Systematic Botany (2019), 44(1): pp. 74–82

DOI 10.1600/036364419X697912
Date of publication February 5, 2019

Molecular and Morphological Analyses Support the Transfer of *Gleadovia kwangtungensis* to *Christisonia* (Orobanchaceae)

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Communicating Editor: Ashley B. Morris

Abstract*—*Christisonia kwangtungensis is a new combination proposed in this study on the basis of both molecular and morphological evidence. The basionym, *Gleadovia kwangtungensis*, was subsumed into *Christisonia hookeri* by Zhang in 1986. Because the type material of *C. hookeri* lacks important diagnostic characters and no fresh materials from Sikkim were available, Chinese plants with white flowers were conservatively treated as C. *sinensis*. Phylogenetic analyses showed that samples of *G. kwangtungensis* from the type locality on Danxia Mountain were monophyletic and sister to a clade including the reciprocally monophyletic C. *sinensis* and *C. siamensis*. Moreover, morphological comparisons showed that ***C. kwangtungensis*** differs from C. *sinensis* by having a conspicuously inflated (vs. scarcely inflated) corolla and clustered hairs in the throat of the corolla (vs. hairs scattered throughout the interior of the corolla). Further, ***C. kwangtungensis*** differs from both *C. sinensis* and *C. siamensis* by the length and color of the corolla.

***Keywords*—*Christisonia hookeri*, *Christisonia kwangtungensis*, *Christisonia sinensis*.**

The earliest delimitations of Orobanchaceae included only holoparasitic taxa (Ventenat 1799; Bentham and Hooker 1897). In other classification systems, the holoparasitic genera were placed in Scrophulariaceae (Fischer 2004; Takhtajan 2009). Phylogenetic analyses have demonstrated that Scrophulariaceae s. l. is polyphyletic (Young et al. 1999; Olmstead et al. 2001), and Orobanchaceae has been expanded to include all parasites in Lamiales as well as three non-parasitic genera from Scrophulariaceae s. l.: *Lindenbergia* Lehm., *Rehmannia* Libosch. ex Fisch. & C.A.Mey., and *Triaenophora* Soler. (Stevens 2001; Wolfe et al. 2005; Bennett and Mathews 2006; Xia et al. 2009; McNeal et al. 2013; Refulio-Rodriguez and Olmstead 2014; Fu et al. 2017; Yu et al. 2018). Holoparasitic genera fall into three clades corresponding to tribes in Orobanchaceae: *Lathraea* L. occurs in clade V corresponding to tribe Rhinantheae; *Aeginetia* L., *Christisonia* Gardner, *Harveya* Hook., and *Hyobanche* L. form a lineage within clade VI corresponding to tribe Buchnereae; the remaining holoparasitic genera including *Gleadovia* Gamble & Prain occur in clade III corresponding to tribe Orobancheae (McNeal et al. 2013; Fu et al. 2017).

Tribe Buchnereae (Clade VI) primarily includes genera of the tropics and subtropics, and so has also been called the "tropical clade" (Morawetz et al. 2010). *Christisonia*, a genus in Buchnereae, occurs in southern China, India, Sri Lanka, Indochina, and part of Malesia (Fischer 2004; Parnell et al. 2014; Van der Ent and Wong 2015). This genus includes about 20 species, most of which are parasitic on the roots of bamboos (Parnell 2001; Fischer 2004), Acanthaceae (Nandikar et al. 2013), or Vitaceae (Benniamin et al. 2012; Tan 2013). Plants are fleshy and covered in sticky translucent slime, and preserve poorly when pressed. Therefore, most herbarium specimens are degraded and floral characters are difficult to recognize, which may cause misidentification.

Including *Christisonia*, nine holoparasitic genera of Orobanchaceae, including 42 species, are treated in the Flora of China (Zhang and Tzvelev 1998). During revision of Chinese *Christisonia*, Zhang (1986) proposed to retain one species, *C. hookeri* C.B.Clarke ex Hook.f., including *C. sinensis* Beck, *Gleadovia kwangtungensis* Hu, and *Gleadovia lepoensis* Hu as synonyms. During investigation on the pollination biology of *G. kwangtungensis* at the type locality in Danxia Mountain (G.-D. Tang et al. unpubl. data), the floral morphology of this population was found to differ from that of *C. hookeri* as described in the Flora of China. Further, doubt has been cast on whether *C. hookeri* occurs in China at all (van der Ent and Wong 2015). Therefore, in this study we have chosen a conservative interpretation of Chinese species of *Christisonia*; Chinese plants are interpreted as either *C. sinensis*, or *G. kwatungensis* if collected in Danxia. To clarify the relationship between *G. kwangtungensis* and *C. sinensis*, we used nuclear ribosomal internal transcribed spacers (ITS) and three chloroplast DNA markers to infer the phylogeny of related members of Orobanchaceae. Multiple species of *Christisonia* were sampled, including specimens from the Danxia type-population of *G. kwangtungensis*, as well as other hemi- and holoparastic species with emphasis on the tropical clade of Orobanchaceae (Morawetz et al. 2010). The main aims of this study were to: 1) reassess the taxonomic status of *G. kwangtungensis* on the basis of both morphological and molecular evidence, and 2) make the appropriate nomenclatural changes.

Materials and Methods

***Sampling and Sequencing*—**We sampled 53 accessions representing 25 genera and 39 subgeneric taxa, with an emphasis on the tropical clade of Orobanchaceae. We included one individual of *G. mupinensis* Hu, three individuals of both *C. sinensis* and *C. siamensis* Craib, and nine individuals of *G. kwangtungensis* from Danxia Mountain which varied in corolla color from white to rose-red. The full list of sampled species and individuals is presented in Appendix 1.

Total genomic DNA of the new samples was extracted from silica-gel dried tissues using a modified CTAB method (Doyle and Doyle 1987). ITS and three plastid DNA markers (*matK*, *rpl16*, *trnL-L-F*) were amplified following Yu et al. (2011). The primers used in PCR and sequencing are listed in Appendix 2.

Sequence Assembly and Phylogenetic Analyses—New raw sequences were assembled using Geneious v. 9.0 (Kearse et al. 2012), and other sequences were downloaded from GenBank (see Appendix 1). MAFFT v. 7.0 (Katoh and Standley 2013) was used to infer a preliminary alignment for each gene, which was then adjusted manually in Geneious. Aligned matrices of four DNA regions were combined using SequenceMatrix v. 1.7 (Vaidya et al. 2011). The ITS and the concatenated plastid datasets were analyzed separately and in combination. Bayesian inference (BI) and maximum likelihood (ML) methods were used to reconstruct the phylogenies following Yu et al. (2015, 2018). DNA substitution models were selected for gene partitions using the Bayesian information criterion (BIC) in jModeltest 2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012). Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations for each dataset, sampling every 1000 generations, with two simultaneous runs, and each run comprising four incrementally heated chains using MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003). The first 25% of trees sampled were discarded as burn-in, and the remaining trees were used to generate a majority-rule consensus tree. The ML tree searches and bootstrap estimation of clade support were conducted with RAxML 8.2.10 (Stamatakis 2014). These analyses used the GTR substitution model with gamma-distributed rate heterogeneity among sites and the proportion of invariable sites estimated from the data. The dataset was partitioned

