Webster. (fruiting specimen, Z. Y. Li & Q. L. Gan 11755, PE).

Chongqing municipality (former eastern Sichuan), Wushan county, Wu Gorge (Wu Xia or Wushan Gorge), bush 3–4 feet, March 1908, E. H. Wilson 3344 (paratypes, A, K). (All male specimens, photos, PE!).

Additional specimens. China: Chongqing municipality, Wuxi county, Jingzhuba, 2009-04-28, Zhen Yu Li & Qi Liang Gan 11751 (female plant, 2 sheets, PE!); 1.c.11755 (female plant, 2 sheets, PE!) (Fig. 1); 1.c. 11758 (male plant, 2 sheets, PE!); and 11759 (male plant, 2 sheets, PE!).

Description. Shrubs, 1–2.5 m high, deciduous. Stems many branched, branches alternate, diverging into an obtuse angle and arranged in two rows, usually with lateral and terminal spines; branchlets of current year's growth copiously hirtellous, angulate, yellow-brown, gray-brown, glabrate and nitid when old. Leaves alternate, distichous, or 1–3 fascicled on upper part of a branchlet; stipules reddish-brown, lanceolate, 0.7–2 mm long, margins ciliate, apex caudate-acuminate, persistent; petioles 1–5 mm long, copiously hirtellous when young, later glabrate; leaf blade oblong-obovate to obovate, 0.5–2 cm long, 0.4–1.5 cm wide, thickly papery in texture, glabrous except young midrib base, base cuneate, margin entire, flat or narrowly revolute when dry, apex retuse, obtuse or rounded, mucronulate, adaxially green, slightly lustrous, abaxially pale green, midrib extending to apex, lateral veins 4–6 on each side of midrib, alternate, rarely opposite, connected near margin, midrib and lateral veins slightly raised on both surfaces, veinlets reticulate. Plants dioecious. Male inflorescence with solitary staminate flower, axillary; bracts inconspicuous; pedicel 2–5(–7) mm long; sepals 6, ovate-oblong or elliptic, 1.2–1.6 mm long, 0.4–1.3 mm wide, apex rounded, margin erose or fimbriate, membranous, imbricate, slightly recurved, disk glands 6, angled, coherent, 0.4–0.5 mm across; petals absent; stamens 6, free, filaments green-yellow, 1.5–2mm long, anthers yellow, 0.6–1 mm long, theca parallel, longitudinally dehiscent, anther connectives not protruding; pistillode with 3(rarely 2) branches fused to the middle or beyond, the fused portion 0.8–1.5 mm high, the sharply recurved tips 0.4–1mm long. Female inflorescence 1-rarely 2-flowered, arising in leaf axil; bracts inconspicuous. Pistillate flower: sepals 6, ovate-oblong or oblong, 1.5–1.8 mm long, 0.6–0.7 mm wide, apex acute, margin membranous, fimbriate; persistent and reflexed after anthesis; pedicels 5–8 (–10) mm long that elongate to 16 mm in fruit, pendulous; disk angular, 0.8–1.3 mm across; petals absent; ovary globose, 3-locules, each with 2 ovules; styles 3, patent, flattened, each 2-lobed to middle, stigmas rounded at apex, styles and stigmas yellow-green. Capsules oblate, somewhat triangular in cross section, glabrous, ca. 5 mm in diam., green, brown when dry with persistent and nigrescent styles and stigmas, dehiscing through the locules when mature, valves and seeds falling off after dehiscing, columella persistent, 2–2.2 mm long. Seeds ovoid-trigonous, brown, 2.3–2.8 mm long, 1.6–1.7 mm broad at back, ca. 1.5 mm thick, glabrous, with raised pigmented minute rectangular testa cells (Figs 2, 3).

Distribution. China: Hubei province (Badong county); Chongqing municipality (Wushan county and Wuxi county), alt. (formerly 30–300 m) 175–300 m.

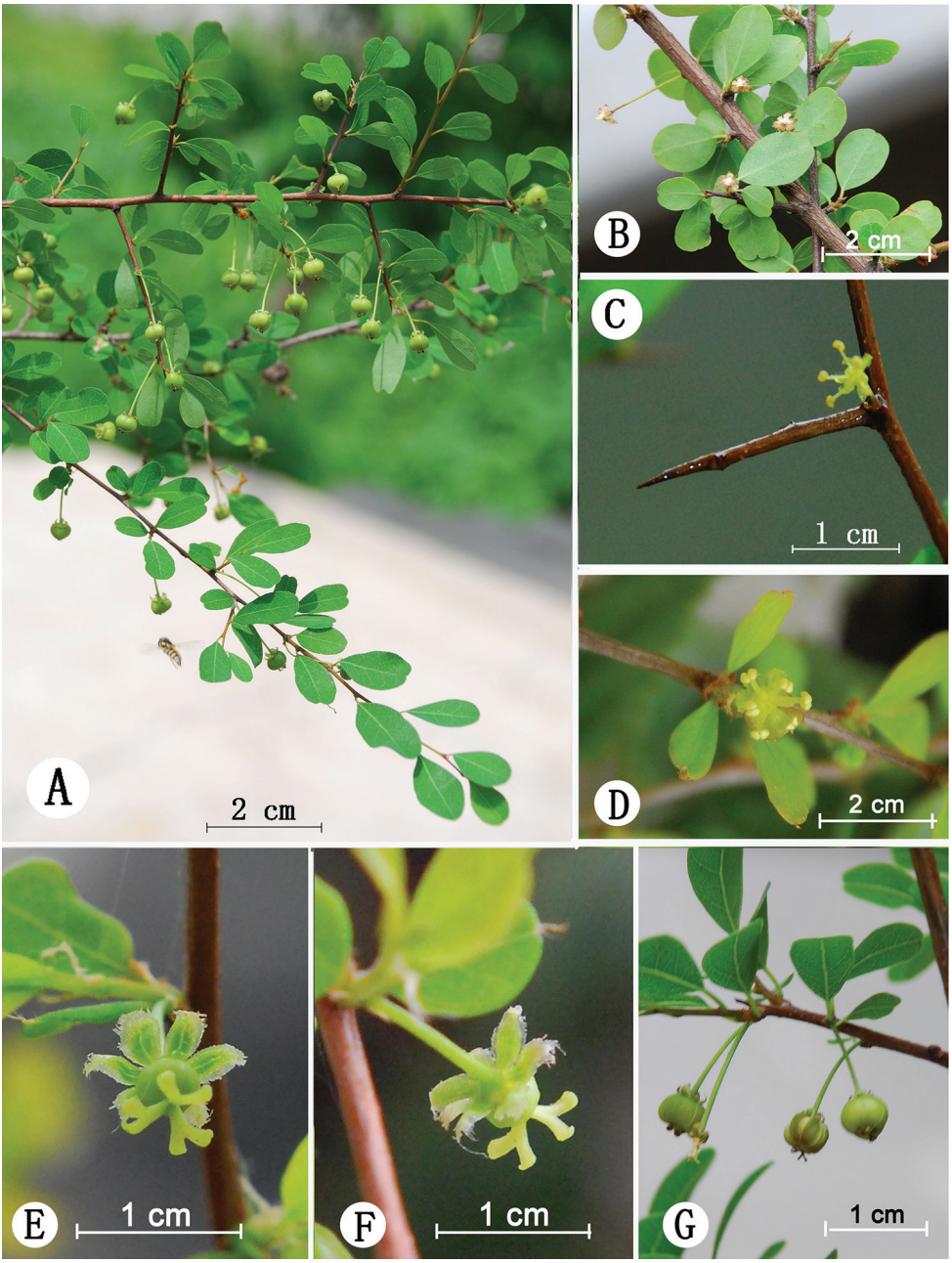

Figure 2. Living plants of *Flueggea acicularis* (Croizat) Webster **A** fruiting branches **B** fruit remnants after dehiscing **C** branches and spines **D** staminate flower **E, F** pistillate flower **G** capsules (note epicarp dehiscence lines in center fruit).

Phenology. Flowering and fruiting from April to May.

Chinese name. Mao bai fan shu (hairy flueggea) (Li 1994), refers to the copiously hirtellous.

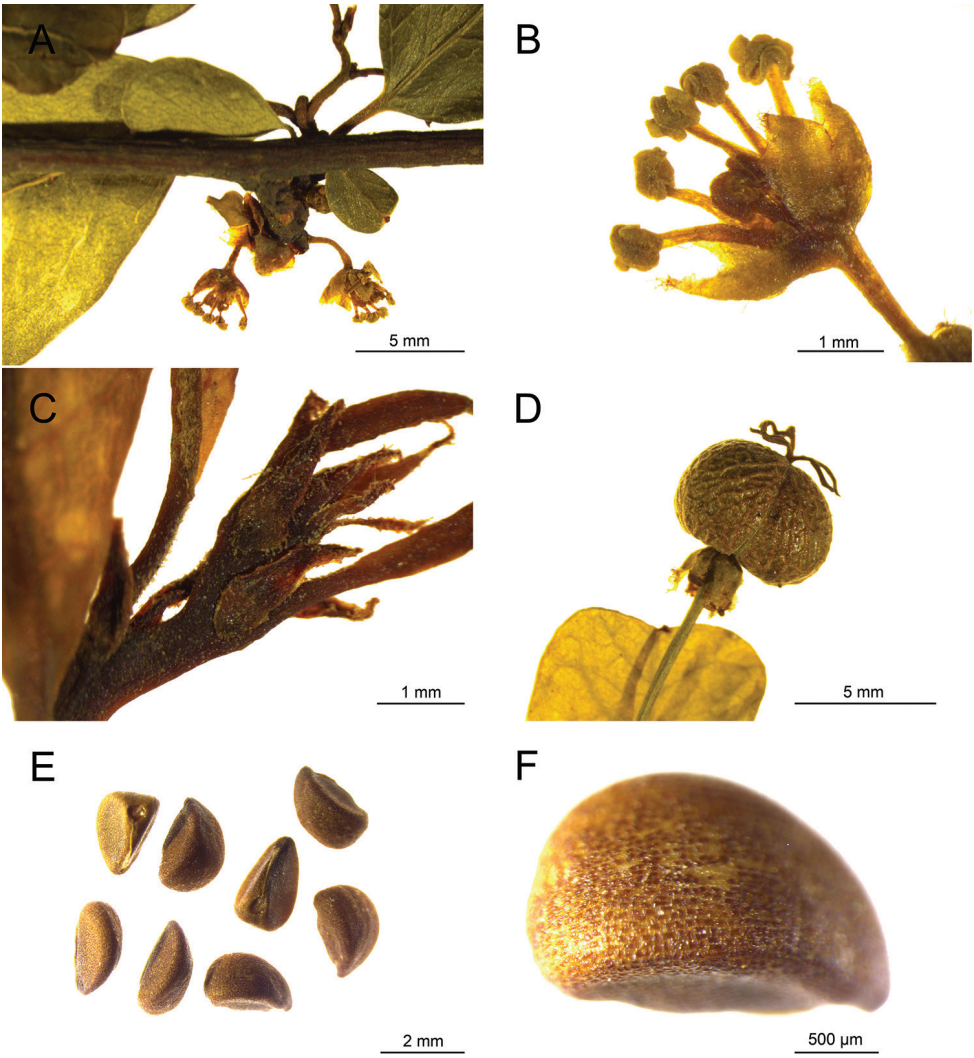

Figure 3. Herbarium collections of *Flueggea acicularis* (Croizat) Webster **A** staminate inflorescence **B** staminate flower **C** young leaves and stipules **D** capsule **E** seeds **F** seed (back view) (**A, B** Z. Y. Li & Q. L. Gan 11758, PE; **C, D, F** Z. Y. Li & Q. L. Gan 11751, PE).

Local name. Yang ci.

Habitat and ecology. Three Gorges reservoir area is located in a subtropical region. The annual average rainfall is 1000–1400 mm, mostly in July and August. The annual average temperature is 18.4 °C (average temperature in January: 7.1 °C; average temperature in July: 29.3 °C), and the extreme maximum temperature is up to 44 °C. The relative humidity is 60%–80%. The photoperiod in this area is short, affected by the canyon landform and the foggy environment.

The population of Badong. In March, 1908, the type specimen was collected from Badong county, in cliffs and rocky places in the bank of Yangtze river at altitudes from

30 to 300 m, without detailed location. In May, 1997, Mingxi Jiang found ca. 2,000 individuals of *F. acicularis* in Mazongshan village (31°2'36"N, 110°9'59"E), Badong county. It grows on a slope located in the bank of Yangtze River, at alt. 100–300 m. The main companion species include shrubs: *Maytenus variabilis* (Hemsl.) C. Y. Cheng, *Viburnum utile* Hemsl., *Vitex regundo* var. *heterophylla* (Franch.) Rehd.; woody liana: *Bauhinia brachycarpa* Wall. ex Benth.

The population of Wushan county. In 1908, Wilson collected specimens of *F. acicularis* here, but without detailed location. In August, 1989, Mingxi Jiang et al. found more than 10,000 individuals of *F. acicularis* in Luyoudong (30°36'24"N, 108°25'30"E), Wushan county. It grows on limestone on the banks of the Yangtze River, at alt. 100–300 m. The main companion species were similar to Mazongshan population. In May, 1990, Zongqiang Xie and Mingxi Jiang et al. found another monodominant community of *F. acicularis* in Bawuxia in the midstream of Daning river, Wushan county, at alt. 50–250m. The main companion species include shrubs: *Euonymus alatus* (Thunb.) Sieb., *Lespedeza formosa* (Vog.) Koehne, *Sageretia thea* (Osbeck) Johnst., *Vitex regundo* var. *heterophylla* (Franch.) Rehd., *Zanthoxylum armatum* DC. etc; herbs: *Arthraxon lanceolatus* (Roxb.) Hochst., *Phyllanthus urinaria* L.; woody liana: *Clematis armandii* Franch., *Millettia reticulata* Benth.; herbaceous vines: *Cayratia japonica* Gagnep. and *Dioscorea oppositifolia* L. (Xie and Jiang 1995).

The population of Wuxi county. This population was located in Jingzhuba, off headwaters of the Daning river, Dahe township, Wuxi county (31°21'40"N, 109°24'34"E). It grows in the cracks of limestone on south slopes, at elevations of ca. 250–350 m. The rock surface is mostly exposed and the soil layer is extremely thin. The vegetation consists of open shrubland with heights lower than 5 m and the main companion species include shrubs: *Boehmeria clidemioides* var. *diffusa* (Wedd.) Hand.-Mazz., *Broussonetia kazincki* Siebold, *Buddleja davidii* Franch., *Cotinus coggyria* var. *pubescens* Engl., *Debregeasia orientalis* C.J. Chen, *Flueggea acicularis* (Croizat) Webster, *Itea illcifolia* Oliv., *Lespedeza floribunda* Bunge and *L. formosa* (Vog.) Koehne; woody lianas: *Ampelopsis aconitifolia* Bunge, *Holboellia fargesii* Reaub., *Parthenocissus dalzielii* Gagnep.; herbs: *Artemisia annua* L., *A. sylvatica* Maxim., *Arthraxon lanceolatus* (Roxb.) Hochst, *Boea hygrometrica* (Bunge) R. Br., *Bothriospermum zeylanicum* (J. Jacq.) Druce, *Carex brevicuspis* C.B. Clarke, *Corydalis ophiocarpa* Hook. f. et Thoms., *Duchesnea indica* (And.) Focke, *Eriophorum comosum* Nees, *Hemistepta lyrata* (Bunge) Bunge, *Miscanthus sinensis* Anderss., *Oxalis corniculata* L., *Setaria viridis* (L.) Beauv., *Stellaria media* (L.) Cyr., *Toridis japonica* (Houtt.) DC., *Youngia heterophylla* (Hemsl.) Babc. et Stebb.; pteridophytes: *Adianthum capillus-veneris* L., *Hypodematium crenatum* (Forssk.) Kuhn, *Pteris vittata* L. and *Selaginella davidii* Franch.; mosses: *Conocephalum conicum* (L.) Dumort; herbaceous vines: *Cayratia japonica* var. *pseudotrifoliata* (W.T. Wang) C.L. Li and *Paederia foetida* L.. There are about 80 individuals including ca. 20 seedlings, and pistillate plants are in the majority. Since the population is close to the road, the alien weeds *Conyza canadensis* (L.) Cronq., *Bidens pilosa* var. *radiate* Sch.-Bip. and *Veronica persica* Poir. have invaded the edge of the population.

Provisional IUCN conservation assessment. In 2009, the Three Gorges water conservancy project, the largest in the world, was completed, located in Yichang city, Hubei province. The altitudes of the dam base and dam top are 4 m and 185 m respectively, and therefore we calculated the populations of Badong and Wushan of *F. acicularis* at alt. lower than 185m have been submerged according to altitude. Based on field investigations and specimens, we are confident that *F. acicularis* is distributed narrowly, and is endemic to the karst region of the Three Gorges Area in Central China. The provisional conservation status is "Near Threatened" (NT) according to the IUCN red list criteria (IUCN 2019).

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Taxonomic note of *Parnassia* (Celastraceae) in China II: population surveys reveal that *P. guilinensis* is conspecific to *P. xinganensis*

Xiao-Song Dai[1,2], Dao-Zhang Min[3], Bo Yang[1], Ding Wu[1], Bo Li[3]

1 *Jiangxi Key Laboratory of Plant Resources and Biodiversity, Jingdezhen University, Jingdezhen 333000, China* **2** *School of Life Sciences, Nanchang University, Nanchang 330031, China* **3** *Research Centre of Ecological Sciences, College of Agronomy, Jiangxi Agricultural University, Nanchang 330045, China*

Corresponding author: Ding Wu (parnassia@126.com); Bo Li (hanbolijx@163.com)

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Abstract

Based on investigation of populations of *Parnassia guilinensis* and *P. xinganensis*, examination of herbarium specimens (including types), as well as consultation of protologues and distributions, *P. guilinensis* is hereby reduced to a synonym of *P. xinganensis*. *P. xinganensis* is endemic to northeastern Guangxi Province of China and characterized by having elliptic to ovate leaves and staminodes 3–5-branched with globose glands. Field photographs and an updated morphological description of *P. xinganensis* are provided.

Keywords

Endemic species, morphology, staminode, synonymy, taxonomy

Introduction

Parnassia L. consists of small, glabrous and rosulate perennial herbs, with several morphologically distinguishable traits, including a solitary, terminal, bisexual and pentamerous flower borne on an unbranched scape; five showy staminodes; and one-by-one stamen movement (Ku 1987; Ku and Hultgård 2001; Sandvik and Totland 2003; Ren 2010; Armbruster et al. 2014; Ren and Bu 2014). Species of *Parnassia* predominantly

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occur in arctic and alpine regions of Europe, Asia, and North America with a center of diversification in mountainous areas in Pan-Himalaya and southwest China (Phillips 1982; Ku 1987; Ku and Hultgård 2001; Wu et al. 2003; Simmons 2004; Wu 2005). According to the most updated checklist, *Parnassia* contains 61 species, 2 subspecies, 11 varieties and 1 form (Shu et al. 2017). However, results from several taxonomic investigations after Shu et al. (2017) have changed the number of species in the genus. These changes include: *P. lanceolata* var. *oblongipetala* T.C. Ku was reduced to a synonym of *P. yunnanensis* Franch. (Shu and Zhang 2017), *P. venusta* Z.P. Jien, *P. degeensis* T.C. Ku and *P. kangdingensis* T.C. Ku were reduced to synonyms of *P. cacuminum* Hand.-Mazz. (Shu and Zhang 2017), *P. chengkouensis* T.C. Ku and *P. dilatata* Hand.-Mazz. were reduced to synonyms of *P. wightiana* Wall. ex Wight et Arn. (Wang et al. 2018), *P. brevistyla* (Brieg.) Hand.-Mazz. and *P. leptophylla* Hand.-Mazz. were reduced to synonyms of *P. delavayi* Franch. (Yu et al. 2018), *P. tibetana* Z.P. Jien ex T.C. Ku, *P. nubicola* subsp. *occidentalis* Schönbeck-Temesy and *P. nubicola* var. *nana* T.C. Ku were reduced to synonyms of *P. nubicola* Wall. ex Royle (Ma et al. 2020). Meanwhile, some new species were described, including *P. zhengyuana* M.X. Ren et J. Zhang and *P. simianshanensis* M.X. Ren, J. Zhang et Z.Y. Liu (Zhang et al. 2019).

In the taxonomy of *Parnassia*, the shape of basal leaves, the characteristics of petals (entire or flat, divided into lobes or filiform rays, respectively), and the shape of staminodes (i.e., number and depth of staminode branches, shape of staminode lobes, with globose glands at apex or not) were considered to be of great significance in species delimitation (Ku 1987; Ku and Hultgård 2001; Wu 2005). *P. guilinensis* G.Z. Li et S.C. Tang (Tang and Li 1999) and *P. xinganensis* C.Z. Gao et G.Z. Li (Gao and Li 1983) were described based on specimens collected from the same mountain: Mao'er Mountain, Xing'an County, Guilin City, Guangxi Province, China. The type specimen of *P. xinganensis* (11 December 1978, *G.Z.Li 62923*) was collected at an altitude of 1200 m on the mountain while that of *P. guilinensis* (23 August 1998, *G.Z.Li and S.C.Tang M93*) was collected at 580 m. Tang and Li (1999) noted that *P. guilinensis* was morphologically similar to *P. xinganensis* but could be distinguished from the latter by the staminodes being 5-lobed and the apex of lobes with globose glands, whereas the staminodes of *P. xinganensis* are 3- or rarely 4-lobed without globose glands at the lobe apex. In both *Flora Reipublicae Popularis Sinicae* (Ku 1995) and *Flora of China* (Ku and Hultgård 2001), the staminodes of *P. xinganensis* were described as eglandular. However, when examining the type specimen of *P. xinganensis* (Fig. 1), we found that the apex of the staminode lobes obviously bear globose glands and the number of lobes range from three to five, strongly indicating that *P. xinganensis* and *P. guilinensis* are the same species. In order to clarify the relationship of the two sympatric taxa, we visited the Mao'er Mountain in September and October of 2020 and conducted population surveys of *Parnassia* species from low to high altitudes. Population observations clearly revealed that there are no differences among individuals at 580 m and 1200 m (Fig. 2), confirming that the later described species *P. guilinensis* is conspecific with the former one, *P. xinganensis*. We thus reduce *P. guilinensis* to a synonym of *P. xinganensis*, and provide an updated description of *P. xinganensis* based on population observations.

Materials and methods

Type specimens of *P. guilinensis* and *P. xinganensis* deposited at IBK, as well as other *Parnassia* specimens collected from Mao'er Mountain, Xing'an County, Guangxi Province, China, preserved in GXMG, IBK, IBSC, KUN and PE (acronyms according to Thiers 2020+) were carefully examined under a stereo dissecting microscope (Stereo Zoom Leica S8 APO, Leica Microsystems 2017). Morphological traits were measured using a ruler and a micrometer based on both herbaria and fresh materials. Field investigations were carried out during September to October of 2020 in Mao'er Mountain and some individuals of *P. xinganensis* collected from different altitudes were transplanted to the greenhouse at Jingdezhen University for further observation and to be photographed. Voucher specimens were deposited at the herbarium of Jingdezhen University.

Taxonomic treatment

***Parnassia xinganensis* C.Z. Gao & G.Z. Li, 1983: 19.**
Figs 1, 2

Type. China. Guangxi: Guilin City, Xing'an County, Mao'er Mountain, streamsides in valleys, alt. 1200 m, 11 December 1978, *G.Z.Li 62923* (holotype: IBK00185227!; isotype: IBK00200466!).

Figure 1. Type specimens of *Parnassia xinganensis* (**A**) and *P. guilinensis* (**B**).

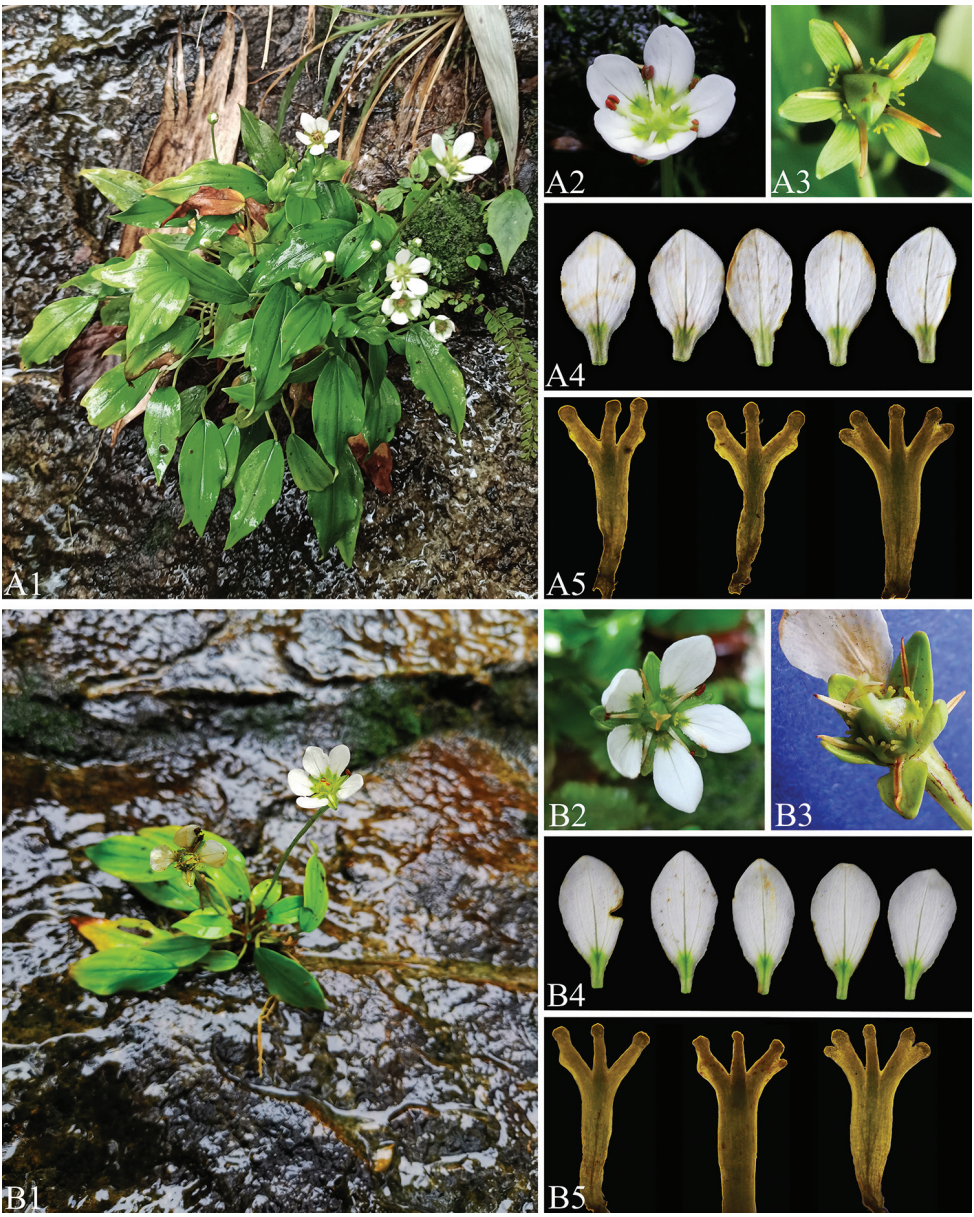

Figure 2. Morphological comparisons between *Parnassia xinganensis* (**A1–A5**) and *P. guilinensis* (**B1–B5**) collected from their type localities of Mao'er Mountain at altitudes of 1200 m and 580 m, respectively **A1, B1** habitat **A2, B2** flower **A3, B3** calyces, staminodes and ovary **A4, B4** petals **A5, B5** variation of staminodes.

=*Parnassia guilinensis* G.Z. Li & S.C. Tang, syn. nov. Type: China. Guangxi: Guilin City, Xing'an County, Mao'er Mountain, streamsides, alt. 580 m, 23 August 1998, *G.Z.Li & S.C.Tang M93* (holotype: IBK00200636!).

Description. Perennial herbs, glabrous. Rhizome sympodial, robust. Stems 1–8, 5–20 cm tall, usually with 1 cauline leaf near middle. Basal leaves (4–) 8–13 (–22); petiole (1–) 4.5–6.5 (–9.5) cm long; leaf blade elliptic, obovate-elliptic, oblong-ovate to ovate, abaxially gray-white, adaxially green, (1–) 3–5.5 (–7.5) × (0.8–) 1.5–2.5(–3) cm, inconspicuously 5–7-veined on both surfaces, midvein prominent, base rounded, subtruncate to cuneate, apex obtuse to acute. Cauline leaf sessile, amplexicaul, ovate or ovate-triangular, 0.9–2.4 × 0.5–1.6 cm. Flowers 1.5–2.3 cm in diam.; hypanthium shortly campanulate or inconspicuous. Sepals green, elliptic to ovate, 3.7–5.2 × 2.3–3.7 mm, 5-veined, margin entire, apex obtuse. Petals spreading, white, elliptic to broadly obovate, 8.5–12 × 6.8–8.3 mm, 5-veined basally, base attenuate into a claw, 2–3 mm long, margin entire or slightly undulate, apex rounded, obtuse or emarginate. Anthers ellipsoid; filaments 2–7 mm long; staminodes flat, 3–3.5 mm long, 3–5-branched to middle, branchedes globose glandular at apex. Ovary superior, greenish, ovoid, slightly sunken into hypanthium; styles short, ca. 1–1.5 mm long; stigma 3-lobed, lobes oblong, spreading. Capsule ovoid, trigonous, 5–10 mm long, 3-valved. Seeds minute, oblong, ca. 1 mm long.

Phenology. Flowering – late June to November; fruiting – August to December.

Distribution and habitat. The species is endemic to northeastern Guangxi Province (recorded only in Xing'an County and Ziyuan County), China, and grows in clefts of the moist rocks along streams or under waterfalls, at an elevation of 400–1350 m.

Additional specimens examined. China. Guangxi: Guilin City, Xing'an County, Mao'er Mountain, streamsides, moist rocks in valleys, alt. 580 m, 23 August 1998, *G.Z.Li & S.C.Tang M211* (IBK00200637!); under forests, alt. 611 m, 29 September 2014, *Xing'an Expedition 450325140929020LY* (GXMG0110865!); Ziyuan County, Mao'er Mountain, on rocks under forests, near streams, 6 December 1980, *G.Z.Li 10120* (IBK!).

Conservation status. At present, *P. xinganensis* has been reported only from two counties in northeastern Guangxi Province of China. Based on our field investigations, there are numerous mature individuals and young seedlings which could be easily discovered along streams and under waterfalls, indicating the population survives and regenerates well. Additionally, the Mao'er Mountain has been projected to a national nature reserve of China in 2003, and ranks among one of the earliest national nature reserves founded in Guangxi Province. It is apparent that the species will not be severely affected by human activities, thus we propose to list *P. xinganensis* as Least Concern (LC) according to the IUCN Red List categories and criteria (IUCN 2017).

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Taxonomic revision of the *Asplenium wrightii* complex (Aspleniaceae) with reinstatement of *A. alatulum* and *A. subcrenatum*

Ke-Wang Xu[1], Lu-Lu Wang[1], Li-Bing Zhang[2]

1 *Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Nanjing Forestry University, Nanjing; 510275, China* **2** *Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166-0299, USA*

Corresponding author: Ke-Wang Xu (xukw10@njfu.edu.cn); Li-Bing Zhang (Libing.Zhang@mobot.org)

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Abstract

The *Asplenium wrightii* complex is morphologically variable and difficult in species delimitation. Owing to lack of comprehensive sampling in phylogenetic studies, the taxonomy of this complex remains unresolved. Based on extensive field observations, specimen examination and our recent molecular data, the present study aims to clarify the identities of three species of *Asplenium* in this complex from Asia. Our study revealed that *A. alatulum* and *A. subcrenatum*, previously treated as synonyms of *A. wrightii*, should be reinstated. A taxonomic revision of the three species, including their type information, detailed descriptions, voucher specimens, distribution, ecology, as well as taxonomic notes, is carried out.

Keywords

species delimitation, spore morphology, stomata

Introduction

Asplenium L. (Aspleniaceae) comprises more than 700 species of epilithic, epiphytic or terrestrial herbs, distributed throughout temperate and tropical regions of the world (Kramer and Viane 1990). A number of systematists worked on the taxonomy of the

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genus, based on morphological characteristics (Presl 1836; Hooker and Baker 1874; Christ 1897; Hayata 1927; Copeland 1947; Holttum 1947; Nayar 1970; Iwatsuki 1975; Mickel 1976; Pichi Sermolli 1977; Tryon and Tryon 1982; Bir et al. 1985; Wu 1989; Kramer and Viane 1990; Lin and Viane 2013). In the late 1980s, Wu (1989) published a comprehensive treatment of the genus in China in which four sections and five series were recognised. *Asplenium* ser. *Wrightiana* Ching & S.H.Wu is one of the five series in *A.* sect. *Asplenium*. Members of this series have 1-pinnate laminae, often falcate pinnae and serrate pinna margins. The wide range of morphological variation in *A.* ser. *Wrightiana* in China has been the basis of different taxonomic treatments. Wu (1999) included 14 species in *A.* ser. *Wrightiana* from China (Table 1), while Lin and Viane (2013) only recognised four species (*A. finlaysonianum* Wall. ex Hook, *A. loriceum* Christ, *A. matsumurae* Christ and *A. wrightii* Eaton ex Hooker) and treated others as synonyms of *A. wrightii* (Table 1). However, *A. finlaysonianum*, *A. loriceum* and *A. matsumurae* were found not to be closely related to the *A. wrightii* complex in a phylogenetic study (Xu et al. 2020). The *A. wrightii* complex was nested within *A. bullatum* clade, while species *A. finlaysonianum* was nested within *Tarachia* clade and species *A. loriceum* and *A. matsumurae* were nested within *Neottopteris* clade (Xu et al. 2020). Therefore, these three species, *A. finlaysonianum*, *A. loriceum* and *A. matsumurae* were excluded from the *A. wrightii* complex.

Asplenium alatulum Ching was first published, based on a single collection from Wuzhishan Mountain in south-central Hainan, China in 1922. In the protologue, Ching and Wang (1964) described *A. alatulum* as a small herb with short pinnae, pinna apices acuminate, pinna margins crenate-serrate and rachises with lateral wings. *Asplenium subcrenatum* Ching ex S. H. Wu (1989) was based on the type material from Malipo County, south-eastern Yunnan, China. He stated that *A. subcrenatum* is similar to *A. wrightioides* Christ, but the stipe and the rachis of the former species are densely covered with red fibrillar scales. These two species were recognised by Wu (1999), but not by Lin and Viane (2013), who treated them as synonyms of *A. wrightii* in light of variable morphological characters within this aggregate. Lin and Viane (2013) further artificially separated the *A. wrightii* complex into four groups, based on a set of morphological characters, such as the venation and the length of the pinnae and the length of sori.

During our taxonomic study on *Asplenium*, we found that *A. alatum*, *A. subcrenatum* and *A. wrightii* could be easily identified and are distinguishable from one another, based on morphological characteristics and geographical distribution. Our recent global phylogeny of *Asplenium* (Xu et al. 2020) supports both *A. alatulum* and *A. subcrenatum* as not conspecific with *A. wrightii*. The *A. wrightii* aggregates were not resolved as monophyletic because the clade also included *A.* × *shikokianum*, a hybrid between *A. wrightii* and *A. ritoense* (Xu et al. 2020) which is not a member of the *A. wrightii* aggregates sensu Wu (1989). In the present study, we evaluate the morphological characteristics of spores and scales of *A. alatulum*, *A. subcrenatum* and *A. wrightii*, in combination with our earlier molecular work, to establish the delimitation and validity of the two species and to produce a taxonomic treatment including descriptions and distributional notes.

Table 1. List of the 14 species included in *Asplenium* ser. *Wrightiana* by Wu (1999) in alphabetical order, references being given in the right-hand column. Species indicated by asterisk were treated as synonyms of *A. wrightii* by Lin and Viane (2013). Four species, recognised by Lin and Viane (2013), are indicated with boldface.

Species	Reference
Asplenium alatulum Ching*	Acta Phytotax. Sinica 9 (4): 359. 1964
A. duplicatoserratum Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 19, f. 4. 1989.
***A. finlaysonianum* Wall. ex Hook.**	Ic. Pl. t. 937. 1854
A. fujianense Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 21, f. 7. 1989
A. lauii Ching*	Acta Phytotax. Sinica 9(4): 360. 1964
***A. loriceum* Christ**	Index Filic. fasc. 2: 119
***A. matsumurae* Christ ex Matsumura**	Bot. Mag. Tokyo 24: 241. 1910
A. neomultijugum Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 21, f. 6. 1989
A. pseudowrightii Ching*	Acta Phytotax. Sinica 9 (4): 360. 1964
A. serratissimum Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 20. 1989
A. subcrenatum Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 19, f. 5. 1989.
A. taiwanense Ching ex S. H. Wu*	Bull. Bot. Res. 9 (2): 18, f. 3. 1989
***A. wrightii* Eaton ex Hook.**	Sp. Fil. 3: 113, t. 182. 1860
A. wrightioides Christ*	Bull. Acad. Int. Géogr. Bot. 11: 238. 1902

Material and methods

Extensive field investigation and careful examination of specimens of *Asplenium wrightii* complex from 21 herbaria (CDBI, CSH, CZH, GH, GXMG, GZTM, HGAS, HUST, HZ, IBK, IBSC, IMC, JIU, JJF, L, MO, NAS, NA, NY, PE and SYS; abbreviations follow Thiers in Index Herbariorum available at http://sweetgum.nybg.org/science/ih/) and our own collections, as well as the study of protologues and other related literature(Lin and Viane 2013; Pham 1999) were carried out.

Rhizome scales were soaked in distilled water for 24 hours and then mounted on glass slides. The morphology of rhizome scales was observed and photographed using a stereo light microscope (LEICA S8APO).

Scanning Electron Microscope (SEM) images were taken of the spores and stomata of *Asplenium alatulum*, *A. subcrenatum* and *A. wrightii*. Spore and pinna samples, obtained from herbarium specimens, were mounted on specimen tabs and then coated with platinum in a sputter coater. Observations were conducted using a JSM-633OF SEM (JEOL Ltd., Akishima, Tokyo, Japan) scanning electron microscope with 10 kV at Sun Yat-Sen University, Guangzhou, China (Figs 1, 2).

The ImageJ software (Rasband 1997–2017) was utilised for measurement on SEM micrographs.

Results

Though the density and shape of stomata are similar amongst *Asplenium alatulum*, *A. subcrenatum* and *A. wrightii*, the size of stomata is distinguishable amongst the three species. *Asplenium alatulum* has the largest stomata (60–70 μm in length and

40–45 μm in width, Fig. 1B, C, E, F), while the other two species *A. subcrenatum* and *A. wrightii* have similar size of stomata (45–65 μm in length and 25–35 μm in width, Fig. 1H, I, K, L). In addition, the rhizomes, scales, rachis wings, pinna pairs, pinna margins and perispores of the three species are very diverse, but relatively stable within species in morphology (Figs 1, 2, 4, 5, 6). Our results confirmed that *A. alatulum* and *A. subcrenatum*, previously treated as synonyms of *A. wrightii*, should be reinstated as distinct species. *Asplenium subcrenatum* is not restricted to their type locality, but it has an extensive geographical distribution in south-western China and northern Vietnam.

A key to *A. alatulum*, *A. subcrenatum* and *A. wrightii*

1 Stipes and rachises densely scaly, scales reddish-brown, pinna margins almost entire to crenate-sinuate, mainly occurs in limestone areas.....***A. subcrenatum***

– Stipes and rachises scaly, scales brown to dark brown, pinna margins serrate to coarsely dentate, mainly occurs in acid soil **2**

2 Rhizomes erect to decumbent, scale cells oblong, rachises with broad lateral wings, pinnae 10–15 pairs ..... ***A. alatulum***

– Rhizomes erect, scale cells quadrangle, rachises only winged towards apex, pinnae (12–)17–25(–34) pairs ..... ***A. wrightii***

Taxonomic treatment

Asplenium alatulum **Ching, Acta Phytotax. Sin. 9(4): 359. 1964.**

Type. China. **Hainan:** Five-Finger Mountain, 19 May 1922, *F.A.McClure 9713* (holotype: PE (PE00059412 [image!])). Fig. 3A.

Description. Plants up to 50 cm tall. Rhizomes erect to decumbent, densely scaly; scales dark brown, narrowly lanceolate or lanceolate-ovate, 5–9 × 0.4–0.8 mm, denticulate glandular margin or long fibrillose (Fig. 2C, F, I). Fronds tufted; stipe dull to semi-shiny, greyish-green to brown or stramineous-green, 12–25 cm, sparsely scaly, scales similar to those on rhizome; lamina triangular-ovate to elliptic, (15–)18–25(–30) × (10–)12–18(–22) cm, base truncate, apex acute, 1-pinnate (Fig. 4A); pinnae 10–15 pairs, basal pinnae subopposite, others alternate, at an angle of ca. 60°–70° to rachis, with stalks (1–)2–4 mm, lower pinnae not reduced, suprabasal pinnae falcate-lanceolate, (5–)6–10(–12) × (0.8–)1.2–1.8(–2.0) cm, base asymmetrical, acroscopic side truncate at an angle of (55°–)65°–75(–85°) to costa, basiscopic side cuneate, becoming decurrent on rachis in apical part of lamina, margin serrate to dentate, apex acuminate (Fig. 4B, C, D). Veins (1 or) 2-forked, with terminal hydathode. Fronds papery, brownish-green when dry, subglabrous; rachis brown to greyish-green or stramineous-green, subglabrous, terete abaxially, with greyish-green lateral wings (Fig. 4E). Sori linear, (2–)5–9(–12) mm, on acroscopic veinlets, medial to supramedial (Fig. 4C); indusia greyish-brown to dark brown, linear, papery, margin entire, opening towards costa, persistent. Spores with average exospore length 40–45 μm, perispore cristato-alate (Fig. 1G).

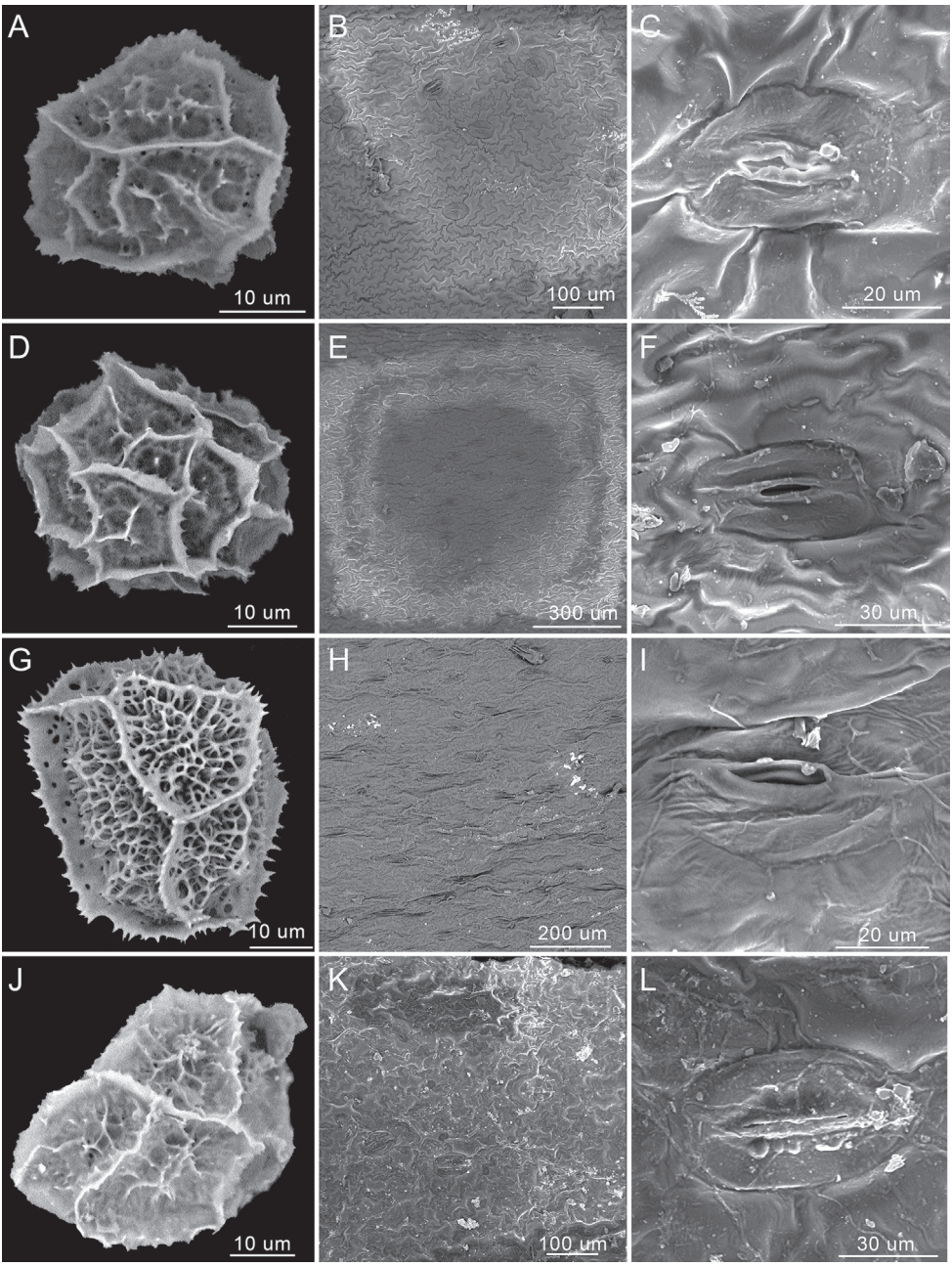

Figure 1. Scanning electron micrographs of spore and stomata of *A. alatulum*, *A. subcrenatum* and *A. wrightii* **A–F** *A. wrightii* **G–I** *A.subcrenatum* **J–L** *A. alatulum*.

Distribution and habitat. *Asplenium alatulum* is apparently restricted to China. It grows on rocks in ravines of broad-leaved forests at an elevation of ca. 500–1600 m. (Fig. 4A).

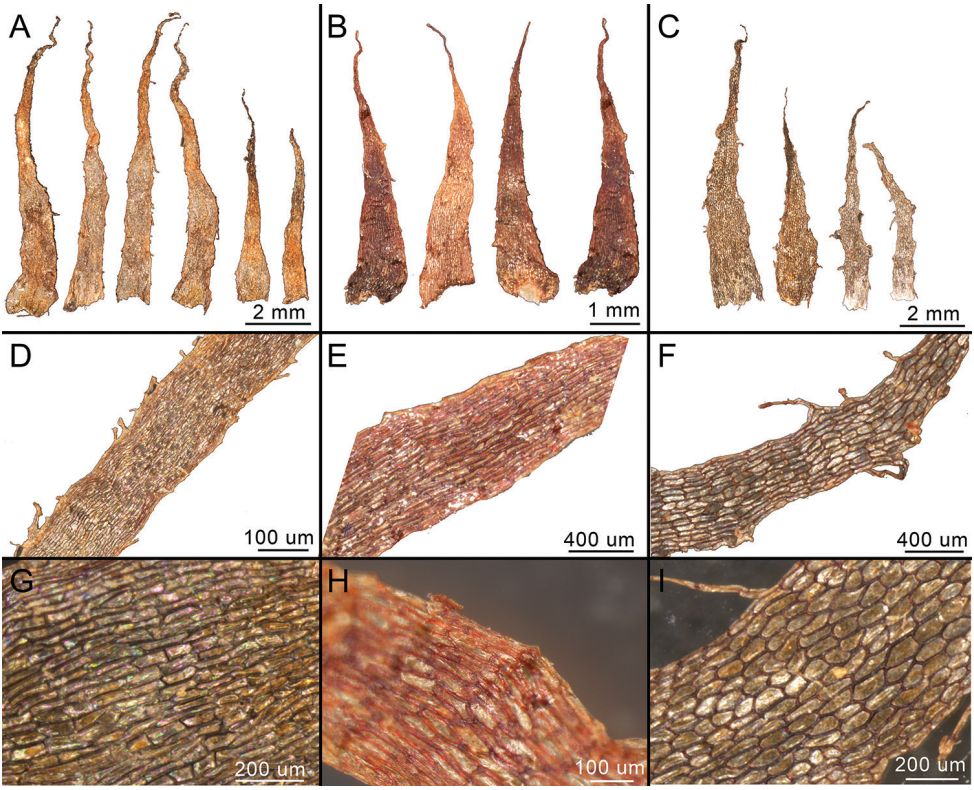

Figure 2. Scales of *A. alatulum*, *A. subcrenatum* and *A. wrightii*. **A, D, G** *A. wrightii* **B, E, H** *A.subcrenatum* **C, F, I** *A. alatulum*.

Additional specimens examined. China. Hainan: Wuzhishan, Shuiman Village, elev. 870 m, 18°54'10.6"N, 109°41'15.6"E, 7 Apr 2016, *K.W.Xu 107* (SYS!); the same locality, elev. 1550 m, 20 Dec 2010, *X.P.Wei & R.Wei WXP113* (PE-2286681!).

Note. Though *Asplenium alatulum* was thought to be an endemic species to the Hainan Island before its synonymisation with *A. wrightii* by Lin and Viane (2013), the morphological distinction between *A. alatulum* and its closely-related species was obscure due to the insufficient field investigations. In the protologue, Ching and Wang (1964) emphasised its small size, the short pinnae, the crenate-serrate pinna margins and whole rachis with lateral wings and stated that *A. alatulum* is markedly different from *A. wrightii*. However, Lin and Viane (2013) recognised that the plant and pinna size and margin shape are variable in the *A. wrightii* complex and included *A. alatulum* in the synonymy of *A. wrightii*. Recently, our study, based on specimen examination and recent field observations of the two species, supports their distinction.

Asplenium alatulum is distinct from *A. wrightii* in having erect to decumbent rhizome (vs. erect rhizome), oblong cells of scales (Fig. 2C, F, I) (vs. quadrangle cells of scales, Fig. 2A, D, G), winged rachis (vs. winged towards apex) and fewer pinna pairs.

Figure 3. Type of *Asplenium alatulum* Ching (**A** barcode PE00059412), *A. subcrenatum* Ching ex S.H.Wu (**B** barcode PE00059494), *A. wrightii* Eaton ex Hooker (**C** barcode NY00128031) and isotype of *A. wrightii* GH (**D** barcode GH00020612).

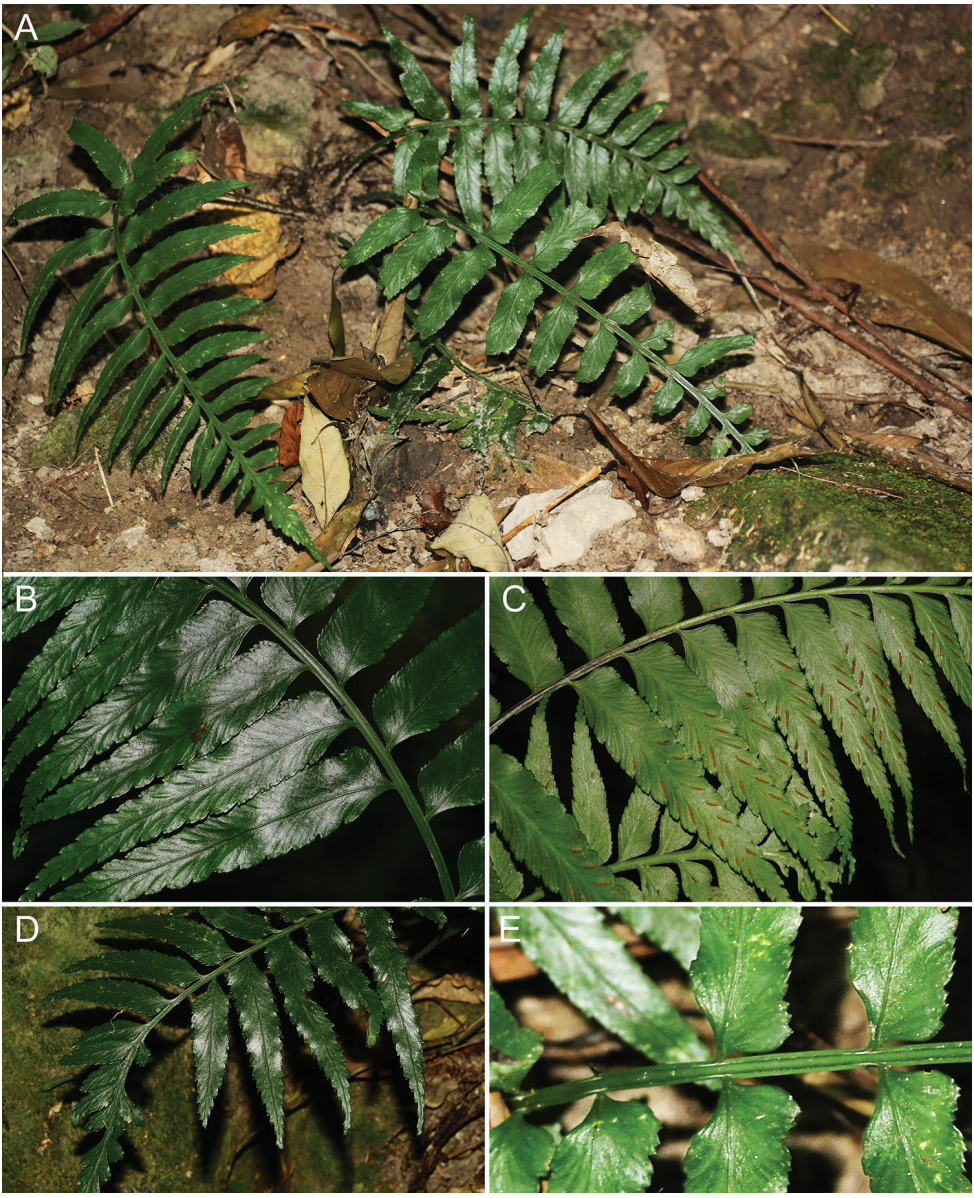

Figure 4. *Asplenium alatulum* **A** Habitat and habit **B** Portion of lamina showing adaxial view of pinna **C** Portion of lamina showing abaxial view of pinna **D** Adaxial view of upper portion of lamina **E** Lower portion of rachis showing wide lateral wings.

Phylogenetically, our earlier molecular work resolved *A. alatulum* in a distinct clade, sister to the clade containing *A. wrightii* and *A.* × *shikokianum* (Xu et al. 2020). One accession from the Taiwan Island was nested within *A. alatulum*, indicating that this species should also be distributed in Taiwan.

Asplenium subcrenatum **Ching ex S.H.Wu, Bull. Bot. Res. 9(2): 86, f. 5. 1989.**

Type. China. **Yunnan:** Malipo, Chung-dzai, in mixed forest, elev. 1600–1800 m, 3 Nov 1947, *K.M.Feng 12803* (holotype: PE (PE00059494 [image!])). Fig. 3B.

Description. Plants up to 30–55(–70) cm tall. Rhizomes erect to decumbent, densely scaly; scales reddish-brown, narrowly triangular, 4–8 × 0.7–1.1 mm, margin near entire (Figs 5E, 2B, E, H). Fronds tufted; stipe stramineous-green or reddish-brown, (10–)15–20(–25) cm, base densely scaly, scales reddish-brown, fibrillose or similar to those on rhizome; lamina oblong-lanceolate, (12–)15–40(–50) × (5–)8–15(–17) cm, base truncate, apex acute, 1-pinnate; pinnae (15–)18–25(–30) pairs, basal pinnae subopposite, others alternate, at an angle of ca. 60°–80° to rachis, with stalks (1–)2–3 mm, lower pinnae slightly reduced, suprabasal pinnae falcate-lanceolate, (3–)6–10 × (0.6–)1–1.5 cm, base asymmetrical, acroscopic side truncate at an angle of (40°–)45°–60(–70°) to costa, basiscopic side cuneate, becoming decurrent on rachis in apical part of lamina, margin almost entire to crenate-sinuate, apex acuminate (Fig. 5B, C, D). Veins (1 or)2-forked, with terminal hydathode. Fronds papery, dark green when dry, subglabrous; rachis reddish-brown to stramineous-green, densely scaly to subglabrous, scales similar to those on stipe, terete abaxially, winged towards apex. Sori linear, (2–)3–8(–10) mm, usually on acroscopic veinlets, medial (Fig. 5C); indusia greyish-brown to dark brown, linear, papery, margin entire, opening towards costa, persistent. Spores with average exospore length 40–45 µm, perispore cristato-alate.

Distribution and habitat. *Asplenium subcrenatum* is distributed in China and Vietnam. It grows as an epiphyte on tree trunks or occurs on rocks by stream-sides in the evergreen broad-leaved forests of limestone areas (Fig. 5A).

Additional specimens examined. China. Gauangxi: Jingxi County, Xinjing Village, Bahong, 23°07'05.56"N, 106°30'24.53"E, 31 Oct 2010, *L.B.Zhang, H.He & Y.Wang 5492* (MO!); Nandan County, Mangchang Village, Lala, 25°10'24.92"N, 107°23'16.26"E, 12 Oct 2010, *L.B.Zhang, H.He & Y.Wang 5492* (MO!); **Guizhou**: Libo County, Jialiang Village, 18 Oct 2018, *JSL6678* (CSH!); Libo County, Shuili Xiang, Shangshuizan, on a dry mountain with mixed pine and broad-leaved forest, 25°28'46"N, 107°47'47"E, 8 Jun 2016, *L.B.Zhang et al.9193* (MO!); Libo County, Wong'ang, Dongduo, elev. 780 m, 16 Sep 2007, *L.B.Zhang 472* (MO!); Guiding County, Houchangbao Xiang, on cliffs by a stream, elev. 1100 m, 26°14'47"N, 107°12'37"E, 10 Jun 2016, *L.B.Zhang, Y.F.Duan, N.T.Lu & X.Y.Miao 9250* (MO!); **Yunnan**: Xichou County, Fadou Village, Xinjing, elev. 1800 m, 10 Jun 2013, *Y.H.Yan YN250* (CSH-0046594!); the same locality, 9 Jan 1962, *S.G.Wu4222-62* (PE-00912376!); Malipo County, elev. 1100 m, 21 Jan 1940, *C.W.Wang 86341* (PE-00912378!); Xiajinchang Village, Huangjinyin, elev. 1416 m, 22°07'28.89"N, 104°51'11.15"E, 29 Oct 2015, Fan 13883 (SYS!); the same locality, 29 Oct 2015, Fan 13884 (SYS!); the same locality, 11 Dec 2015, the same locality, *Xu TTJ-YN-031* (SYS!); 11 Dec 2015, *Xu TTJ-YN-032* (SYS!); Tianbao County, Tianbao Village, Bajiaoping, elev. 1135 m, 22°58.6607'N, 104°50.8035'E, 30 Oct 2015, Fan 13884 (SYS!); Maguan County, Bazhai, Lvditang, 7 Apr 2017, *X.C.Zhang et al. 8219* (PE-02236348).

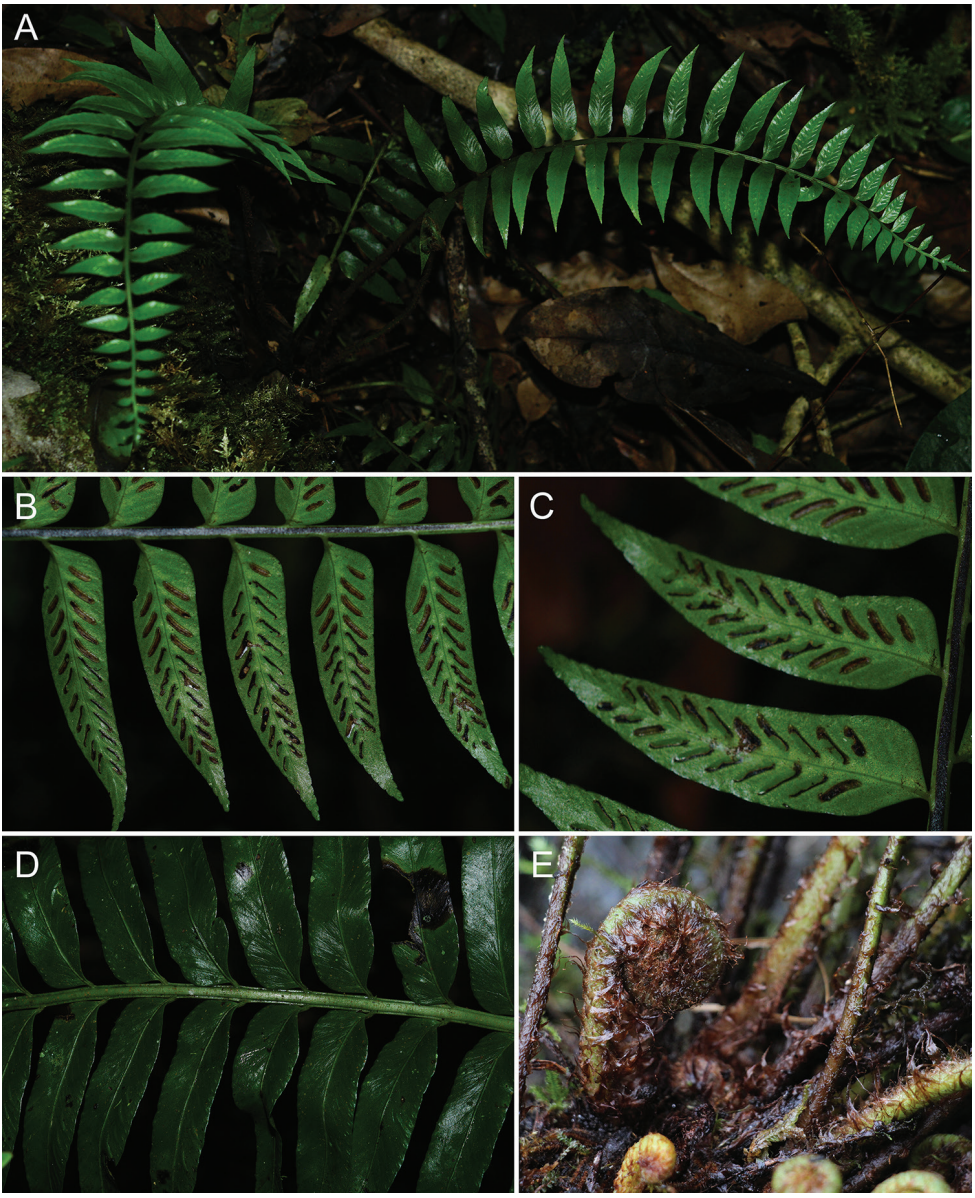

Figure 5. *Asplenium subcrenatum* **A** Habitat and habit **B** Portion of lamina showing abaxial view of pinna **C** Abaxial view of pinna showing the serrate to coarsely dentate margin **D** Adaxial view of upper portion of lamina **E** Lower portion of stipes showing the reddish-brown scales.

Vietnam. Ha Giang: Yen Minh District, Lao Va Chai Municipality, vicinities of Chi Sang Village, elev. 1450 m, 23°06'20"N, 105°04'25"E, 09 Dec 2005, *Averyanov, Leonid V. HAL8420* (MO-3136047!); Vi Xuyen District, Tung Ba Community, Khau Ca Nature Reserve, Hom Mountain, elev. 900 m, 13 Dec 2013, *L.B.Zhang, L.Zhang*

& L.T.Ngan 6966 (MO!); Quan Ba District, Nghia Thuan Community, Bat Dai Son Nature Reserve, 10 Dec 2013, *L.B.Zhang, L.Zhang & L.T.Ngan 6882* (MO!).

Note. Just like *Asplenium alatulum*, a comprehensive taxonomic study of *A. subcrenatum* was scarce after this species was described. Lin and Viane (2013) treated it as a synonym of *A. wrightii*, based only on macromorphological data. Both our molecular data (Xu et al. 2020) and micromorphological data in this study (Figs 1, 2) support the distinction of *A. subcrenatum*.

Asplenium subcrenatum is different from *A. wrightii* in having its stipe and rachis covered with reddish-brown scales (Fig. 2B, E, H) (vs. brown to dark brown scales, Fig. 2A, D, G), scale margins nearly entire (vs. glandular margins or long-fibrillose) and pinna margins almost entire to crenate-sinuate (vs. serrate to coarsely dentate). Meanwhile, the perispores of *A. subcrenatum* are different from those of *A. wrightii*. The folds of *A. subcrenatum* are cristato-alate and areolofenestrate and the margins of the folds are echinulate, while the folds of *A. wrightii* are cristato-alate and imperforate and the margins of the folds are echinulate or approximately laevigate.

Geographically, *Asplenium subcrenatum* was only known from the type locality Malipo County and Yanshan County, southern Yunnan, China (Wu 1999). Our study, based on specimen investigations and the field survey, shows that this species is mainly distributed in limestone areas of south-western China and northern Vietnam, while *A. wrightii* is widely distributed in acidic soils in southern China and Japan and it might occur in Korea.

Asplenium wrightii **Eaton ex Hooker, Sp. Fil. 3: 113. pl. 182. 1860.**

Asplenium duplicatoserratum Ching ex S.H. Wu, Bull. Bot. Res., Harbin 9(2): 86, f. 4. 1989.
Asplenium fujianense Ching ex S.H. Wu, Bull. Bot. Res., Harbin 9(2): 21, 88–89, f. 7. 1989.
Asplenium laui Ching, Acta Phytotax. Sin. 9(4): 360–361. 1964.
Asplenium neomultijugum Ching ex S. H. Wu, Bull. Bot. Res. 9 (2): 21, f. 6. 1989.
Asplenium pseudowrightii Ching, Acta Phytotax. Sin. 9(4): 360. 1964.
Asplenium serratissimum Ching, Bull. Bot. Res., Harbin 9(2): 87. 1989.
Asplenium taiwanense Ching ex S.H. Wu, Bull. Bot. Res., Harbin 9(2): 85–86, f. 3. 1989.
Asplenium wrightioides Christ, Bull. Acad. Int. Géogr. Bot. 11(153–154): 238–239. 1902.

Type. Japan. **Kagoshima:** Ryukyu Islands, 1853–1856, *C. Wright, #s.n.* (holotype: NY (NY-00128031 [image!], isotype: GH (GH00020612 [image!])). Fig. 3C, D.

Description. Plants 35–70(–100) cm tall. Rhizome erect, short, scaly; scales brown to dark brown, lanceolate-triangular, 5–12 × 0.6–1.2 mm, denticulate glandular margin or long fibrillose. Fronds tufted; stipe greyish-green to brown, (18–)23–29(–31) cm, base densely scaly, scales brown, lanceolate to linear-lanceolate, (4.6–)6.1–7.8(–8.8) mm long, with multicellular hairs, subglabrous towards apex; lamina ovate-lanceolate to lanceolate, (19–)39–70(–88) × (9–)17–27(–35) cm, base truncate, apex acute, 1-pinnate; pinnae (12–)17–25(–34) pairs, basal pinnae subopposite, others alternate, at an angle of

ca. 50°–60° to rachis, with stalks (2–)4–8 mm, lower pinnae slightly reduced, suprabasal pinnae narrowly oval-lanceolate and often falcate, (6–)9.1–13(–20) × (0.9–)1.2–1.8(–2.5) cm, base asymmetrical, acroscopic side truncate at an angle of (40°–)55°–75°(–85°) to costa and often auriculate, basiscopic side cuneate, at an angle of (20°–)30°–40°(–60°), becoming decurrent on rachis in central part of lamina, margin serrate to coarsely dentate, apex acuminate. Veins (1 or)2-forked, with terminal hydathode (Fig. 6B, C, D). Fronds papery, green to brownish-green when dry, subglabrous; rachis dull green to reddish-brown, terete abaxially, winged towards apex. Sori linear, (3–)6–10(–12) mm, on acroscopic veinlets, medial (Fig. 6B, C); indusia brown, linear, papery, opening towards costa, persistent. Spores with average exospore length 32–45 μm, perispore cristato-alate.

Distribution and habitat. *Asplenium wrightii* is commonly distributed in China and Japan and it might occur in Korea. This species is found in damp valleys under evergreen broad-leaved forests where it grows as a lithophyte by streams (Fig. 6A).

Additional specimens examined. China. Anhui: Qimen County, Xifengsi, Nov. 1957, *M.B.Deng 5308* (HZ-028581); Jing County, Dingxi Village, Suhong, 22 Sep 1959, *0431* (NA-S00153661); Tongling County, Xiaokeng, Jun 1985, *X.L.Liu 85053* (HUST-00008926); **Chongqing**: Pengshui County, Hanjia Village, elev. 370 m, 29°25'137.98"N, 108°18'19.1"E, 16 Oct 2012, *Pengshui expedition 500243-003-167* (IMC-0003680); Yangshan County, Nanling Nature Reserve, Xinerkeng, 29 Sep 2007, *X.L.Zhou & H.F.Chen 785* (HUST-00008767); **Fujian**: 22 Jul 2015, *ZXL05538* (CSH-0101629); 10 Apr 2014, *H.Shang & Y.F.Gu SG134* (CSH-0034578); Wuyishan, elev. 750 m, 15 Aug 2011, *X.F.Zeng 11215* (CZH-0006554); Zhaoan County, Wushan, 12 Apr 2015, *X.F.Zeng ZXF20041* (CZH-0012412); Nanping City, Mangdangshan, 17 Feb 1999, *G.S.He 9643* (PE-00913318); Jianning County, 20 Nov 1977, *Z.Y.Li 10619* (PE-0913321); Jiangle County, Longxishan, 27 May 1991, *Longxishan expedition 0359* (PE-01555190); **Guangxi**: Lingyun County, Yuhong Village, Donglan, elev. 1303 m, 24°24'13.19"N, 106°29'03.17"E, 16 Aug 2013, *Lingyun expedition 451027130816103* (GXMG-0117634); Longshen County, Heping Village, elev. 615 m, 25°41'12.10"N, 110°03'22.40"E, 6 Mar 2013, *Longshen expedition 450328130306038LY* (IBK-00362003); Xingan County, Maoershan, 5 Oct 2007, *L.Wu & X.X.Xu 1042* (HUST-00019436); Sanjiangdongzu County, Sanxingpo, Dudong Village, elev. 700 m, 18 Dec 2007, *X.X.Xu & L.C.Qin 255* (HUST-00011550); **Guangdong**: Heping County, Daba Village, Dafukeng, 2 Jan 2007, *C.M.Tan et al. Y06630* (HUST-00004651); Lechang County, 23 Nov 1931, *Z.Huang 31490* (IBK-00035278); the same locality, 21 Jun 1942, *S.Q.Chen1611* (IBK-00035299); Boluo County, Luofushan, 18 Aug 1930, *N.Q.Chen 41631* (IBK-00035279); Xinyi, 13 Aug 1931, *Z.Huang 31164* (IBK-00035285); Dawuling, 4 Aug 2003, Y.H.Yue et al. 1568 (PE-01784537); 21 Mar 1931, *Z.Huang 31756* (PE-00913341); Ruyuan, Daxiagu, 2 Aug 2005, *B.R.Liu 05100* (PE-01785865); Yingde, Shimentai Nature Reserve, Oct 2001, *Y.H.Yue & F.W.Xing 13265* (PE-01784538); Mei County, Jiaying, 4 Aug 1932, Tsang, *W.T. 21466* (PE-00913338); **Guizhou**: Yongshun County, Xiaoxi Village, elev. 621 m, 28°82'21.10"N, 110°25'11.00"E, 14 Jan 2014, *D.G.Zhang zdg9949* (JIU-04159); Liping County, Pingjia, elev. 670 m, 16 Dec 2007, *X.X.Xu & L.C.Qin 349* (HUST-00011558); Libo County, Maolan Nature Reserve, 25 Apr 2015; *X.C.Zhang*

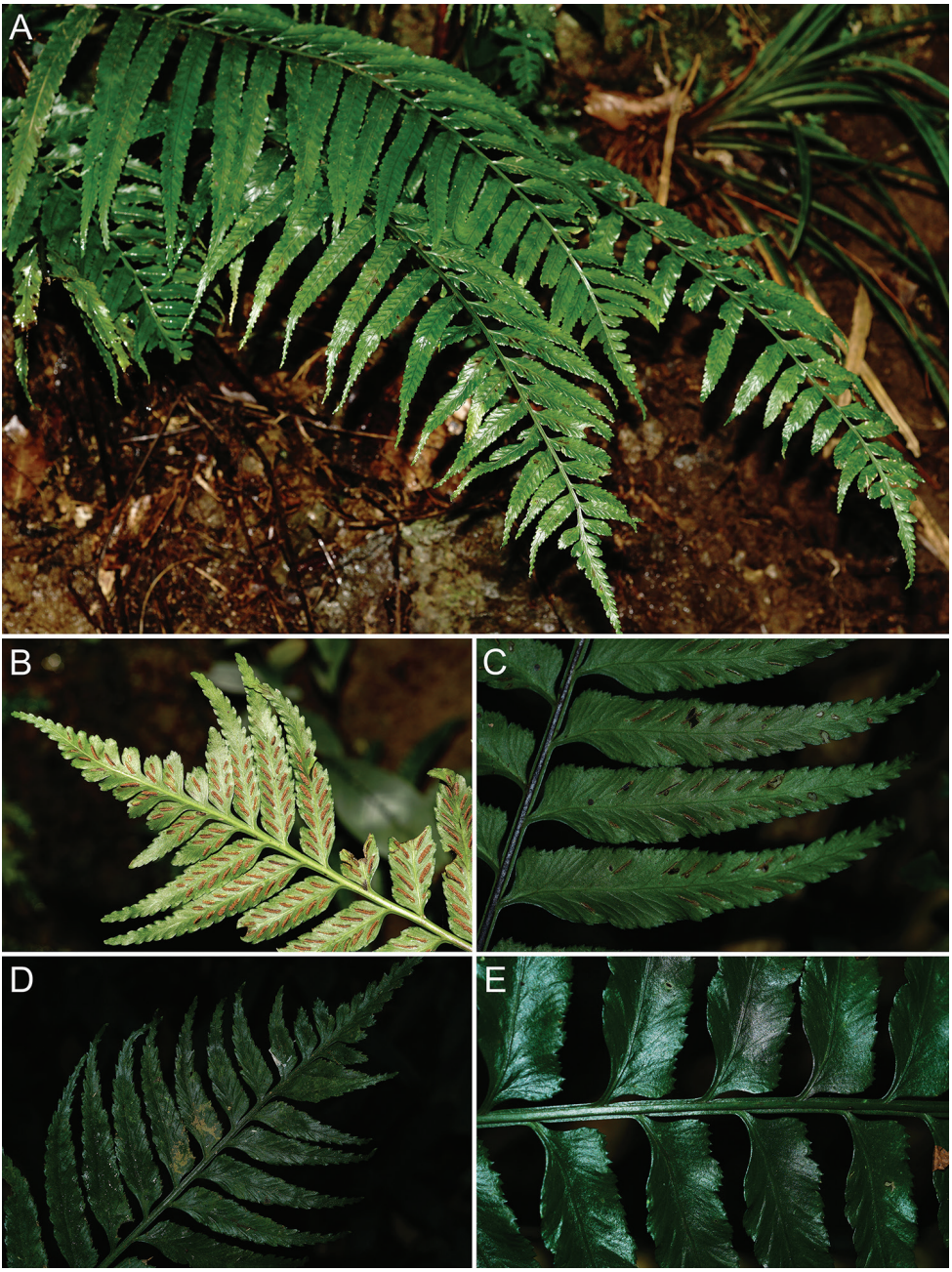

Figure 6. *Asplenium wrightii* **A** Habitat and habit **B** Abaxial view of upper portion of lamina **C** Abaxial view of pinna showing the almost entire to crenate-sinuate margin **D** Adaxial view of upper portion of lamina **E** Upper portion of rachis showing the lateral wings.

7259 (PE-02051530); Shiqian County, Qiangyang Village, Longdong, 30 Jul 1988, *Wulingshan expedition 2355* (PE-01557785); Jiangkou County, 30 Aug 1986, *B.Bartholomew et al. 595* (PE-00913309); Jianhe County, Nanjiaqu, 30 Apr 1992,

F.Wang 651 (HGAS-055247); Jinping County, Gaodengpo, 27 Nov 1991, *fern expedition 91474* (HGAS-055248); Rongjiang County, Shuiwei Shuizu Village, 15 Oct 2014, *Wei et al. WYG036* (CSH-0043860); Shibing County, Maohao Village, 30 May 2016, *D.Y.Zhou 522623160530476LY* (GZTM-0066044); Danzhai County, 18 Oct 2012, *Hou GZDZ201210180003* (GYBG-0009834); **Hunan**: Sangzhi County, Bamaoxi Village, 21 Oct 2014, *X.L.Zhou et al. ZXL09673* (CSH-0045103); Baojing County, Baiyunshan Nature Reserve, elev. 397 m, 28°37'51.90"N, 109°17'11.34"E, 11 Aug 2012, *X.J.Su. & H.B.Liu 433125D00030810086* (JIU-06613); Yongshun County, Xiaoxi, Daping, 12 Sep. 2009, *L.Xu 090912005* (JIU-02238); Dongzu County, Pingyang Village, Yangdongtan, elev. 300 m, 10–15 Jan 2008, *L.C.Qing & H.B.Ouyang 1080* (HUST-00012147); Shangyan, Sanxingpo, elev. 750 m, 14 Dec 2004, *L.C.Qing. & X.X.Xu 691* (HUST-00011548); Suining County, Huangsang Nature Reserve, elev. 450 m, 10 Jan 2008, *J.M.Xi & Y.B.Qin 07919* (HUST-00012150); Yanling County, Taoyuandong Nature Reserve, Jul 2008, *X.L.Zhou &Z.L.Zhu 2154* (PE-01964123); Rucheng County, Donggangling, Shuinishan, elev. 110 m, 31 Jul 2007, *L.Wu & H.B.Ouyang 88* (HUST-00008785); Pingjiang County, Sicun Village, Wudeng, elev. 500 m, 15 Dec 2007, *L.Wu & S.X.Qi W467* (HUST-00011555); Huaihua County, Bamianshan, 15 Dec 2007, *X.L.Zhou & Y.Q.Yao 1744* (HUST-00011562); **Hubei**: Laifeng County, Dahe Village, Lianghekou, elev. 590 m, 25 Jul 2013, *W.Z.Zhu BLF077* (CCAU-0002976); **Jiangxi**: Xingzi County, Wenquan Village, Xiufeng, 26 Jul 2013, *A.M.Dong 2082* (JJF00010847); Wanzai County, Jiulong Forest Park, elev. 190 m, 28°21'38"N, 114°30'47"E, 15 Aug 2013, *H.G.Ye & F.Y.Zeng LXP10-2161* (IBSC-0773610); Wuning County, Yuangkou Village, Dongkeng, elev. 400 m, 16 Oct 2012, *J.H.Zhang 2045* (JJF-00010846); Chongyi County, Fengzhou Village, Changlongao, elev. 1000 m, 8 Jul 2007, *L.Y.Wang W.181* (HUST-00008778); Jianggangshan, elev. 800 m, 10 Apr 2004, *Y.H.Yan & J.S.Zhou 3415* (HUST-00001166); **Jiangsu**: Yixing, Longchi, Shuizhushan, 1956, *Liu & Huang 2874* (NAS-00091223); **Sichuan**: Emeishan, 15 Sep 1963, *G.X.Xing & K.Y.Lang 1788* (PE-13307); the same locality, 21 Sep 1979, *Z.R.Wang C122* (PE-96543); Zhulian County, 1 Jun 1978, *X.R.Kong 5204* (CDBI-003584); **Taiwan**: Wulai, Fushan-Hapen, 13 Oct 1984, *Y.Tateishi et al. 20492* (PE-01451621); Takao, 27 Jan 1939, *M.Tagawa 2049* (PE-00913326); *W.Hancock 141* (PE-00913329); Taito, 4 Mar 1940, *M.Tagawa 3168* (PE-00913330); Huanlian County, Changliang, 8 Jun 2001, *T.C.Chen 11303* (PE-01451613); Taibei County, Lujiaokengxi, 28 Jan 2000, *T.C.Chen 10393* (PE-01451614); **Zhejiang**: Tonglu County, Luci, Changzhou, 8 Nov 1989, *L.Hong s.n.* (HZ-028576); Shouchang Zhen, 19 Jun 1959, *Zhejiang expedition 27803* (HZ028578); Taishun County, Liguang, Huangshikeng, 27 Nov 1958, *Anonymous 23818* (HZ-028579); Lishui, Suichang Nature Reserve, elev. 780 m, 25°59'11"N, 116°25'11"E, 5 Jun 2012, *Q. Tian TQ01773* (CSH-0002430); 25 Nov 2015, *H.J.Wei JSL3466* (CSH-0110219); 29 Jul 1958, *Shan 5578* (PE-00913289);

Japan. Kyushu: Chikushi-yabakei, Nakagawa-cho, Chikushi-gun, Fukuoka Pref., 27 Mar 1975, *T.Nakaike et al. 105* (PE-01708808); Is. Yakushima (Kagoshima Pref.), from Kurio to Segiri through Ookonotaki, elev. 30–100 m, 16 Jul 1979, *T.Yamazaki et al. 2327* (PE-01708809); Kagoshima Pref., Okuchi-shi, Jusso, 6 Sep 1959, *Tetsuji Yamanaka s.n.* (PE-01964369); Prefecture Kagoshina, Yakushina Island, Yaku-

cho, Tainoko-gawa River, 10-11 Oct 1977, *Boufford, David Edward 20118* (MO-3136075); Along trail in gorge, foot of Mt. Awa. Motobu Peninsula, Okinawa Island, 10 Dec 1953, *Walker, EH 7614* (L-3508507); **Honshu**: Owase City, Kuki, Namera Valley, 12 Nov 1991, *K.H.Shing 31* (PE-01708810); Pref. Mie,Kuki, Omse-shi, 5 Oct 1971, *Kato, Masahiro-259* (MO-3136077); Pref. Schizouka: Aono, Minamiizu-cho, Kamo-gun, 30 Dec 1972, *T.Nakaike 5* (L-3508506); **Shikoku**: Nakaohsaka Ohsakadani, Nakatosa Town, Takaoka Distr. Kouchi Pref., elev. 60–80 m, 33°18'48"N, 133°23'48"E, 6 Jan 2012, *Taku Miyazaki 1201164* (PE-02002704); Yatabe, Nakatosa Town, Takaoka District, Kouchi Pref., elev. 40–60 m, 33°15'70"N, 133°15'37"E, 3 Jan 2007, *T.Miyazaki 0701137* (PE-01963957); Takanoyama-rindou Yamauchi, Nakatosa Town, Takaoka Distr. Kochi Pref., elev. 60–90 m, 33°17'04"N, 133°12'18"E, 27 Mar 2009, *Taku Miyazaki 0903138* (PE-01963829); Ohkawauchi Kaminokae, Nakatosa Town, Takaoka District, Kouchi Pref., elev. 20 m, 33°16'67"N, 133°14'15"E, 29 Dec 2006, *Taku Miyazaki 0701025* (PE-01962310).

Note. *Asplenium wrightii* is described based on the type material from Japan and it is widely distributed in China. The morphology of this species is variable, which resulted in the taxonomic chaos amongst *A. wrightii* and its closely-related species. Several ploidy levels (e.g. tetraploid, octoploid, decaploid and dodecaploid) have been reported for the *A. wrightii* complex, but no correlation was found between ploidy levels and morphological characters within the complex (Mitui 1967; Wang 1988; Lin and Viane 2013). *Asplenium* × *shikokianum* is a natural hexaploid hybrid between octoploid *A. wrightii* and tetraploid *A. ritoense* and *Asplenium* × *wangii* is another hybrid between *A. wrightii* and *A. bullatum*. These hybrids are usually uncommon in terms of their distributions where their parents grow together (Kuo 1988; Lin and Viane 2013). Due to hybridisation and polyploidisation, there are a large number of recognised species complexes with ambiguous boundaries between species in Aspleniaceae (Reichstein 1981; Dyer et al. 2012). More comprehensive taxonomic studies are still needed to elucidate the species delimitation in this species complex.

Asplenium wrightii was also documented in Vietnam (Pham 1999; Lin and Viane 2013). However, we checked specimens identified as *A. wrightii* from Vietnam and found none of these specimens is true *A. wrightii*. It is possible that the name *A. wrightii* has been used erroneously for species *A. subcrenatum* in Vietnam.

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SHORT COMMUNICATION

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Lectotypification of *Tsuga longibracteata* W.C.Cheng (Pinaceae)

Yong Yang[1], Keith Rushforth[2]

1 *College of Biology and Environment, Nanjing Forestry University, 159 Longpan Road, Nanjing 210037, Jiangsu, China* **2** *The Shippen, Ashill, Cullompton, Devon, EN15 3NL, UK*

Corresponding author: Yong Yang (ephedra@ibcas.ac.cn)

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Abstract

W.C.Cheng did not clearly indicate the herbarium repository of the type specimen (*Y.Tsiang 7712*) when he described *Tsuga longibracteata* W.C.Cheng. Later, researchers suggested that the type is either in NAS or in PE. However, we found more than one duplicate of the type collection in both NAS and PE. Following the *Shenzhen Code*, we lectotypify the name *T. longibracteata* with *Y.Tsiang 7712* (PE00003223) that bears a handwritten identification of W.C.Cheng.

Keywords

Lectotype, nomenclature, *Nothotsuga*, Pinaceae, *Tsuga longibracteata*

Introduction

Cheng (1932) described an unusual species of *Tsuga* (Endl.) Carrière: *Tsuga longibracteata* W.C.Cheng. This species differs from all known species of *Tsuga* in both vegetative and reproductive characters (Cheng 1932). Its leaves are radially arranged and have stomatal lines on both surfaces; pollen cones are clustered in umbels, and pollen grains possess paired air-bladders; its pedunculate seed cones are more or less erect, and the apical cusp of subspathulate bracts are slightly exserted (Page 1988; Fu et al. 1999; Farjon 2010).

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Hu (1951) proposed separating *T. longibracteata* from *Tsuga* and established a new genus "*Nothotsuga*", but this name was not validly published because Hu did not provide a Latin diagnosis. Page (1988) validated the generic name *Nothotsuga* Hu ex C.N.Page by providing a Latin diagnosis. This segregation has been justified by subsequent molecular systematic studies: *Nothotsuga* diverged from *Tsuga* in the Late Cretaceous (ca. 90 mya, Havill et al. 2008). *Nothotsuga* is now recognized as a distinct genus, with the only extant species being distributed in southern China including Fujian, Guangdong, Guangxi, Guizhou, Hunan, Jiangxi, and Yunnan (Fu et al. 1999; Farjon 2010).

Cheng (1932) designated *Y.Tsiang 7712* as the type of the species name *Tsuga longibracteata*, but did not clearly indicate which specimen is the holotype or where the type specimen is deposited. We found 12 specimens deposited in eight international herbaria, i.e. three specimens in PE (PE00003225, PE00003224 and PE00003223), two in NAS (NAS00070064 and NAS00070063), two in HUH (A00052508 and A00052510), one each in E (E00215871), IBSC (IBSC0012857), K (K000288277), NY (NY00001279), and S (S-C-4796) respectively. Farjon (2010) indicated that the holotype is in NAS, but Lin (2014) recorded the specimen in PE (PE00003223) as the holotype. Under Art. 7.11 of the *Shenzhen Code* (Turland et al. 2018), neither designation can be considered an effective lectotypification because their books were published after 1 January 2001 and did not include the phrase "designated here" (hic designatus) or an equivalent. The two specimens in NAS are poorly preserved and have printed labels without any handwriting. However, one of the two specimens in PE (PE00003223) bears Cheng's handwritten identification and is relatively well preserved. Accordingly, we choose to lectotypify *Tsuga longibracteata* with this specimen.

Typification

***Tsuga longibracteata* W.C.Cheng, Contrib. Biol. Lab. Sc. Soc. China, Bot. Ser. vii. 1 (1932).**
Fig. 1.

≡*Nothotsuga longibracteata* (W.C.Cheng) Hu ex C.N.Page, Notes Roy. Bot. Gard. Edinburgh 45(2): 390 (1988, published in 1989).

Type. China. Guizhou (贵州): Yinjiang Tujiazu Miaozu Zizhixian (印江土家族苗族自治县, as "Yin-Kiang"), Fanjing Shan (梵净山, as "van-ching-shan"), in densely shaded ravine, alt. 400–500 m, 19 December 1930, *Y.Tsiang* (蒋英) *7712* (Lectotype: PE00003223, designated here; isolectotypes: A00052508, A00052510, E00215871, IBSC0012857, K000288277, NAS00070063, NAS00070064, NY00001279, PE00003225, PE00003224, S-C-4796).

Figure 1. Lectotype of *Tsuga longibracteata* W.C. Cheng [≡*Nothotsuga longibracteata* (W.C.Cheng) Hu ex C.N.Page]: *Y.Tsiang* (蔣英) *7712* (PE00003223).

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RESEARCH ARTICLE

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Electronic identification keys for species with cryptic morphological characters: a feasibility study using some *Thesium* species

Natasha Lombard[1,2], Margaretha Marianne le Roux[1,2], Ben-Erik van Wyk[2]

1 *Biosystematics Research and Biodiversity Collections Division, South African National Biodiversity Institute, Private Bag X101, Pretoria, 0001, South Africa* **2** *Department of Botany and Plant Biotechnology, University of Johannesburg, PO Box 524, Auckland Park, 2006, South Africa*

Corresponding author: Natasha Lombard (n.lombard@sanbi.org.za)

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Abstract

The popularity of electronic identification keys for species identification has increased with the rapid technological advancements of the 21st century. Although electronic identification keys have several advantages over conventional textual identification keys and work well for charismatic species with large and clear morphological characters, they appear to be less feasible and less effective for species with cryptic morphology (i.e. small, obscure, variable characters and/or complicated structures associated with terminology that is difficult to interpret). This is largely due to the difficulty in presenting and illustrating cryptic morphological characters unambiguously. When taking into account that enigmatic species with cryptic morphology are often taxonomically problematic and therefore likely exacerbate the taxonomic impediment, it is clear that species groups with cryptic morphology (and all the disciplines dependent on their correct identification) could greatly benefit from a user-friendly identification tool, which clearly illustrates cryptic characters. To this end, the aim of this study was to investigate and develop best practices for the unambiguous presentation of cryptic morphological characters using a pilot interactive photographic identification key for the taxonomically difficult plant genus *Thesium* (Santalaceae), as well as to determine its feasibility. The project consisted of three stages: (1) software platform selection, (2) key construction and (3) key evaluation. The

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proposed identification key was produced with Xper³ software and can be accessed at http://www.xper3.fr/xper3GeneratedFiles/publish/identification/1330098581747548637/mkey.html. Methodologies relating to amongst others, character selection and delineation, visual and textual descriptions, key construction, character coding and key evaluation are discussed in detail. Seventeen best practices identified during this study are subsequently suggested for future electronic key compilation of species with cryptic morphology. This study indicates that electronic identification keys can be feasible and effective aids for the identification of species with cryptic morphological characters when the suggested best practices are followed.

Keywords

Best practice, interactive key, key construction, photographic key, Santalaceae, South African plants, taxonomic impediment, Xper³

Introduction

Species identification underpins the majority of biological sciences (Stevenson et al. 2003; Farr 2006; Farnsworth et al. 2013). Attributing a name to a specimen is central to, amongst others, the classification of groups of organisms, ecology, species and habitat conservation, ecological restoration and the management of biological collections (Lawrence and Hawthorne 2006; De Carvalho et al. 2007; Joly et al. 2014; Joly et al. 2019). Traditionally, textual dichotomous keys have been the main tools used for species identification (Walter and Winterton 2007; Nimis et al. 2012; Seo and Oh 2017). More recently, rapid technological advancements of the 21st century have resulted in the production of a wide array of electronic identification guides (Stevenson et al. 2003; Farnsworth et al. 2013) that range from simple textual electronic dichotomous keys (e.g., Leistner 2000; Beuk 2019) to interactive mobile identification applications (apps) with access to large multimedia databases (e.g., De Vaugelas et al. 2011; Merlin Bird ID App, https://merlin.allaboutbirds.org/) and automatic visual recognition apps (e.g., Zhao et al. 2015; PlantSnap, https://www.plantsnap.com/). Electronic identification keys have become commonplace (e.g., Kirchoff et al. 2011; Nimis et al. 2012; Seo and Oh 2017; Jouveau et al. 2018; Bodin et al. 2019; Reeb and Gradstein 2020), are relatively easy to produce and are aimed at enhancing the accessibility and usability of identification keys, as well as the efficiency and accuracy of identifications (Drinkwater 2009; Kirchoff et al. 2011).

Although electronic identification keys have several advantages over conventional identification keys [as detailed in Farr (2006) and Dallwitz et al. (2000)], the few studies comparing the performance of these different identification keys have showed mixed results. Stagg et al. (2015) showed that the accuracy and speed of woodland moss identification was higher using a traditional dichotomous key than an electronic key, while Seo and Oh (2017) found that orchid species were more accurately identified by senior college students when using an electronic identification key than when using a textual dichotomous key or a guide book based on flower colour. Stagg and Donkin (2017) showed that identifications of United Kingdom (UK) wild flowers

were significantly more accurate using an electronic app than a guide book, but that the identification accuracy of UK winter trees was significantly lower when using an electronic app than when using guide books. Stagg and Donkin (2017) posited two reasons for their contrasting results. First, the number of tree species was less than the number of wild flowers so that browsing for tree species in the printed guides was more time efficient than browsing for wild flowers of which there are many species (Drinkwater 2009; Stagg and Donkin 2017). Second, winter tree character states were perceived as subjective, ambiguous and overall cryptic compared to wild flower character states which were clear and concise (Stagg and Donkin 2017). These comparative studies indicate that while electronic identification keys such as interactive photographic keys are effective when identifying charismatic species with large and clear morphological characters, they are often ineffective when identifying enigmatic species with cryptic morphological characters. Here cryptic characters (not to be confused with cryptic species) refer to any morphological character which might cause uncertainty or confusion during the identification process due to one or a combination of the following: (1) very small size [e.g., characteristics of leaf margins and venation in mosses (Stagg et al. 2015); minute characters of armoured scale insects (Schneider et al. 2019)], (2) obscure nature [e.g., subtle differences in bud colour of winter trees (Stagg and Donkin 2017); metasternum related characters in some parasitoid wasps (Klimmek and Baur 2018)], (3) intra-specific variation [e.g., flower colour variation in the carnivorous plant genus *Drosera* L. (Drinkwater 2009); pronotum colour variation in ladybirds (Jouveau et al. 2018)], and (4) complicated structures associated with terminology that is difficult to interpret [e.g., inflorescences of grasses (Fish et al. 2015); thorax morphologies of Brazilian sand flies (Rocha et al. 2019)]. The challenge remains to determine which aspects are critical to produce electronic identification keys that can successfully identify species with cryptic morphological characters.

Enigmatic species with cryptic characters such as many plants, insects, bryophytes and microorganisms are common and are often surrounded by much taxonomic uncertainty (Convention on Biological Diversity 2007). This is partly due to a research bias towards charismatic species and partly due to the difficulty in finding and describing characters with which to delimit and identify enigmatic species. Often only one or a few specialist taxonomists can accurately identify them. All of these aspects add to the taxonomic impediment (Convention on Biological Diversity 2007; Walter and Winterton 2007; Dar et al. 2012) and it is clear that species groups with cryptic characters (and all the disciplines dependent on their correct identification) could greatly benefit from a user-friendly identification tool that clearly illustrates cryptic characters. To address this need we investigated the use of a multi-access interactive photographic identification key as an identification aid for selected species of the morphologically difficult and near cosmopolitan genus *Thesium* L. [Santalaceae (The Angiosperm Phylogeny Group 2016)].

Thesium is a hemi-parasitic plant genus of ± 350 species that has its centre of diversity in southern Africa, with ± 175 species (Lombard et al. 2020). Some *Thesium* species are of economic importance (Lombard et al. 2020). For instance, *T. humile* Vahl

has caused substantial losses to cereal crops in the Mediterranean region (Belakhdar et al. 2014), while *T. chinense* Turcz. is sold commercially in Asia as an herbal medicine to treat a wide array of ailments (Lombard et al. 2020). Species of this genus are notoriously difficult to identify due to, amongst others, the extreme intra-specific variation observed in vegetative morphology, as well as their diminutive flowers (< 10 mm) which contain several important diagnostic characters (Hill 1915). Identifications are further complicated by the large number of species in the genus and the superficial similarities among species (Hill 1915). Current identification keys for South African *Thesium* species are textual keys (e.g., Hill 1925) that are often very difficult to use due to the overlap of character states between couplets (to account for intra-specific variability), as well as the difficulty in describing subtle differences in the general impression, size and shape (GISS) of species (García et al. 2018). *Thesium* is therefore an ideal group in which to study cryptic characters and their representation in an electronic identification key.

The aim of this study was to investigate and develop best practices for the unambiguous presentation of cryptic morphological characters using a pilot interactive photographic identification key. The project was developed by (1) identifying practical, easy-to-use software with which to construct a photographic identification key, (2) producing a pilot identification key for 25 *Thesium* species found in the eastern part of South Africa and (3) evaluating the effectiveness of the identification key with a target group of users from different backgrounds. We subsequently propose a multi-access interactive photographic identification key produced with Xper^3 software.

Materials and methods

Taxa

As the intent of this study was to investigate and demonstrate principles behind the unambiguous presentation of cryptic characters and not to produce a comprehensive field-ready identification key, a subset of 25 species (Table 1) from the morphologically difficult genus *Thesium* were selected as a case study. These species are among ± 60 *Thesium* species that occur in the eastern part (summer rainfall area) of South Africa and were chosen, firstly because they have been observed, collected and photographed by the authors in their living state and natural habitat. Information and media collected in the field is advantageous when constructing photographic identification keys and circumvents several problems associated with electronic key construction from literature and preserved collections (see Morse et al. 1996; Drinkwater 2009). Second, the majority of the 25 species are notoriously difficult to identify as is evidenced by the numerous identification queries the authors received, as well as by the mixed specimen collections encountered in several South African herbaria. This indicates that even trained taxonomists responsible for curating these collections had considerable difficulty in identifying the species in question. Third, recent (and ongoing) taxonomic stud-

Table 1. The 25 *Thesium* species included in the pilot interactive photographic identification key, as well as the most recent taxonomic treatment for each species.

Species	Taxonomic treatment used
Thesium angulosum A.DC.	Hill 1925
Thesium asterias A.W.Hill	Hilliard 2006
Thesium confine Sond.	Mashego and le Roux 2018
Thesium costatum A.W.Hill	Hill 1925
Thesium cupressoides A.W.Hill	Hill 1925
Thesium davidsoniae Brenan	Brenan 1985
Thesium durum Hillard & B.L.Burtt	Mashego and le Roux 2018
Thesium goetzeanum Engl.	Visser et al. 2018
Thesium gracilarioides A.W.Hill	Visser et al. 2018
Thesium gracile A.W.Hill	Visser et al. 2018
Thesium gypsophiloides A.W.Hill	Visser et al. 2018
Thesium impeditum A.W.Hill	Hill 1925
Thesium magalismontanum Sond.	Hill 1925
Thesium multiramulosum Pilg.	Hilliard 2006
Thesium natalense Sond.	Lombard et al. in prep.
Thesium ovatifolium N.Lombard & M.M.le Roux	Lombard et al. 2019
Thesium pallidum A.DC.	Hill 1925
Thesium procerum N.E.Br.	Visser et al. 2018
Thesium racemosum Bernh.	Hill 1925
Thesium resedoides A.W.Hill	Visser et al. 2018
Thesium scirpioides A.W.Hill	Lombard et al. in prep.
Thesium transvaalense Schltr.	Hill 1925
Thesium utile A.W.Hill	Hill 1925
Thesium vahrmeijeri Brenan	Visser et al. 2018
Thesium zeyheri A.DC.	Hill 1925

ies of 12 of the 25 species (Mashego and le Roux 2018; Visser et al. 2018; Lombard et al. 2019, Lombard et al. in prep.) prompted the compilation of user-friendly identification keys and a platform for information dissemination to non-taxonomist users. Fourth, the identification key contributes to research on *Thesium* that is considered a high priority for taxonomic research in South Africa (Victor et al. 2015; Victor 2020).

Software platform: Xper^3

Xper^3 was chosen as the platform for the present study as it is a free access self-controlled programme where no external data storage or servers are needed, and which includes all of the functionalities required by the authors (e.g., multi-access keys, visual and text descriptors and species profiles) (Vignes-Lebbe et al. 2016; Vignes-Lebbe et al. 2017; Pinel et al. 2017). The platform allows for remote access, is intuitive and user friendly for both authors and users, allows for multiple contributors (including concurrent data editing), has the option for both web-based and mobile interfaces and can function as a taxonomic data management programme. The Xper^3 home page can be accessed at http://www.xper3.fr/ and detailed user documentation at http://wiki.xper3.fr/lib/exe/fetch.php?media=wiki:xper3documentation.pdf.

Key construction

Construction of the identification key was completed in four steps: 1) data collection, 2) taxonomic and character backbone construction, 3) character coding and 4) species profile compilation.

Data collection

Characters and character states used

A total of 26 characters were used in the key (Table 2), including 24 discrete characters and two range characters. In the absence of a published or widely accepted list of morphological characters for the genus, morphological characters and character states were adapted from an unpublished character list by, and initial discussion with, Daniel Nickrent (pers. comm.). All informative vegetative and reproductive characters that could clearly be shown with photographs were included. The maximization of the number of morphological characters available to choose from facilitates use by both non-specialist and specialist users. The majority of morphological characters included can be observed with the naked eye, but a 10x hand-lens or light microscope is needed for some of the diminutive floral characters such as style length, stigma position and placental column shape. Character states were delineated and presented in such a way as to facilitate unambiguous interpretation by users and following the guidelines provided in Walter and Winterton (2007); also see Results and Discussion. Each of the provinces of South Africa were also included as characters in the key (see Table 2), as this proved to be the most efficient and user-friendly way to account for the geographical distribution of each species.

Images

Live material was photographed in the field during the flowering seasons (September to February) of 2016, 2017 and 2018 using a Canon EOS 400D camera and Canon EF 100 mm/2.8 USM macro lens. Where live material could not be accessed for certain characters or species, photographs of herbarium material were used. One of the advantages of electronic identification keys is that they can continuously be updated and current images can be replaced with superior images as they become available. Flowers from herbarium material were rehydrated by placing them in Windowlene (cleaning agent) for 15 min before being photographed. Herbarium material were photographed with standard smartphone cameras (Huawei P9 lite, Samsung S7) by aiming the smartphone camera lens at the eyepiece of a light microscope (Nikon SMZ 745 T stereo microscope, Nikon Corporation) so that the enlarged image becomes visible through the eyepiece and then taking the photo. Photographs were later ed-

Table 2. The 26 characters and their respective character states used to distinguish between selected *Thesium* species in a pilot interactive photographic identification key. Definitions of characters and character states are given in the identification key (http://www.xper3.fr/xper3GeneratedFiles/publish/identification/1330098581747548637/mkey.html).

Character	Character state							
	1	2	3	4	5	6	7	8
Distribution in South Africa (Province)	Eastern Cape	Free State	Gauteng	KwaZulu-Natal	Limpopo	Mpumalanga	Northwest	Northern Cape
Habit 1 (shape)	Erect	Virgate	Decumbent or procumbent					
Habit 2 (woodiness)	Woody	Herbaceous						
Habit 3 (branching position)	Unbranched	From the lower third	From the middle third	From the upper third				
Root system	Branched	Underground stem						
Vegetative scales	Present	Absent						
Plant height	(Actual measurement in m)							
Stem cross-section	Smooth	Ribbed (sulcate)	Winged (alate)					
Plant hairiness (indumentum)	Hairs absent (glabrous)	Hairs present (pubescent)						
Foliage type	Leaves	Scales						
Leaf orientation	Appressed	Spreading	Not applicable					
Leaf attachment	Fused to stem (decurrent)	Not fused to stem (not decurrent)						
Inflorescence 1 - apex	Indeterminate	Determinate						
Inflorescence 2 - structure	Raceme-like	Cymes	Spike-like	Solitary				
Inflorescence 3 - synflorescence flower arrangement combinations	Monochasium	Dichasium	Not applicable					
Flower shape	Cup-shaped (stellate/ patelliform)	Bell-shaped (campanulate)	Tubular					
Involucral bracts	Absent	Present						
Bract fusion to flower stalk (bract recaulescence)	Not fused	Partially fused	Fully fused	Not applicable				
Bract shape	Lanceolate	Linear	Ovate	Deltoid				
Corolla lobe shape	Triangular	Linear						
Flower disc	Present	Absent						
Corolla lobe margin hairiness (indumentum)	Dense hairs	Sparse hairs	Lacinulate	Papillose (ciliate or erose)	Smooth (glabrous)			
Style length	Sessile	Short	Long					
Stigma position	Below the anthers	In line with the anthers	Above the anthers					
Placental column shape	Straight	Twisted						
Fruit length	(Actual measurement in mm)							

ited where necessary to enhance characters using Microsoft PowerPoint software v. 14.0.7229.5000 (Microsoft Corporation). All photographs included here and in the key were taken by the authors unless stated otherwise. Bract shape and placental column shape photographs were supplemented with illustrations to ensure unambiguity (Leggett and Kirchoff 2011).

Taxonomic and character backbone construction

Taxonomic backbone

The first data to be uploaded into Xper[3] were the scientific names of the 25 *Thesium* species included in this study (Fig. 1; Table 1). Sound taxonomy is a crucial prerequisite for identification key construction. Therefore, species concepts and names were taken from the most recent taxonomic treatments of each species, which are provided in Table 1.

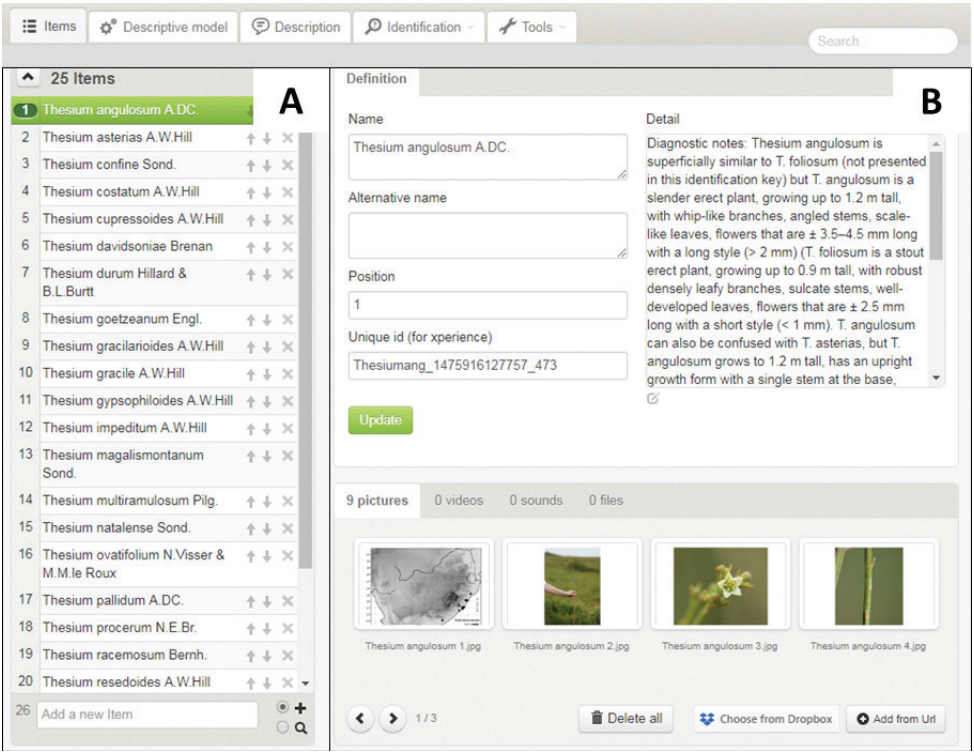

Figure 1. Xper[3] author interface showing **A** the list of 25 *Thesium* species (items) which forms the taxonomic backbone of the interactive photographic identification key, as well as **B** an example of the supplementary information provided for *T. angulosum*.

Character backbone

After the taxonomic backbone was completed the character backbone was compiled. This was done by adding each character and its corresponding character states into Xper[3]. Each character and character state was listed in the key using descriptive terms such as flower shape, style length etc. (e.g., Fig. 2). Generalist terminology was sometimes used and specialist terminology added in brackets where applicable to cater for specialist users. Where needed, terminology was supplemented with textual descriptions further explaining what was being shown (e.g., Fig. 2).

In addition to terminology and textual descriptions, each character and character state was also visually represented with a figure plate containing representative photographs. For example, the character vegetative scales, was illustrated with three photographs; two plants with vegetative scales and one without vegetative scales (Fig. 3). Where possible, each character state was illustrated with multiple photographs to enhance clarity. For example, the branched character state of the root system character was illustrated with three photographs and the underground stem character state with six photographs (Fig. 4). Due to the small and cryptic nature of many morphologi-

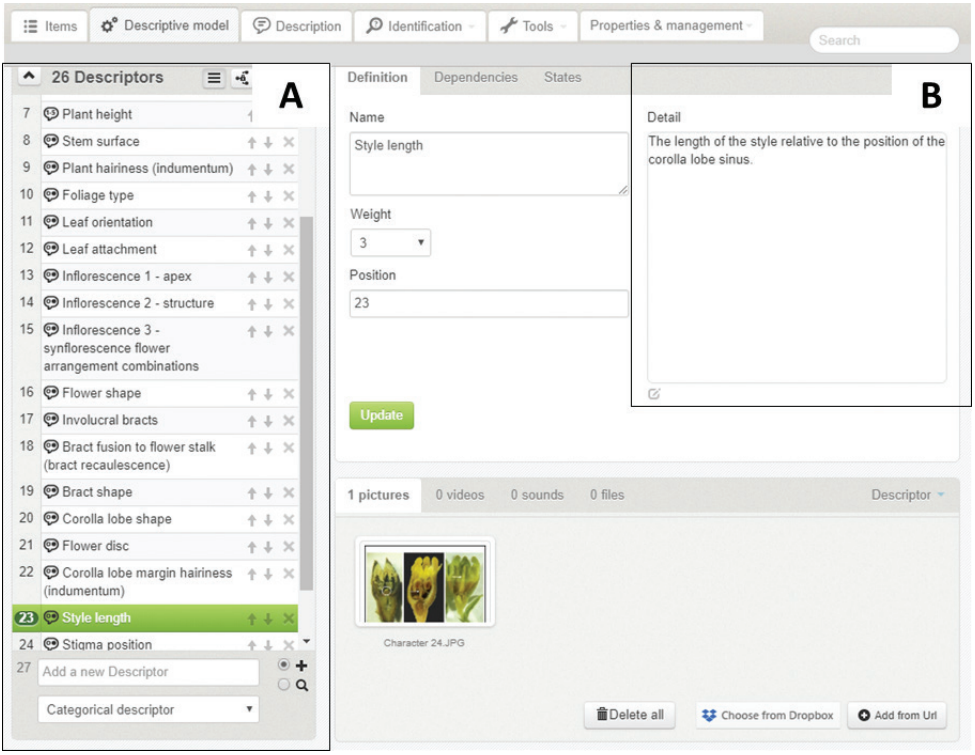

Figure 2. The Xper[3] author interface showing **A** the list of 26 characters (descriptors) which forms the character backbone of the interactive photographic identification key, as well as **B** an example of the supplementary information provided for the character, style length.

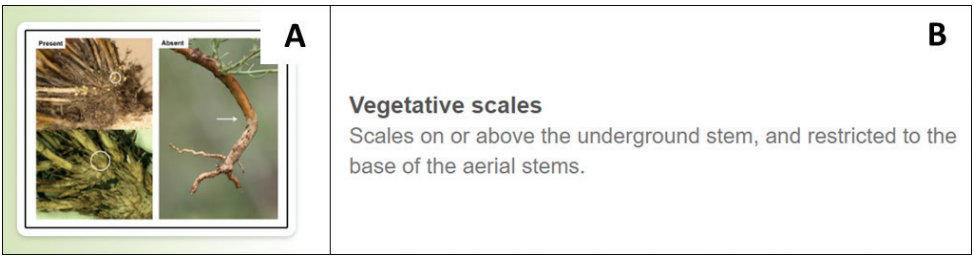

Figure 3. An example of the visual and textual presentation of a character, vegetative scales, in the user interface of the interactive photographic identification key. **A** representative images of each character state (present and absent) of the character, with the relevant structures further emphasized using circles and arrows **B** a textual description of the character.

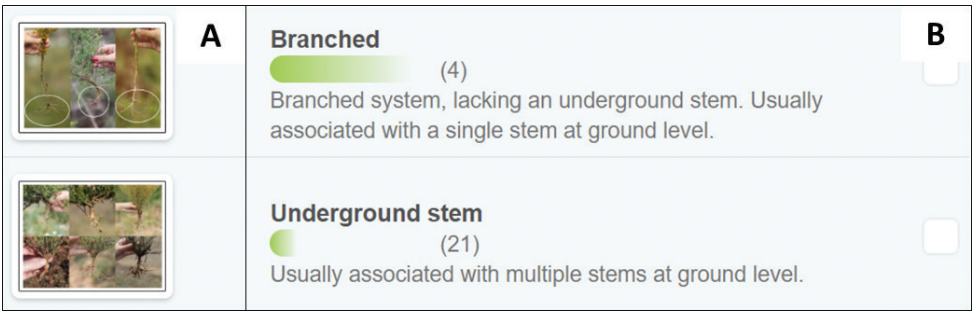

Figure 4. An example of the visual and textual presentation of character states in the user interface of the interactive photographic identification key. For the character, root system, each character state (branched and underground stem) is **A** illustrated with multiple photographs to show variation, as well as **B** a textual description.

cal structures, relevant characters and/or character states were highlighted in certain images, either by a circle or an arrow. Individual images were labelled where needed for clarity. All figure plates were compiled in Microsoft PowerPoint software v. 14.0.7229.5000 (Microsoft Corporation), exported as JPEG files, resized to a standard height of 1000 pixels using FastStone Photo Resizer 3.8 software (FastStone Soft), saved to Dropbox and uploaded into Xper^3 by copying the Dropbox link for each figure plate to the "Add from Url" feature in Xper^3. All photographs and figures were compiled based on the best practices provided by Leggett and Kirchoff (2011).

Character coding

After constructing the taxonomic- and character backbones of the key, character states were manually coded for each species in Xper^3, for example, the style length of *T. angulosum* is long (Fig. 5). The appropriate character states for each species were determined by the authors through examining species in the field, as well as studying herbarium material at the National Herbarium in Pretoria (PRE), South Africa. Subsequent

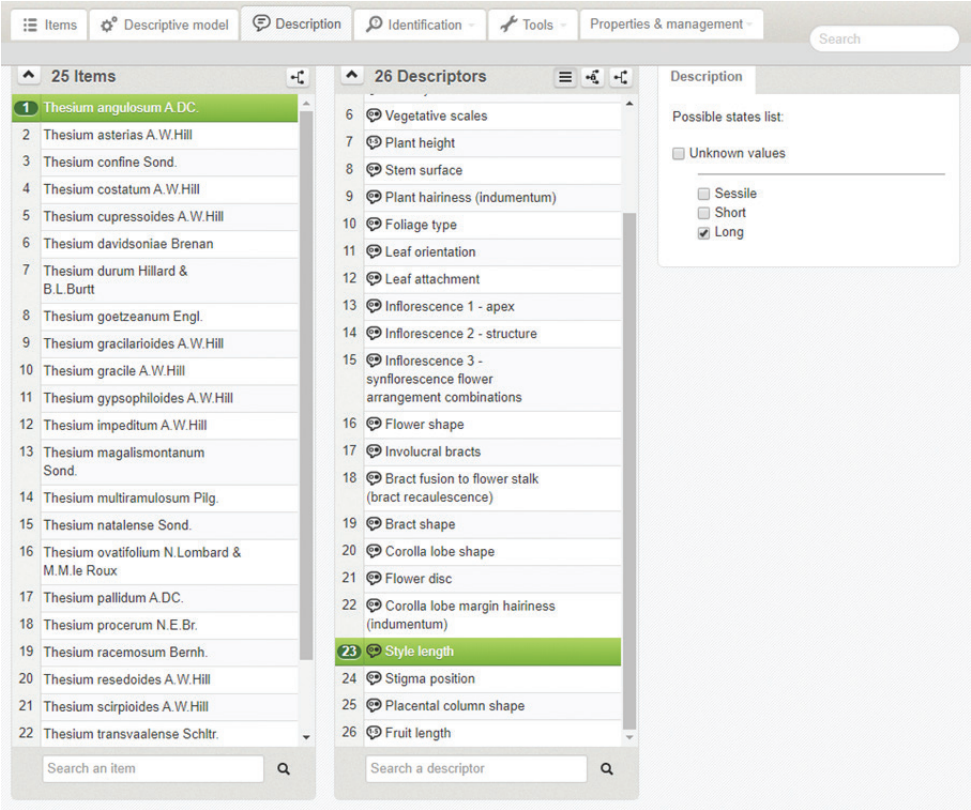

Figure 5. The Xper[3] author interface showing an example of character state coding in the interactive photographic identification key, where the style length of *Thesium angulosum* is coded as long.

knowledge gaps were filled using the most recent taxonomic description available for each species (Table 1). It was occasionally necessary to select more than one character state for a species to account for the intra-specific variation observed in *Thesium* species, as well as differences in user interpretation (see Results and Discussion). Character weighting was not utilized in this study.

Species profiles

The final step in key construction was to create a profile for each species (Fig. 6) that includes contextual photographs, a detailed distribution map, a short diagnosis and a list of character states for that species (automatically generated by Xper[3]). Photographs included here show important diagnostic characters, as well as other general impressions of each species (e.g., the habit and flowers) to aid identification. The short diagnoses give notes on separating morphologically similar species. Where needed, comparisons with similar species not presented in the key were also included to ensure correct identifications despite the nature (pilot study) of the key.

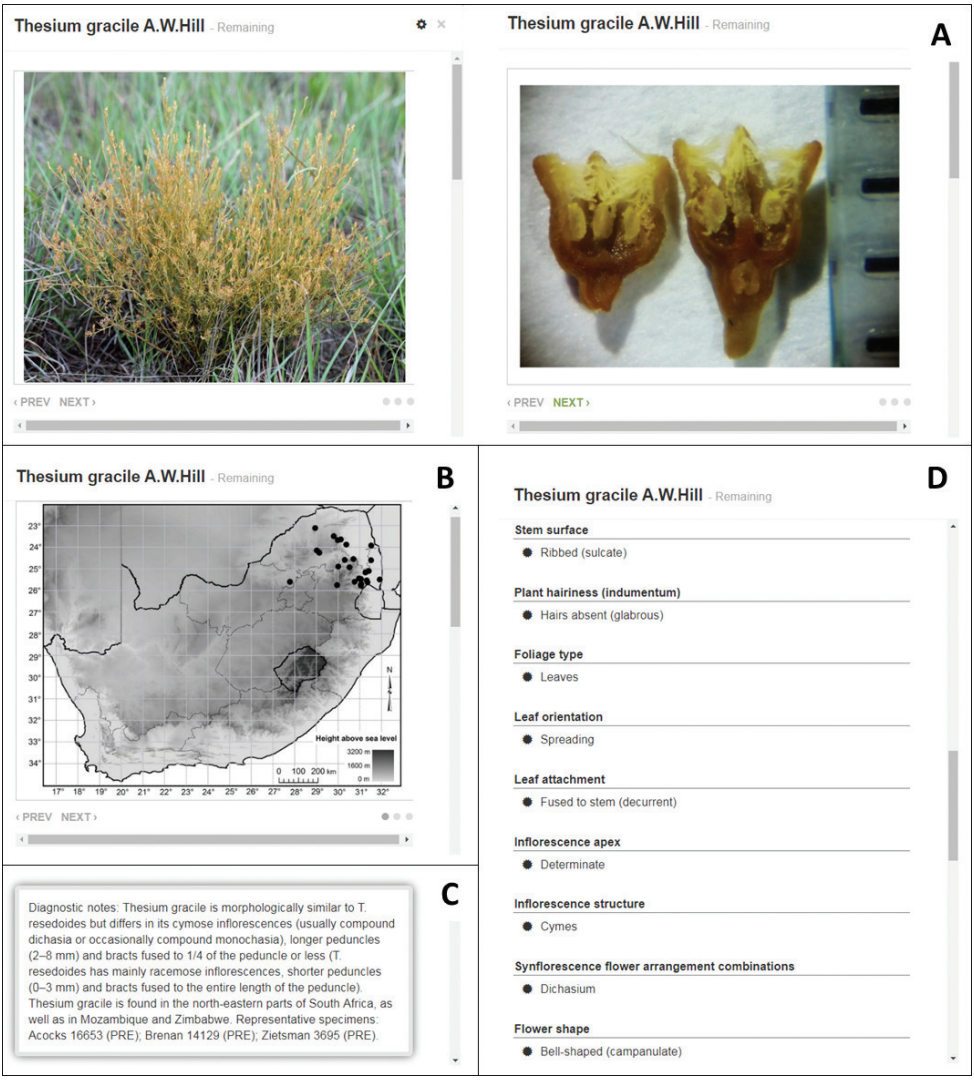

Figure 6. An example of a species profile with supplementary information in the user interface of the interactive photographic identification key, showing **A** representative photographs, **B** a distribution map, **C** diagnostic notes and **D** a list of character states of *Thesium gracile*.

Key evaluation

Target group testing

A target group testing was done at a Plant Specialist Group meeting at Buffelskloof Nature Reserve, South Africa. The 22 participants included amateur botanists, conservationists, ecologists, environmental consultants, horticulturists and taxonomists. Partici-

pants were divided into six groups of three or four and provided with access to the Xper^3 key on their smartphones and laptops, a printed dichotomous key (automated by the Xper^3 platform), a microscope and a flower dissecting kit (a razor blade, tweezers and dissecting needle). Each group was given ample fresh material of three *Thesium* species (*T. confine* Sond., *T. procerum* N.E.Br. and *T. utile* A.W.Hill) and was asked to identify them accurately at their own pace. During the exercise participants provided feedback which allowed characters, character states, terminology and photographs that caused confusion and/or uncertainty to be identified. Participants also gave general feedback on the usability of the key and all of these suggestions were incorporated into an improved key (discussed below). The authors aim to continuously improve the key by trial and revision and also expand the key by systematically adding more *Thesium* species.

Checkbase

In addition to the target group testing, the identification key was also evaluated using Checkbase, a build-in tool provided by Xper^3. Checkbase provides information on discrimination between (1) items (species), (2) descriptions (characters) and (3) character states, as well as (4) missing character states.

Results and discussion

In the current age of information and digital technology more emphasis is being placed on the development of electronic resources to advance the identification of species, which is vital for all practices related to or dependent on biological studies (Walter and Winterton 2007; Kirchoff et al. 2011). One of the remaining challenges in electronic identification key development, namely the effective presentation of cryptic morphological characters to ensure successful identifications in morphologically difficult species groups, was addressed in a pilot study using a *Thesium* identification key. The identification key is accessible through the following link: http://www.xper3.fr/xper3GeneratedFiles/publish/identification/1330098581747548637/mkey.html.

Software platform selection

While Xper^3 software provided a pragmatic platform for key construction in this study, the general principles and best practices discussed hereafter, can be applied to any software with the relevant functionalities (e.g., DELTA, http://www.delta-intkey.com/; Lucid, http://www.lucidcentral.org/). Walter and Winterton (2007), Drinkwater (2009) and Dallwitz et al. (2000) amongst others provide summaries of the general advantages of interactive keys, which are applicable to all electronic keys but not the specific focus of this study.

Multi-access keys

Software platforms with a multi-access approach, where a user can choose any of the available characters throughout the key, circumvent multiple problems associated with the identification of species, especially those with cryptic characters (Walter and Winterton 2007; Drinkwater 2009). Compared to single-access keys, where characters follow on each other in a predetermined order, multi-access keys allow users to select the characters that are available and that they are most confident about, thereby optimizing identification accuracy (Morse et al. 1996; Drinkwater 2009). It also decreases the chances of a user abandoning the identification process altogether due to the cryptic nature of some characters (e.g., a minute ovary that can only be accessed by dissecting a flower) or guessing character states, which might lead to misidentifications (Morse et al. 1996).

Multi-access keys furthermore cater for the unambiguous presentation of cryptic characters and character states by allowing authors to utilize numerous character divisions. For example, the inflorescence structure of *Thesium* species is an important distinguishing character, but often varies considerably and is notoriously difficult to interpret. Its incorporation into traditional textual keys (Hill 1915, 1925) has resulted in several overlapping character states between divisions, the use of vague phrases such as "more or less" and other complex terminology. When taking into account that inflorescence type is only the second division in Hill's key, it is understandable that users have struggled to identify *Thesium* species correctly. In contrast, the electronic key proposed here clearly delineates different inflorescence types using three characters and nine character states with no overlap and also illustrates each division with both visual (including multiple photographs) and text aids.

Updatable keys

Software allowing for updates and changes to be made to identification keys after publication is pertinent for species groups with cryptic morphology as these groups are likely to be taxonomically problematic and subject to ongoing taxonomic study. For example, subsequent to the construction of the identification key presented here, a *Thesium* species new to science was described (Lombard et al. 2019) and a taxonomic revision of two species in the key completed (Lombard et al. in prep.). Information from both these studies was easily incorporated into the database in Xper[3]. The addition of new species to electronic keys is especially important in enigmatic species groups with cryptic characters as the electronic key might be one of the only user-friendly information sources available to non-specialist users. Furthermore, minimizing the lag time between taxonomic research and its availability to the end user, for example through an identification key, might contribute somewhat to alleviating the taxonomic impediment (Walter and Winterton 2007). In addition to research, user feedback and its incorporation is central to a study like the one presented here as user experience is the ultimate measure of both the success of cryptic character presentation and species identifications, and allows for continual improvement of the identification key.

Species profiles

Species profiles with supplementary information and media on each species form part of many software platforms and contribute considerably to accurate identifications (Kumar et al. 2012). Xper^3 species' profiles include, amongst others, contextual photographs, detailed distribution maps and short diagnoses, and can easily be accessed at any point in the identification process. In dealing with species with cryptic morphology, it is recommended that users follow the key until they are uncertain about all of the remaining characters. If more than one species remains, the profiles of the remaining species should be consulted for a final identification (one can flip from one profile to the next in Xper^3) (Drinkwater 2009). Detailed distribution maps are also very useful as they are unambiguous and instantly allow a user to determine whether the species in question occurs in the applicable geographical area. Furthermore, the value of contextual photographs displaying the general impression, size and shape (GISS) of a species should not be overlooked. While two species might differ in only one or two particularly cryptic characters, they are often easily distinguishable by their GISS. "A picture is worth a thousand words" and relays information which is difficult to capture in words. For species with cryptic morphology, photographs are the crux of resolving confusion originating from traditional textual identification keys. Lastly, the short diagnosis provided for each species further streamlines the identification process by providing information on similar species and how they differ from one another (Stevenson et al. 2003). Species profiles can also be used independently of the identification key to confirm species identities or for additional information on a particular species.

Key construction

Character and character state delineation

In this study, maximizing the number of valuable characters while minimizing the number of associated character states proved most pragmatic. Contrary to species groups with clearly defined morphological characters (e.g., Jouveau et al. 2018), maximizing the number character options in morphologically difficult groups provides more opportunities for users to select characters that they are certain about (Walter and Winterton 2007; Drinkwater 2009). One caveat of this approach is that it is time consuming to work through many characters (Stagg et al. 2015). However, algorithms giving continual preference to characters with the most discriminatory power, as is the case in Xper^3, offsets this limitation to some degree (Walter and Winterton 2007; Drinkwater 2009). Furthermore, in challenging species groups, increased identification accuracy should arguably take preference over identification time. In the case of *Thesium* specifically, identification time using the interactive photographic key is unlikely to exceed identification time using the traditional textual keys provided by Hill (1915, 1925).

The electronic key further improves identification efficiency by subdividing particularly confusing and cryptic characters into more digestible units (Drinkwater

2009). During the reconstruction and revision of the identification key, this approach not only resulted in a more user-friendly key but also allowed for more precise character coding (Drinkwater 2009). For instance, the habit (growth form) of *Thesium* species, although often variable (Cohn 2004; Luo et al. 2012; Gamoun 2014), is an important distinguishing character. To improve the unambiguity of this valuable character, habit was divided into three separate characters namely, shape, woodiness and branching position. Furthermore, minimizing the number of character states that users are presented with at each character facilitated ease of use and decreased the chances of incorrect user interpretation.

Character and character state presentation

One of the main advantages of electronic identification keys when identifying species with cryptic characters is the illustration of characters using multiple aids, which greatly reduces ambiguity (Lawrence and Hawthorne 2006; Drinkwater 2009; De Vaugelas et al. 2011). Optimal visual presentation of each character and character state ideally requires sufficient photographs to illustrate the full range of variation, thereby leaving little to no room for user misinterpretation (see Kirchoff et al. 2011). For species with cryptic morphology, electronic key construction therefore goes hand in hand with field observations and photographs of live material. Unfortunately, in the majority of cases, acquiring the necessary photographs remains a major challenge due to resource and logistical constraints, especially in groups with many or rare species. Nevertheless, without adequate visual aids, the efficient and accurate identification of species with cryptic characters is improbable.

It is also true that images may contain only partial information (Joly et al. 2019) and should thus be supplemented with textual aids that are tailored to the requirements of the target audience. In the case of *Thesium* (and likely other species groups with cryptic morphology) the need for a user-friendly identification guide that can be used by both specialist and non-specialist users was immediately apparent. While generalist terminology saves non-specialist users the time and resources needed to familiarize themselves with the workings of a specific group, specialist terminology allows specialists to cross-reference information in the key with other taxonomic literature.

Key evaluation

Checkbase

The Xper[3] evaluation tool Checkbase showed that five species pairs were only partially discriminated: (1) *T. racemosum* Bernh. and *T. costatum* A.W.Hill, (2) *T. gracilarioides* A.W.Hill and *T. multiramulosum* Pilg., (3) *T. gracilarioides* A.W.Hill and *T. resedoides* A.W.Hill, (4) *T. gracile* A.W.Hill and *T. utile* A.W.Hill, and (5) *T. asterias* A.W.Hill and *T. ovatifolium* N.Lombard & M.M.le Roux. These species pairs are morphologically

similar and the coding of multiple character states to account for variation resulted in partial, but not full, overlap in some characters. While this result highlights the challenge of successfully separating species with cryptic morphology using electronic keys (as well as traditional keys), these species can nonetheless be successfully identified using their respective species profiles as discussed before. All of the characters and character states included in the key provided full discrimination between species (as opposed to only partial discrimination or no discrimination). One exception was the Western Cape Province character state under the geographical distribution character, as none of the species included in the key occur in the province. It was, however, retained along with the other eight provinces of South Africa for completeness and to allow for future expansion in the scope of the key.

Target group evaluation

The target group evaluation indicated that the proposed key could be useful for identifying species with cryptic morphological characters and provided valuable suggestions for improvement that were subsequently incorporated. Differences in user interpretation of character states had to be addressed and subsequently, following Kirchoff et al. (2011) and Leggett and Kirchoff (2011), some arrows and/or circles were added. Furthermore, we replaced images causing uncertainty with superior images and incorporated labels.

During the evaluation, it was clear that some characters were problematic. Participants had very subjective interpretations of the degree of woodiness of plants (originally divided into herbs, suffrutices and shrubs) and consequently had trouble identifying the correct character state. To address this unambiguity, the number of character states was reduced to two: plants that were obviously herbaceous (including suffrutices) and robust woody plants. Corresponding textual descriptions were also revised and expanded, and clearer photographs were used to illustrate each character state. Similarly, the difficult-to-interpret inflorescence structure was simplified from six complex character states (e.g., monotelic racemose inflorescence with a terminal dichasial cyme, and simple or compound dichasial and monochasial cymes) to four, more general types (raceme-like, spike-like, cymes and solitary). The majority of participants were not able to utilize the placental column shape (generally < 2 mm) as they could not successfully dissect flowers to access this structure. Although there is little that can be done to improve this hurdle, the character was retained in the key as it is valuable for specialist use, and it is not crucial for species identification so that non-specialist users can simply forgo it.

The last method employed to improve the accuracy of the identification key was the coding of multiple character states (multiple correct answers) where necessary. This step is crucial as it accounts for intra-specific variation in characters, characters with continuous character states and also for user subjectivity (Drinkwater 2009; Stagg et al. 2015). For example, participants had difficulty determining the character state for corolla lobe hairs, partly due to user subjectivity and partly due to the fact that there

is an almost continuous range of character states, from dense hairs to sparse hairs to papilla to smooth lobes. It is suggested that multiple character states are coded where a character state of a species is intermediate between two character states in the key, thereby resolving the problem of continuous characters, as well as subjective user interpretation. This flexibility in coding optimizes the chances of correct identifications without jeopardizing the discriminatory power of the key, as species are separated based on a combination of many characters. However, this approach should be applied conservatively to ensure that overall distinguishing power is not significantly reduced and that characters do not become redundant (see Jouveau et al. 2018). In Xper[3], the key can easily be checked for redundancies using the item 'comparison tool', which indicates whether each character provides full discrimination, partial discrimination or no discrimination between species.

Suggested best practices

Based on the pilot electronic identification key presented here, the following best practices are suggested for the unambiguous presentation of cryptic morphological characters and their character states in electronic identification keys: (1) maximization of the number of valuable characters; (2) minimization of the number of character states associated with each character; (3) division of difficult/complex characters into multiple simpler characters; (4) illustration of characters and character states using multiple aids such as visual and text descriptions; (5) illustration of character states using multiple photographs to show the entire range of variation (if applicable); (6) use of photographs of live material (as opposed to preserved material) and plants in situ where possible; (7) addition of labels and accents such as arrows or circles to photographs to highlight relevant characters; (8) tailoring text descriptions to the target audience(s) (generalist or specialist terminology, or both); and (9) coding for multiple character states (multiple correct answers) where intra-specific variation is present or if a species falls on or close to the border between two character states (to ensure that the discriminatory power of characters is not lost).

Other general best practices include: (10) ensuring sound taxonomy and clearly defined species concepts prior to key construction; (11) using software that allows for updates and improvements (as necessitated by user feedback and ongoing research), including the replacement of images with superior ones as they become available; (12) utilizing a multi-access key approach [as opposed to a single-access approach (dichotomous or polychotomous)]; (13) using species profiles with representative photographs and supplementary information including (14) photographs of diagnostic features and the general impression, size and shape (GISS); (15) detailed distribution maps (if species are geographically separated) and (16) diagnostic notes separating morphologically similar species; and (17) evaluation of proposed identification keys by participants from the target audience and the subsequent incorporation of feedback prior to publication.

Conclusions

Electronic identification keys are valuable resources for species identification, which underpins all biological sciences. This study contributes to the rather limited body of knowledge on the successful identification of enigmatic species with cryptic morphologies using contemporary identification aids. It has shown that well-constructed electronic identification keys are feasible and offer the possibility of accurate identifications, in particular for species with cryptic characters, despite apparent contradictory reports in the literature. We have gained valuable insights into not only the problems and challenges associated with the successful identification of *Thesium* species (as a practical example of species groups with cryptic morphology) but also possible solutions and circumventions for difficulties in electronic key construction.

Ultimately a sound knowledge of the taxonomy and diagnostic characters of the taxa will determine the quality and efficacy of the identification key, regardless of the technology used in its construction and presentation. High attention to the presentation of the characters and their respective states are critical. There is no substitute for careful field studies of live organisms in their natural environment to overcome the typical limitations imposed by preserved specimens. This means a much greater effort in data collection but also a much greater reward in achieving a high level of discriminatory power in the identification key. Such electronic identification keys maximise the benefits that can be derived from the use of digital images and undoubtedly increase the accuracy of identification and reduce ambiguities that lead to a more user-friendly product for both specialist and generalist users. This might be especially valuable in economically important species groups such as grasses, which are characterised by cryptic morphological characters, by expanding the suit of potential users to farmers, conservationists, ecologists and so forth. The gap between research and users can also be minimised by adding the latest information on subjects such as synonyms, ecology and potential uses to species profiles.

To our knowledge, the best practices suggested here (although a combination of novel and previously known guidelines) are the first guidelines on electronic identification key construction tailored to species with cryptic morphology. While these guidelines work well for *Thesium*, similar studies of other species groups with cryptic morphologies will test these best practices, and likely reveal additional challenges and guidelines. This study therefore serves as a starting point for similar studies.

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RESEARCH ARTICLE

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Thismia belumensis (Thismiaceae), a remarkable new species from The Royal Belum State Park, Gerik, Perak, Peninsular Malaysia

Mat Yunoh Siti-Munirah[1], Zainol Suhaimi-Miloko[2], Mohammad Ismail Zubir Ahmad[3]

1 *Forest Research Institute Malaysia, 52109, Kepong, Selangor, Malaysia* **2** *No 49, Taman Desa Damai, 33300, Gerik, Perak, Malaysia* **3** *The Royal Belum State Park, Perbadanan Taman Negeri Perak, Hentian Amanjaya Royal Belum, Pulau Banding, 33200, Gerik, Perak, Malaysia*

Corresponding author: Mat Yunoh Siti-Munirah (sitimunirah@frim.gov.my)

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Abstract

This report describes *Thismia belumensis* Siti-Munirah & Suhaimi-Miloko, a novel species of achlorophyllous herb discovered in the Royal Belum State Park, Peninsular Malaysia. This new species is unlike any previously described species of *Thismia*. In particular, *T. belumensis* possesses a unique annulus, which has been expanded and modified into a cucullate (hood-like) structure. This structure covers the apical floral tube and has an opening on one side facing a thickened part of the annulus, and the off-centre floral aperture confers a zygomorphic symmetry to the flower, indicating *T. belumensis* is more similar to *Thismia labiata* J.J.Sm. This morphological detail makes this new species distinct from all other described species of *Thismia*. In this report, we provide descriptions, illustrations, colour plates, and the provisional conservation status of *Thismia belumensis*.

Keywords

Conservation status, endemic, Peninsular Malaysia, Perak, *Thismia belumensis*, zygomorphic

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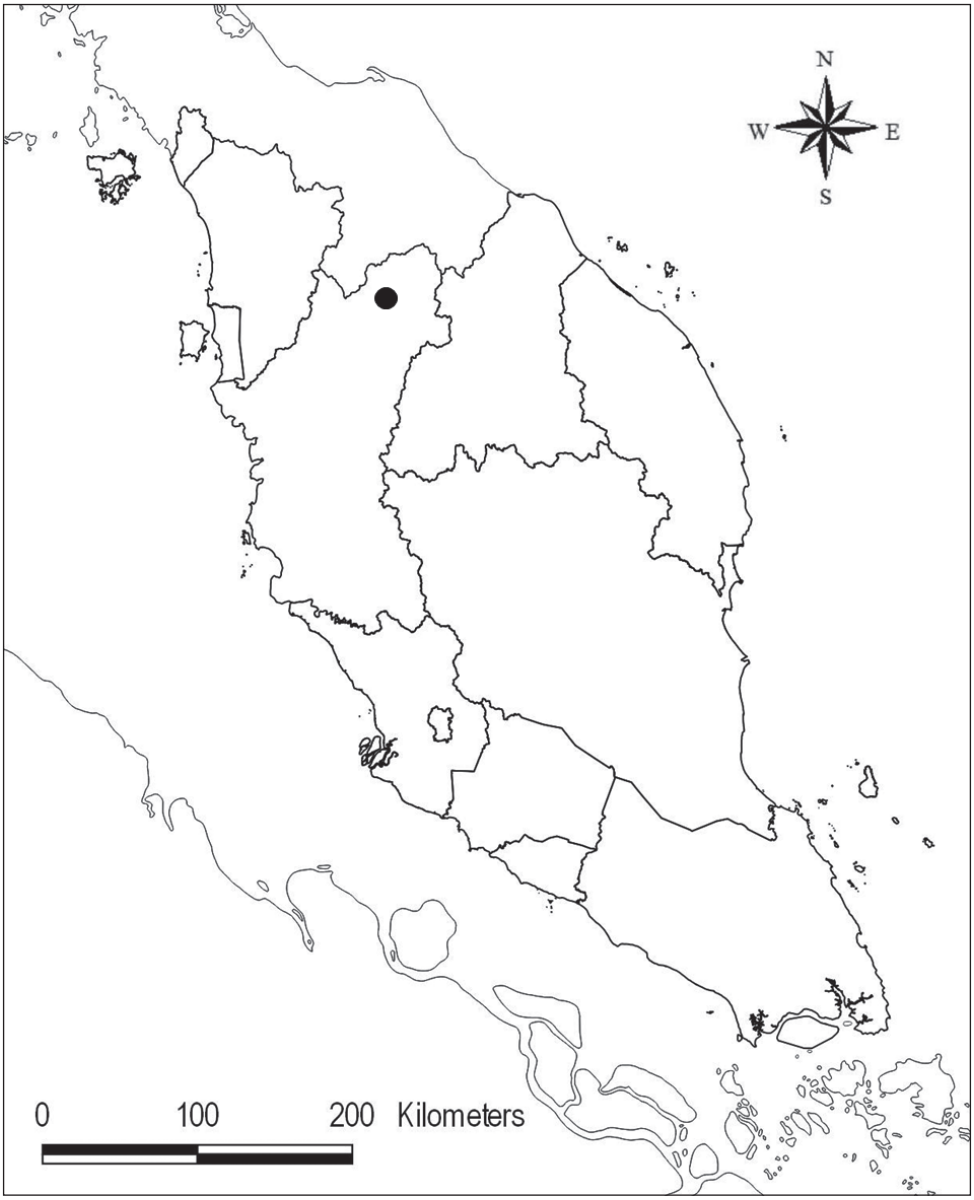
Introduction

Thismia Griff. (Thismiaceae) is a genus of small mycoheterotrophic herbs, currently comprising approximately 80–90 species (Dančák et al. 2020a; Nuraliev et al. 2020; Shepeleva et al. 2020). Plants of this genus are primarily distributed in the tropical regions of Asia, Australia, and South America, and extend into the subtropical and temperate regions of Japan, New Zealand, Australia, and the USA (Merckx et al. 2013; Dančák et al. 2020b). In Peninsular Malaysia, little is known about most *Thismia* species as they are normally only noticeable when in flower, and the flowers can be small, fragile, and ephemeral. The plant is also easily dehydrated and can rapidly degrade if taken from its original habitat. Studying these plants is challenging, as careful and rapid care must be taken to retain the plant's true appearance and structure. Due to their fast degradation, *Thismia* species cannot be handled and studied like typical pressed herbarium specimens. Therefore, to obtain accurate images of their morphology, they must be dissected in the field, or immediately stored in an airtight container for transport to the research station for further investigation, or immediately preserved in 70% ethanol.

For many decades, the knowledge of *Thismia* species in Peninsular Malaysia has not been updated, with Jonker (1948) the most recent taxonomic. Seventy years later, in 2018, a new species description was published – *Thismia kelantanensis* Siti-Munirah (Siti-Munirah 2018). Since then, two more species have been described – *Thismia domei* Siti-Munirah and *Thismia terengganuensis* Siti-Munirah (Siti-Munirah and Dome 2019). It is likely that more novel species will be discovered in the future (Siti-Munirah and Dome 2019). The most recent publication describing Malaysian *Thismia* is from Sarawak (Borneo) and describes *Thismia minutissima* Dančák, Hroneš & Sochor (Dančák et al. 2020a). Overall, throughout Malaysia, there are currently approximately 30 *Thismia* species, of which – including *T. belumensis*, described in this report, – 14 of these are found in Peninsular Malaysia. These are: *Thismia alba* Holttum ex Jonker, *Thismia arachnites* Ridl., *Thismia aseroe* Becc., *Thismia chrysops* Ridl., *Thismia clavigera* F. Muell., *Thismia crocea* (Becc.) J.J.Sm, *Thismia domei* Siti-Munirah & Dome, *Thismia fumida* Ridl., *Thismia grandiflora* Ridl., *Thismia javanica* J.J. Sm, *Thismia kelantanensis* Siti-Munirah, *Thismia racemosa* Ridl. and *Thismia terengganuensis* Siti-Munirah (Jonker 1948; Siti-Munirah 2018; Siti-Munirah and Dome 2019).

The *Thismia* species described here was first discovered in 2017 by Mr. Suhaimi-Miloko during a guided nature tour in the Royal Belum State Park (SP), Perak, Peninsular Malaysia (Map 1). The majority of the Royal Belum SP is covered by pristine primary tropical rainforest and was established as a 117,500 ha forest park in 2007 by the Perak state government. Royal Belum SP is the second-largest protected area in Peninsular Malaysia – after the Taman Negara (431,435 ha) (Schwabe et al. 2015). The new species of *Thismia* was discovered in the Sungai Gadong forest area, in the southern part of the Royal Belum SP. This location is also an important habitat of *Rafflesia azlanii* Latiff & Wong, and *R. cantleyi* Solms, which possess the world's largest



Map 1. Map of Peninsular Malaysia indicating the location of *Thismia belumensis* (●).

flowers (Siti-Munirah 2012, 2020). In October 2019, we visited the site, and several specimens of the new *Thismia* species were collected for taxonomic study. After careful examination, some novel characteristics of the annulus and tepals were identified in the specimens. These traits formed a unique combination of characteristics that did not match any described species of *Thismia*. Hence, it is described here as a new species.

Materials and methods

This study is based on material collected by M.Y. Siti-Munirah in October 2019 from The Royal Belum SP, Gerik. The specimens were preserved in 70% ethanol in the Kepong (KEP) herbarium collections. Morphological characteristics were studied using a stereomicroscope and high-resolution macro photography. Measurements were taken from both living and alcohol-preserved material. The specimen details were thoroughly compared with drawings and descriptions in the protologues of *Thismia* species worldwide.

Taxonomic account

Thismia belumensis **Siti-Munirah & Suhaimi-Miloko, sp. nov.**
urn:lsid:ipni.org:names:77215193-1
Figs 1–3

Diagnosis. Most similar to *Thismia labiata* J.J.Sm. but differing in the floral tube partially covered by a massively expanded cucullate bilabiate annulus, longer outer tepals appendage, obovate to spathulate rather than linear to filiform inner tepals and the supraconnective apex curved outwards like a skirt rather than straight.

Type. Malaysia. Peninsular Malaysia: Perak, Gerik Distr., Royal Belum State Park, ca. 290 m alt., 22 October 2019, *M.Y. Siti-Munirah FRI 94758* (holotype: KEP!, spirit collection, No. barcode 279998).

Description. Terrestrial, achlorophyllous, brownish-whitish-green herbs up to 8 cm tall. ***Roots*** vermiform white-brownish. ***Stems*** erect (sometimes ascending), unbranched, 2–4 cm long, glabrous. ***Leaves*** scale-like, simple, translucent white, 5 mm long, 1–2 mm wide, triangular, apex acute or acuminate, base appressed. ***Involucral bracts*** 3, white, up to ca. 1 cm long, lanceolate, apex acute to acuminate, margin entire, glabrous, base appressed. ***Pedicel*** 2–4 mm long (post anthesis). ***Flowers*** terminal, zygomorphic, solitary, 5–8 cm long (including appendages); ***floral tube*** **(hypanthium)** 5–7 mm long, 3–6 mm wide, narrowed just above the ovary ca. 3 mm wide, widest on upper part ca. 6 mm wide, slightly shorter at one side (lower on the floral aperture side), ***outer surface*** verrucose covered with very short minute warts (papillae); basal half green, apical half with 12 dark brown and 12 pale brown (almost translucent) vertical stripes; ***inner surface*** smooth without transverse bars and other ornamentation; emerald green and translucent; ***outer tepals*** 3, pale brown each 3 × 2 mm, apex acute, each with forming a distal filiform, tentacle-like appendage, the two on the opposite side of the annulus opening erect and the one below the thickened annulus slender, ca. 1.5–2.5 cm long, ca. 1 mm wide, cylindrical, brownish-greenish and sometimes whitish towards the subulate tip; ***inner tepals*** 3, brownish to pure white, glabrous (smooth), obovate to spathulate, tightly adpressed and almost completely overlapping the cucullate part of annulus, basally with long and erect appendages, ca. 3 cm long, brownish-greenish and sometimes whitish towards clavate tip. ***Annulus*** expanded and modified into a cucullate (hood-like) structure and thickened part of

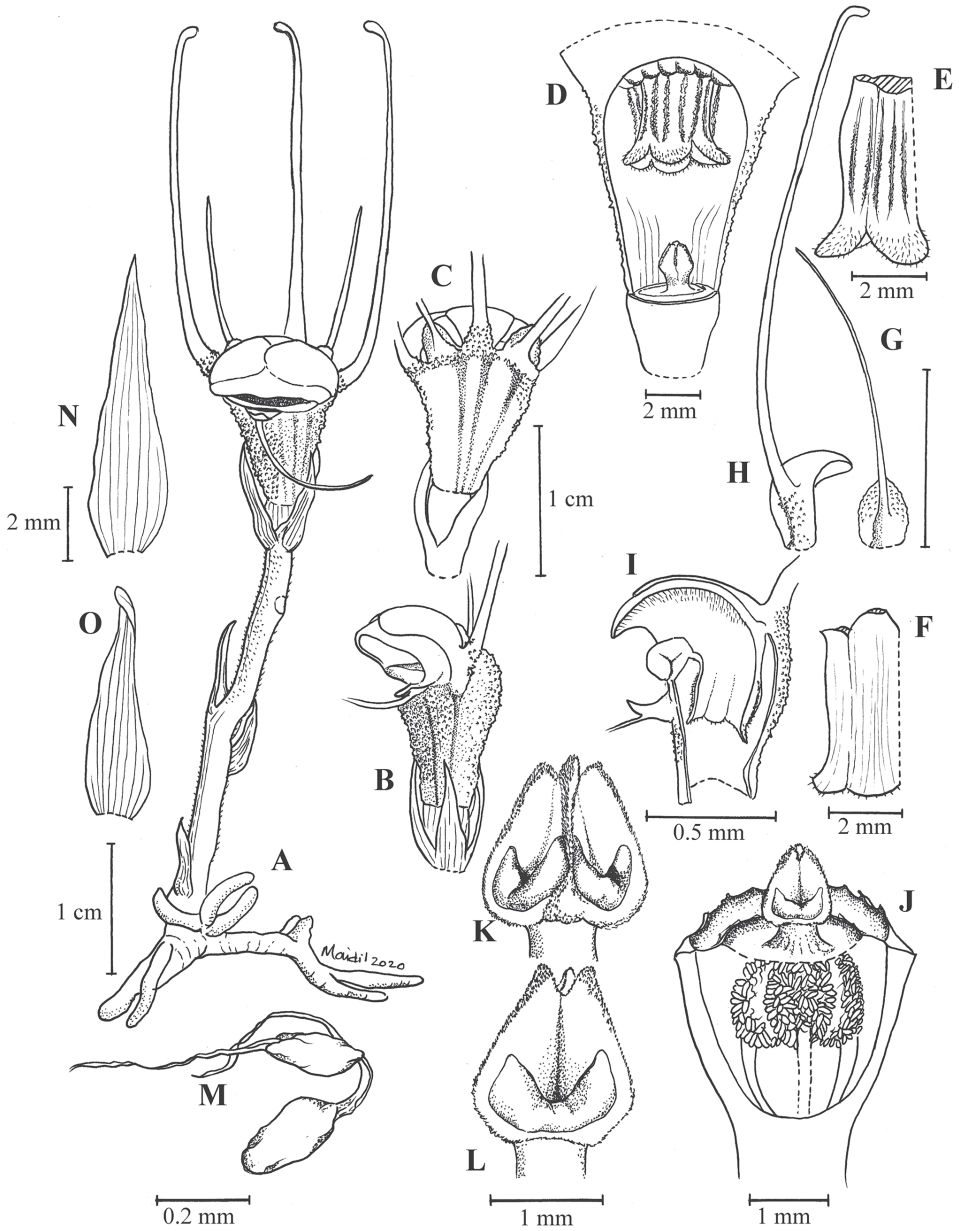


Figure 1. Illustration of *Thismia belumensis* Siti-Munirah & Suhaimi-Miloko **A** habit **B** side view of flower **C** back view of flower **D** longitudinal section of the floral tube showing pendulous stamen with ovary and stigma **E** outer view of stamens **F** inner view of stamens **G** outer tepal **H** inner tepal **I** longitudinal section of hood (annulus) and pendulous stamens **J** longitudinal section of ovary **K** stigma **L** pistil **M** ovules **N** bract **O** leaf. All from *FRI 94752* & *FRI 94758*, drawn by Mohamad Aidil Noordin.

the annulus, the cucullate covering the apical part of the floral tube and forming a downwards floral aperture and facing thickened part of the annulus on one side of the flower; cucullate outer surface white with 3 black lines, glabrous; cucullate inner

surface white to brownish-peach, covered with numerous white translucent trichomes pointing inwards. ***Stamens*** 6, pendent from the apical part of the floral tube; each connective ca. 3.3 mm long, peach; outer surface with two linear to filiform thecae, each 1.8 mm long, facing the inner wall of the floral tube; inner surface smooth; supraconnective apex blunt and rounded, curved (like a skirt) outwards, covered with transparent trichomes on margin (apparent only in the living state); lateral appendage, apical appendages and interstaminal glands absent; ***filaments*** short, connected to floral tube and annulus. ***Ovary*** inferior, unilocular, cup-shaped, ca. 3 mm × 4.7 mm, whitish brown outer surface covered with numerous warts; ***placentas*** 3, bearing numerous ovules; ***style*** ca. 0.4 mm long; ***stigma*** triangular-pyramidal, dark greenish, ca. 1.55 mm long, papillate, unusual whitish thickened part on each surface (sometimes difficult to see), apex truncate (trilobed). ***Fruit*** cup-shaped, white or pale brown with white operculum, pedicel not elongated.

Distribution. Endemic to Perak, Peninsular Malaysia. Currently known only from the type locality (Map 1).

Ecology. Lowland dipterocarp forest, under shade, 260–290 m altitude. Flowering from June to October. *Thismia belumensis* was found growing within tree leaf litter and between the buttress roots of large trees (Fig. 4). Two other *Thismia* species were also discovered within a radius of about 350 meters, *T. javanica* and *Thismia* sp. 1 (see Fig. 5), which is currently suspected by the authors to be *Thismia arachnites* Ridl., but further examination is still needed. All plants were found close to the walking trail.

Etymology. The species is named after The Royal Belum State Park, the type locality of this species.

Conservation status. Critically Endangered (B1B2ab(iii)). Following the IUCN Standards and Petitions Committee (2019), this species is assessed as critically endangered as it is only known from the type locality and is certainly endemic and rare. Fewer than ten specimens were observed, including flowering and fruiting individuals. Although the locality is within the State Park forest reserve, a protected area, the area is located beside the walking trail, which is a common visiting site for tourists in the Park. The site where *T. belumensis* was found is currently designated as a Tourist Zone in the Royal Belum State Park Management Plan 2018–2027. All guides can freely bring tourists to the area without special permission from the Perak State Park Corporation. Due to the small size of *T. belumensis*, it may not be noticed, and so has a high chance of being stepped on. Without official reminders or guidance, the existence of *T. belumensis* might be threatened by ecotourism activities. *Thismia belumensis* qualifies for CR (B1B2ab(iii)) for its single location, EOO less than 100 km^2, and AOO less than 10 km^2. Its habitat quality is also threatened by wild boars (personal observation by Suhaimi-Miloko) and other destructive activities, such as regular visits by tourists to the area. Together, these have the potential to cause a population reduction.

Additional specimen examined. Peninsular Malaysia. Perak: Gerik, Royal Belum State Park, ca. 260 m alt., 22 October 2019, *M.Y. Siti-Munirah FRI94752* (KEP, spirit collection, No. barcode 279997).

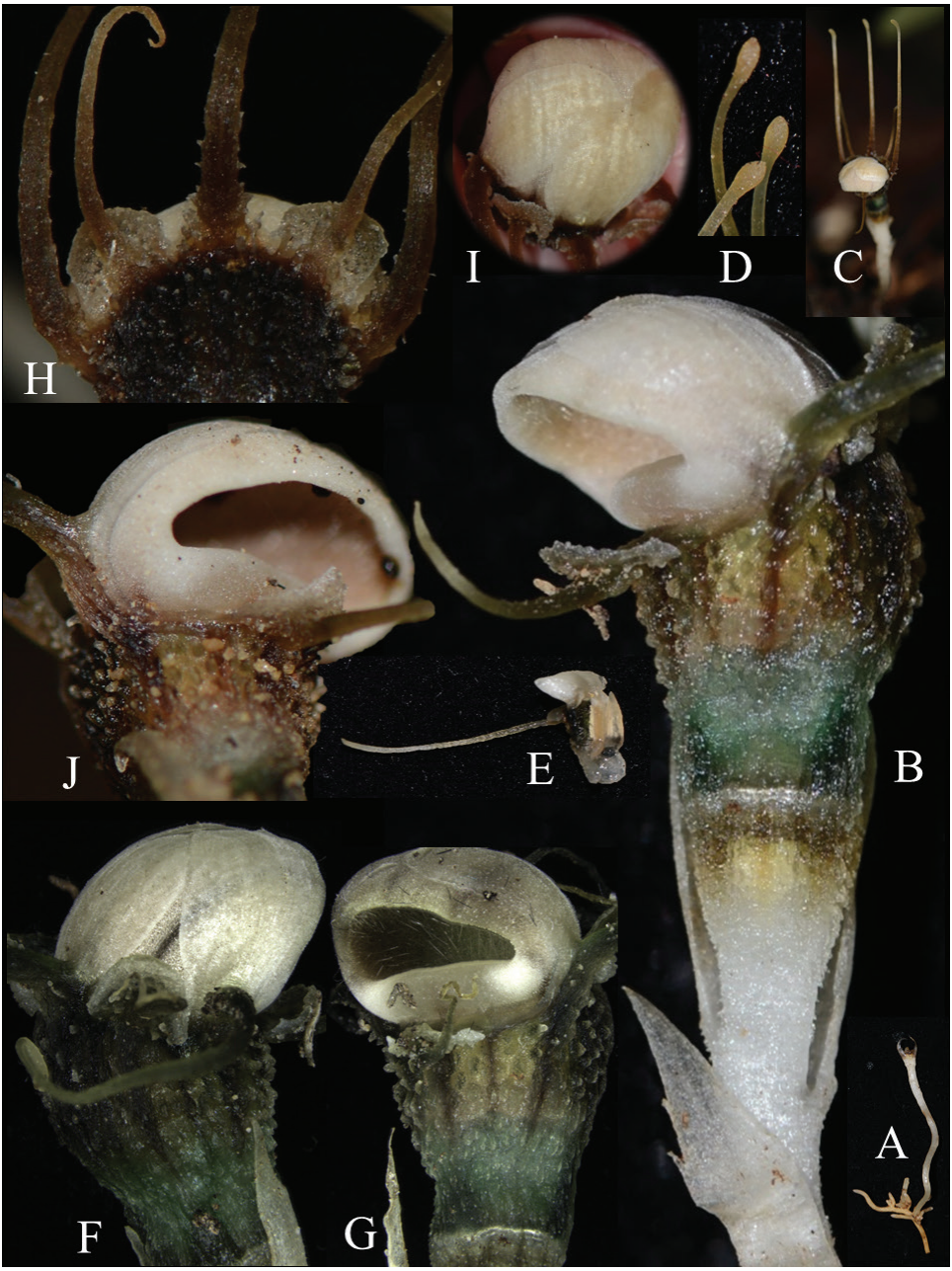


Figure 2. Outer appearance of *Thismia belumensis* Siti-Munirah & Suhaimi-Miloko **A** root and stem **B** flower from side view **C** flower showing the erect tepal appendages of the live plant **D** tip of erect tepal appendages **E** slender appendage of outer tepal **F** side view showing inner tepals overlaying the cucullate structure **G** floral tube with the floral aperture of the expanded annulus **H** outer tepals alternate with inner tepals, each with distal or dorsal appendage **I** top view of flower showing inner tepals overlapping on cucullate structure **J** semi-round shape of the aperture of the annulus (All photos by Siti-Munirah MY, *FRI 94752* & *FRI 94758*).

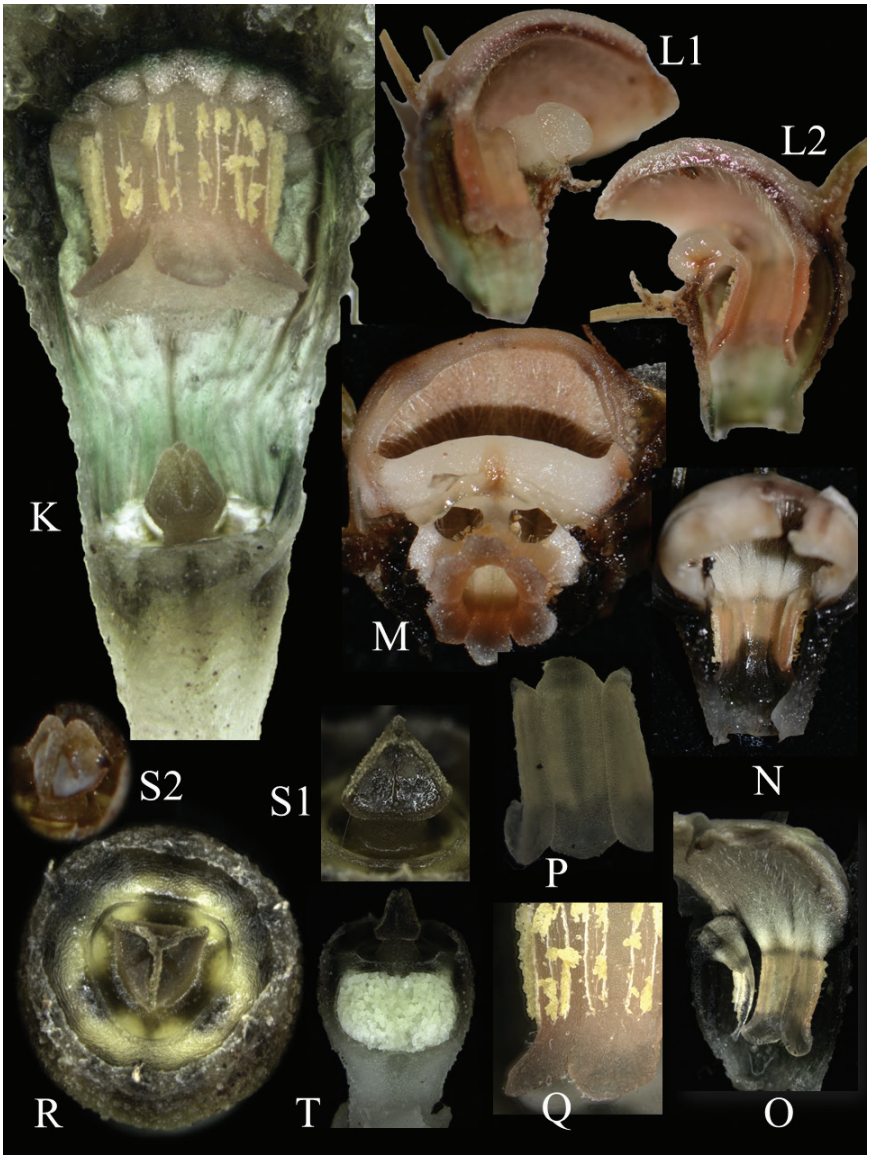


Figure 3. Inner flower appearance of *Thismia belumensis* Siti-Munirah & Suhaimi-Miloko **K** longitudinal section showing the internal parts of flower **L** longitudinal section of floral tube and apical part (**L1** left side **L2** right side) showing pendulous stamen attached to the filament, expanded annulus developing a cucullate (hood-like) structure, thickened part of the annulus at one-side, and outer surface of hood overlaid by inner tepals **M** transverse section of floral tube at lower part showing the apex of fused pendulous stamen and the floral aperture with the thickened part of the annulus in the centre **N** inner view of stamens and filament attached to the expanded annulus, which is covered with white trichome hairs **O** inner view of two stamens and hood inner surface **P** stamen (from inner view) **Q** stamen displaying linear to filiform thecae (outer view) and supraconnective apex curved outwards **R** top view of ovary and stigma **S** stigma from side view (**S1** shows triangular-pyramidal **S2** shows whitish part on surface) **T** cross-section of ovary showing young seeds (All photos by Siti-Munirah MY, *FRI 94752* & *FRI 94758*).

Figure 4. *Thismia belumensis* Siti-Munirah & Suhaimi-Miloko **A** habitat **B** *T. belumensis* growing in leaf litter, *FRI 94758* **C** habit of the young flowering plant and the young fruit of *T. belumensis* **D** fruits of *T. belumensis*, in-situ (Photos by **A–C** Siti-Munirah MY **D** Suhaimi-Miloko Z).

Discussion

This report represents the first description of a novel, notable, and very rare plant – *T. belumensis* – from The Royal Belum SP, Perak, Malaysia. This new species is significantly different from other "fairy lantern" species found in the Malay Peninsula and Borneo. Morphologically, *T. belumensis* is strikingly different from other species by its annulus structure, tepals with an appendage, and floral colours. Nuraliev et al. (2020), describe the annulus as a large, fleshy ring-like structure. However, *T. belumensis* possesses an unusual form of annulus. *T. belumensis* is characterised by most of the annulus being hugely expanded and modified into a cucullate (hood-like) structure covering the apical part of the floral tube, with an aperture on one side facing a thickened part of annulus. The off-centre floral aperture changes the flower symmetry to bilateral, or zygomorphic. In this way, *T. belumensis* is unlike all other known *Thismia* species in Malaysia, which have radially-symmetrical flowers in which the annulus, when developed, has an apical aperture (Fig. 5).

Thismia belumensis is morphologically similar to *Thismia labiata* J.J.Sm. (Smith 1927) and *Thismia sahyadrica* Sujanapal, Robi & Dantas (Sujanapal et al. 2017). All three species share several unique characters, such as a zygomorphic flower with a sideways facing annulus orifice (*T. belumensis* and *T. labiata*), filiform and linear thecae (*T. belumensis*, *T. labiata* and *T. sahyadrica*), a curved supraconnective (*T. belumensis* and *T. sahyadrica*), and a lack of a lateral appendage and interstaminal glands (*T. belumensis*, *T. labiata* and *T. sahyadrica*). However, most other morphological characteristics of *T. belumensis* are unique. Both *T. belumensis* and *T. labiata* have two types of tepals and appendages; however, these differ between the species. *Thismia belumensis* tepals are unique for the false mitre formed by the inner tepal (loose mitre, if the cucullate structure is absent) (Fig. 2F). With the presence of the cucullate structure, the inner tepals of *T. belumensis,* therefore, completely overlay the outer surface of the cucullate structure (Fig. 2F, I). In *T. labiata*, the annulus forms a labiate structure – a thick, fleshy, upper lip bent over the opening of the floral tube (Fig. 6A), with one inner and two outer tepals of the back of this lip (Fig. 6A, B). There are two more inner tepals between the two lips – one on either side of the flower – and the third outer tepal is inserted in the middle of the lower lip (Fig. 6A, B). In contrast, the outer tepal of *T. belumensis* appears upright (Figs 1G, 2H) with the inner tepals (with erect appendages, Fig. 2C), and with only one slender tepal and appendage (Fig. 2E), attached below the thickened part of the annulus (Figs 2B, 3L2). Both types of tepal in *T. belumensis* have their own form of appendage, and both were much longer compared to appendages of *T. labiata* (Figs 1G, H, 6B–D). Based on other records, the morphology of the outer tepals (also call lateral tepals, Smith 1927) and the appendage of *T. labiata* are more similar to the tepals of *T. sahyadrica*. Additionally, while the inner tepals of *T. labiata* are linear to filiform (appearing as a long appendage without a tepal, Fig. 6A, B), this characteristic is different in *T. belumensis*, inner tepals obovate to spathulate with a ca. 3 cm long appendage (Figs 1H, 2F). Furthermore, the *T. belumensis* stigma morphology is nearly similar to *T. labiata* but completely different from *T. sahyadrica*. Finally, all

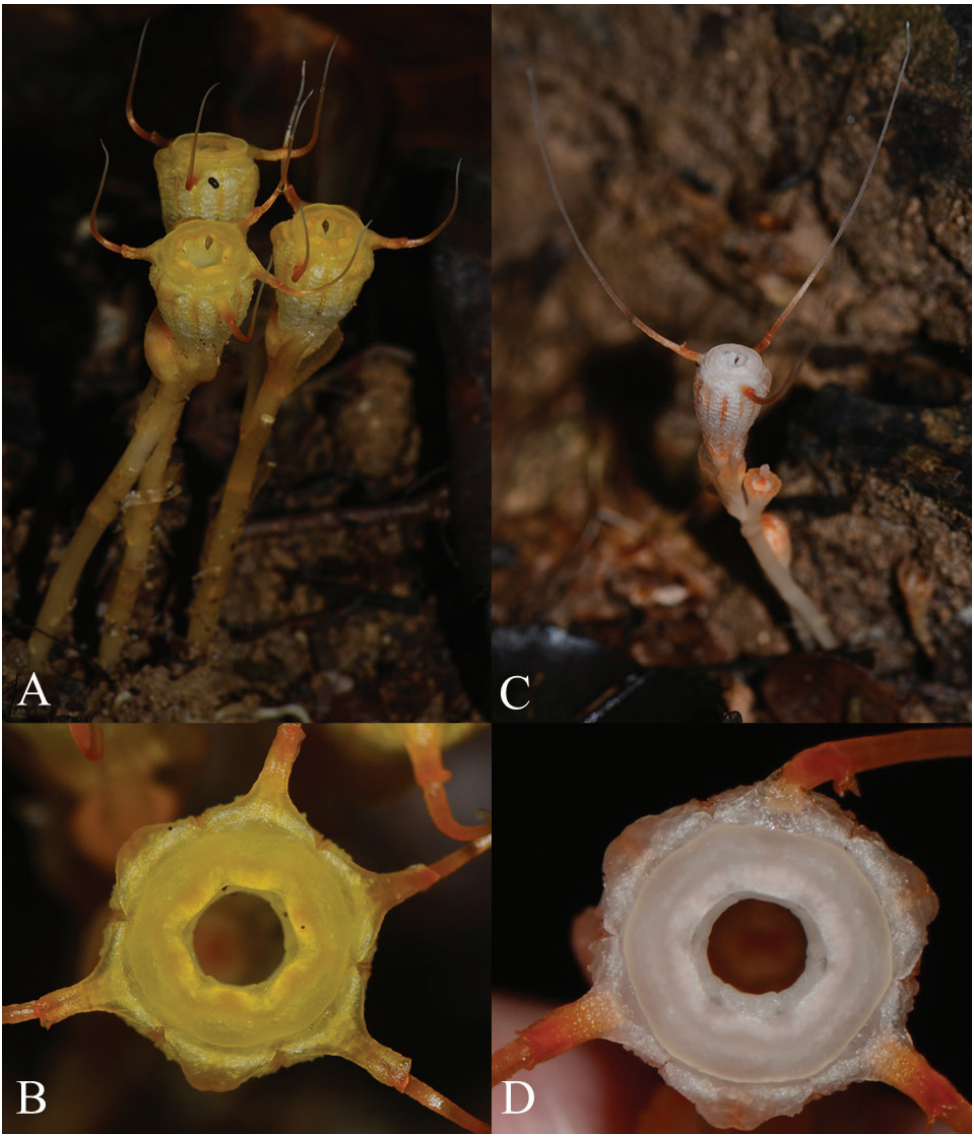


Figure 5. Other *Thismia* species found in the same area as *T. belumensis* **A** *Thismia* sp. 1, undescribed species **B** top view of *Thismia* sp1. showing the annulus and tepals **C** *Thismia javanica* **D** top view of *T. javanica* showing the annulus and tepals (symmetrical flower) (All photos by Siti-Munirah MY).

three species have vermiform roots, with both *T. belumensis* and *T. labiata* possessing simple vermiform roots with and single axis. By contrast, *T. sahyadrica* possesses vermiform roots with clustering at the base of many stems. In summary, there is no doubt that *T. belumensis* is unique, but morphologically closest to *T. labiata* – especially with its zygomorphic flower. However, *T. belumensis* has certain other morphological characteristics that indicate a different position and relatives in *Thismia*.

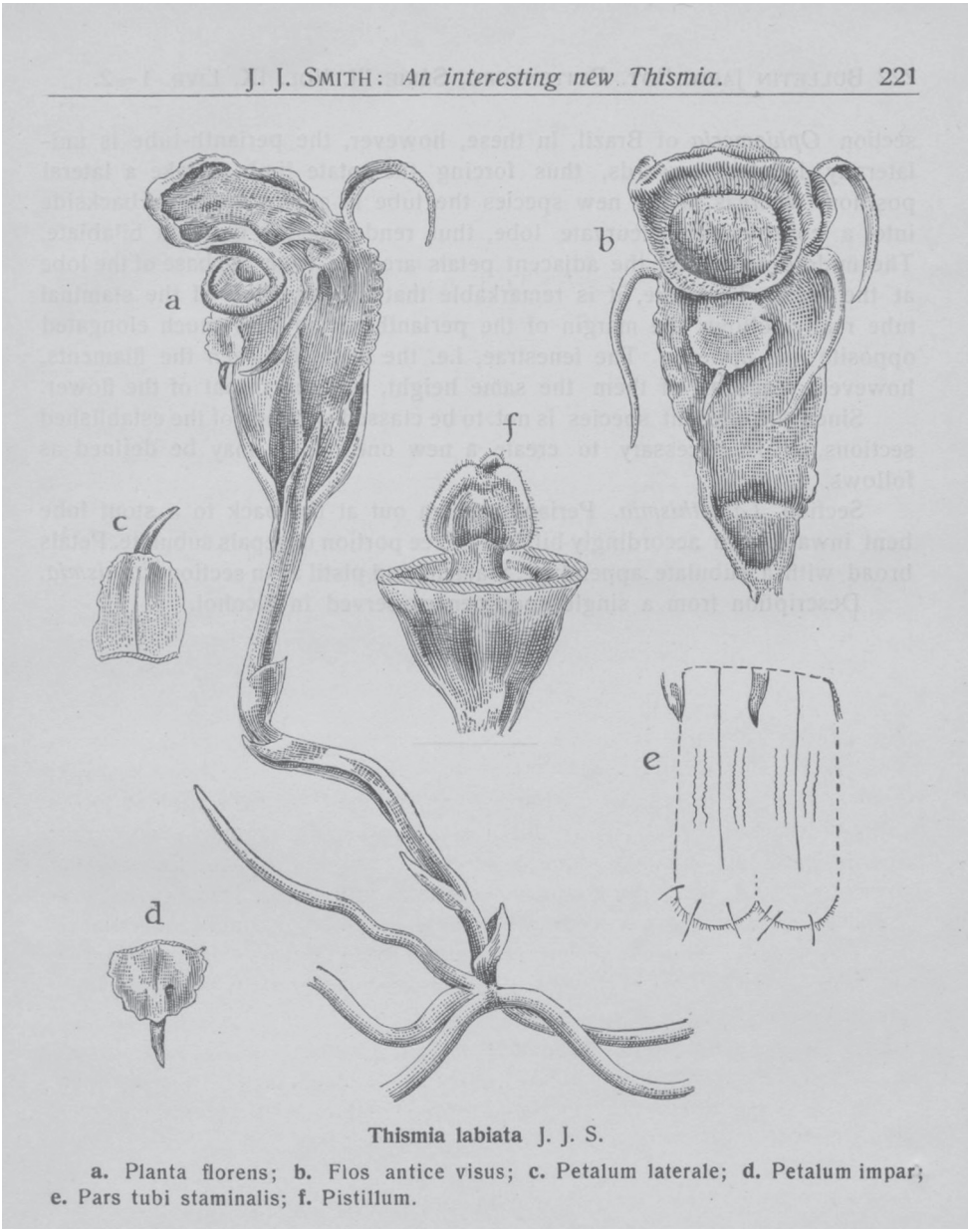


Figure 6. Drawing from the original protologue of *Thismia labiata* (Smith 1927: 221) **A** plant blossoming **B** flower in front view **C** lateral tepal (outer tepal) **D** odd tepal (outer tepal) **E** part of the stamen tube **F** pistil.

As discussed above, *T. belumensis* resembles *T. labiata* (Fig. 6) – a Sumatran (Indonesia) species for which own section *Labiothismia* was proposed (Smith 1927) – in its growth and overall appearance (i.e., bilaterally symmetrical flowers and stamens with elongated thecae). However, it must be noted that the *Labiothismia* section is not recognised by Kumar et al. (2017), which instead places *T. labiata* in subsection *Brunonithismia*.

Thismia belumensis also belongs to subgenus *Thismia* and potentially belongs to subsection *Brunonithismia* along with *T. labiata* (Kumar et al. 2017). However, the accuracy of this placement is uncertain, and *T. belumensis* probably cannot be included in subgeneric classification proposed by Kumar et al. (2017). Further investigation is greatly needed.

Thismia belumensis could also potentially belong in section *Glaziocharis* (Shepeleva et al. 2020). The most prominent trait of species in this section is an absence of the lateral appendage, but other traits also suggest this placement; for example, the presence of a false mitre and the absence of transverse bars inside the hypanthium, both observed in *T. belumensis*. The absence of interstaminal glands and a lateral appendage (also called a wing-like appendage) suggest that all three species' (*T. belumensis*, *T. labiata* and T. *sahyadrica*) are closely related to species forming clade 1 in Shepeleva et al. (2020). Whether they are encapsulated within this section or form a distinct sister group must be resolved by phylogeny studies.

In conclusion, the significant differences and unique traits of *T. belumensis* – i.e., the floral tube detail, the annulus structure, the morphology of the tepals and appendages, and the stamens – compared to other *Thismia* species worldwide, strongly supports the recognition of this remarkable new species. Further study of this new species, such as, its life cycle, pollination system, and including molecular analysis, is crucial to gain new knowledge and understanding of the phylogenetic and biogeographic of *Thismia* of the world.

Acknowledgements

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Notes on leaf micromorphology of the rare herbaceous bamboo *Buergersiochloa bambusoides* Pilg. (Olyreae, Poaceae) from New Guinea and its taxonomic implications

Jamile F. Lima[1], Kelly Regina B. Leite[2], Lynn G. Clark[3], Reyjane P. Oliveira[1]

1 *Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Avenida Transnordestina s/n, Novo Horizonte, 44036-900, Feira de Santana, BA, Brazil* **2** *Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, 668, Campus de Ondina, 40170-115, Salvador, BA, Brazil* **3** *Department of Ecology, Evolution, and Organismal Biology, Iowa State University, 251 Bessey Hall, Ames IA 50011-4009, USA*

Corresponding author: Jamile F. Lima (jamilefelima@gmail.com)

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Abstract

We present notes on the leaf micromorphology of *Buergersiochloa bambusoides*, a rare species from New Guinea and included in Buergersiochloinae, one of three subtribes of the herbaceous bamboos (tribe Olyreae). We used scanning electron microscopy and light microscopy to analyze the microcharacters of both adaxial and abaxial leaf surfaces. Within the Olyreae, saddle-shaped silica bodies in both the costal and intercostal zones are considered unique to Buergersiochloinae. Simple, circular and very small papillae are observed on the adaxial surface, and for the first time, branched papillae on the abaxial surface are observed in *B. bambusoides*. On the abaxial surface, there are papillae on long cells associated with the stomatal complexes. Bicellular microhairs are the only trichomes present and they are found almost exclusively on the abaxial surface. The saddle-shaped silica bodies are the most taxonomically important among the microcharacters observed on the leaf surface of *B. bambusoides*.

Keywords

Branched papillae, bicellular microhair, Buergersiochloinae, saddle-shaped, silica body

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Introduction

Buergersiochloa bambusoides Pilg. is the only species of this genus historically included in Buergersiochloinae (Olyreae, Bambusoideae), which is endemic to the northern coastal rainforests of the Indonesian island of New Guinea (Fijten 1975) and Papua New Guinea (Judziewicz and Clark 2007; BPG 2012). This subtribe is sister to the clade composed of the other two lineages (Olyrinae and Parianinae), which are essentially restricted to the Neotropics (Oliveira et al. 2014; Ferreira et al. 2019). However, the recircumscription of Buergersiochloinae is in progress based on current phylogenetic studies (Carvalho et al. in press).

This is a rare, poorly collected and monoecious perennial species, with scaly branched rhizomes, leafy sterile culms and leafless fertile culms, and a rather dense paniculate synflorescence with male spikelets borne on the lower and female spikelets on the upper branches (Fijten 1975). Interestingly, although unisexual spikelets, cruciform silica bodies in the costal zone, and crenate silica bodies (olyroid-type) in the intercostal zone have been considered synapomorphies of the Olyreae, these types of silica bodies are absent in *B. bambusoides* (BPG 2012). A few previous observations on the leaf anatomy and micromorphology of this species were published by Renvoize (1985), in comparison to some genera of Olyrinae, using light microscopy (LM). However, there are no studies of this species using scanning electron microscopy (SEM), which clearly offers important information for understanding the foliar micromorphology in Olyreae (e.g. Oliveira et al. 2008a, b; Ferreira et al. 2013; Leandro et al. 2016; Lima et al. 2020), especially when compared to LM.

Papillae and trichomes can also inform general bamboo systematics (Calderón and Soderstrom 1967; Soderstrom and Ellis 1987; Leandro et al. 2020) and other microcharacters have been used in taxonomic studies within Olyreae (Metcalfe 1960; Calderón and Soderstrom 1967, 1973; Renvoize 1985; Jesus et al. 2012; Ferreira et al. 2013; Leandro et al. 2016; Lima et al. 2020), which remain undescribed for this species.

For these reasons, we present updated information on the foliar surfaces of this species, which was historically the single representative of the Buergersiochloinae. We also aim to answer the following specific questions regarding the leaf micromorphology of *B. bambusoides*: Do the silica bodies of the costal zone have the same orientation, with respect to the longitudinal axis of the leaf, like those of the intercostal zone? What is the morphology of the papillae and how are they distributed? On which cell type(s) are the papillae associated with the stomata found? What types of trichome occur? Do these characters have potential taxonomic utility?

Materials and methods

Samples used in this work were obtained from the U.S. National Herbarium of the Smithsonian Institution (US) from the vouchers *Croft et al. 68692* and *Dransfield et al. 1382* [acronym according to Thiers (2020+)]. The epidermal micromorphology of the

leaves of *B. bambusoides* was analyzed using scanning electron microscopy (SEM) and light microscopy (LM).

The SEM analysis was performed with two samples of 0.5 cm^2 from the median region of fully developed dried leaf blades, to observe both adaxial and abaxial surfaces. Samples were submerged in xylene for ca. 10 minutes, eliminating the epicuticular wax to allow better observation of the microcharacters (Dávila and Clark 1990), and mounted on small steel cylinders with metallic adhesive tape and covered with platinum in a Denton Desk II sputter coater. They were imaged using a JEOL JSM-5800LV scanning electron microscope at the Roy J. Carver High Resolution Microscopy Facility at Iowa State University.

For the LM analysis, two other similar samples of the same size and from the same region of the leaf blades were used. The epidermises were dissociated using the Jeffrey method (Johansen 1940) and stained with 1% alcoholic safranin. Semipermanent slides were mounted with glycerinated gelatin, analyzed, and photomicrographed in a Zeiss Axio Scope A1 optical microscope with a Canon EOS attached digital camera. The description of the epidermal microcharacters followed the terminology proposed by Ellis (1979).

Results

Epidermal cells: long and tabular in the intercostal zones, and nearly equidimensional in the costal zones, walls anticlinally sinuous (Fig. 1A, B). **Short cells:** silica cells containing a silica body and cork cells with wall impregnated with suberin, the latter paired with silica cells (Fig. 1B) or the basal cells of bicellular microhairs. **Bulliform cells:** anticlinally sinuous wall, wider and shorter than long cells, in bands in the intercostal zone of the adaxial epidermis (Fig. 1A). **Stomatal distribution:** hypostomatic (Fig. 1C). **Silica bodies:** saddle-shaped and transversely elongated in the intercostal and costal zones (Fig. 1B). **Adaxial surface:** very small papillae on the bulliform cells; bicellular microhairs scarce, prickles, and macrohairs absent (Fig. 1D). **Abaxial surface:** stomata in 2 rows on each side of a costal zone; subsidiary cells dome-shaped, papillae absent; long cell papillae encircling the stomata in the stomatal cell rows (Fig. 1F); long cells bearing small, branched, abundant papillae in the costal and interstomatal cell rows; panicoid type bicellular microhairs present along the edge of stomatal cell rows; prickles and macrohairs absent (Fig. 1E, G, H).

Discussion

The leaf blade epidermis of *B. bambusoides* is consistent with other members of Olyreae and indeed the whole subfamily, considering both long and short cells, in addition to the rows of bulliform cells in the adaxial intercostal zones (Vieira et al. 2002; Oliveira et al. 2008a, b; Jesus Junior et al. 2012; Leandro et al. 2016, 2020; Lima et al. 2020). The short cells observed include silica or cork ones, paired with each other or the lat-

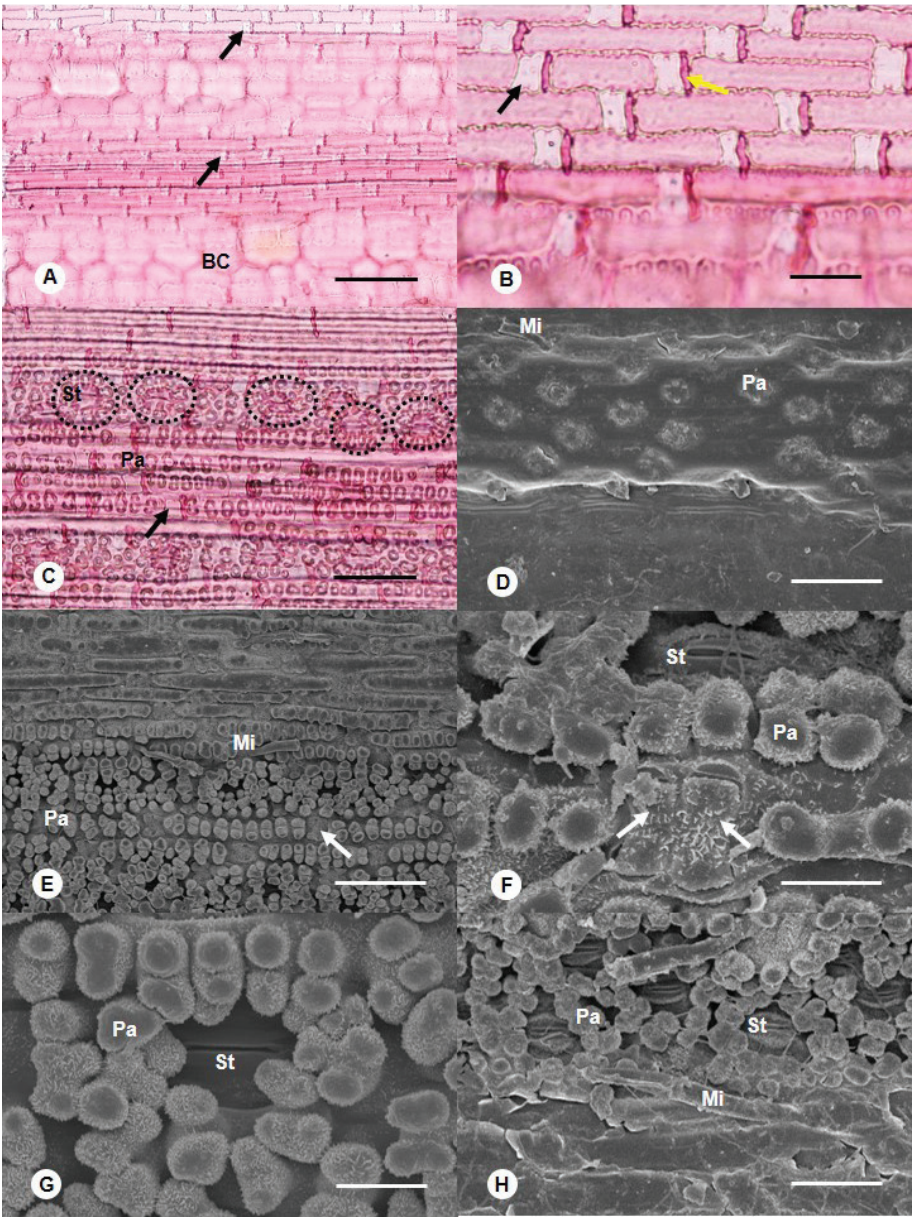

Figure 1. Leaf micromorphology of *Buergersiochloa bambusoides* **A–C** leaf surface observed under LM **A** adaxial surface showing long cells, bulliform cells with sinuous anticlinal walls and saddle-shaped silica bodies **B** detail of saddle-shaped silica bodies and cork cells **C** abaxial surface showing silica bodies, abundant papillae and papillae encircling the stomata (dotted circles) **D–H** leaf surface observed under SEM **D** adaxial surface with very small papillae and the basal cell of a broken bicellular microhair **E** abaxial surface showing microhairs, abundant papillae on costal and stomatal cell rows, and long cell papillae encircling the stomata **F** detail of two adjacent saddle-shaped silica bodies, papillae and stoma on the abaxial surface **G** detail of papillae encircling a stoma **H** detail of panicoid type bicellular microhairs. BC: Bulliform cell; Mi: bicellular microhair; Pa: papilla; St: stoma. Black or white arrows: silica body; yellow arrow: cork cell. Scale bars: 100 μm (**A, C**); 25 μm (**B, H**); 10 μm (**D, G**); 50 μm (**E, F**)

ter paired with the basal cells of trichomes, as is common to Poaceae (Calderón and Soderstrom 1973; Soderstrom and Ellis 1988; Vieira et al. 2002; Lima et al. 2020).

The saddle-shaped silica bodies of *B. bambusoides* have been recognized as characteristic of many woody bamboos of the Arundinarieae and Bambuseae tribes (Renvoize 1987; Yang et al. 2008; Zhang et al. 2014; Leandro et al. 2020), but within the Olyreae are exclusive to Buergersiochloinae, as noted here and confirming previous studies (Renvoize 1985; Lima et al. 2020). For this reason, the presence of saddle-shaped silica bodies is here considered as an important taxonomic character for this subtribe since it does not have crenate silica bodies (olyroid-type) in the intercostal zone, which have been considered synapomorphies of the Olyreae (BPG 2012). In addition to the shape, the orientation of the silica bodies on the leaf surface is also considered important information for the identification and taxonomic utility of these microcharacters (Rudall et al. 2014). In *B. bambusoides* the silica bodies are transversely elongated (i.e. perpendicular to the long axis of the leaf), and have the same orientation in both the costal and intercostal zones. Thus, we confirm that they are the same type in both zones.

The simple and very small papillae on the adaxial leaf surface, found only on the outer periclinal wall of the bulliform cells in *B. bambusoides* may be related to the environment in which the plants occur (Lee 1986; Sahuquillo and Lumaret 1995; Oliveira et al. 2008a, b; Glover and Whitney 2010; Thomas et al. 2010; Ferreira et al. 2013). This species is endemic to lowland primary forests of New Guinea (Fijten 1975) and considering the shaded environment of the understory, these papillae can help to enhance the active capture of light (Lee 1986; Glover and Whitney 2010; Thomas et al. 2010) as suggested for *Eremitis afimbriata* F.M.Ferreira & R.P.Oliveira and *E. magnifica* F.M.Ferreira & R.P.Oliveira, the first registrations of this characteristic in bamboo leaves (Ferreira et al. 2013).

The stomatal complexes with dome-shaped subsidiary cells of *B. bambusoides* are very common in Bambusoideae (Metcalfe 1960; Ellis 1979; Zuloaga et al. 1993; Oliveira et al. 2008a, b; Jesus et al. 2012; Ferreira et al. 2013; Leandro et al. 2016, 2020; Lima et al. 2020), occurring in well-defined rows in the intercostal zones on either side of a costal zone. Although papillae on the subsidiary cells have been documented for some Olyreae, mainly members of subtribe Olyrinae (Lima et al 2020), and are also characteristic of Chusqueinae (Clark 1986; Soderstrom and Ellis 1987; Fisher et al. 2009, 2014), these are here indicated as absent in *Buergersiochloa*. The papillae associated closely with the stomatal complexes in this species are from the long cells of the stomatal rows, as is common in many woody bamboos (Yang et al. 2008; Guerreiro et al. 2013). However, these papillae on the long cells are branched and since they are observed for the first time on the abaxial surface of the leaf blade of *B. bambusoides*, this is a micromorphological novelty, not only for Buergersiochloinae, but also for Olyreae.

Bicellular microhairs observed on the abaxial surface were also similar to those often found in Bambusoideae as a whole (Prat 1936; Metcalfe 1960; Calderón and Soderstrom 1973; Ellis 1979; Vieira et al. 2002; Oliveira et al. 2008a, b; Jesus et al. 2012; Leandro et al. 2016; Lima et al. 2020), therefore classified as panicoid, having two cells that are longer than thick (Amarasinghe and Watson 1988), and the distal cell has a cellulosic wall that is sometimes lost (Tateoka et al. 1959).

Conclusions

Among the microcharacters observed on the leaf surface of *B. bambusoides*, saddle-shaped silica bodies are the most taxonomically important, since this type of silica body was not observed in any genus of Olyrinae or Parianinae within Olyreae (Lima et al. 2020; Lima et al. unpubl. data).

The leaf surface of *B. bambusoides* presented simple and very small papillae on the adaxial surface, and branched papillae on the abaxial surface. This first record of branched papillae in *B. bambusoides* is a novelty for Olyreae. On the adaxial surface, the papillae are only on bulliform cells, and on the abaxial surface, the papillae of the long cells adjacent to stomata encircle the stomatal complexes. In *B. bambusoides* microhairs on the adaxial surface are very scarce but are more common on the abaxial surface, but because it is a type of trichome common in Bambusoideae, this character does not offer relevant taxonomic information.

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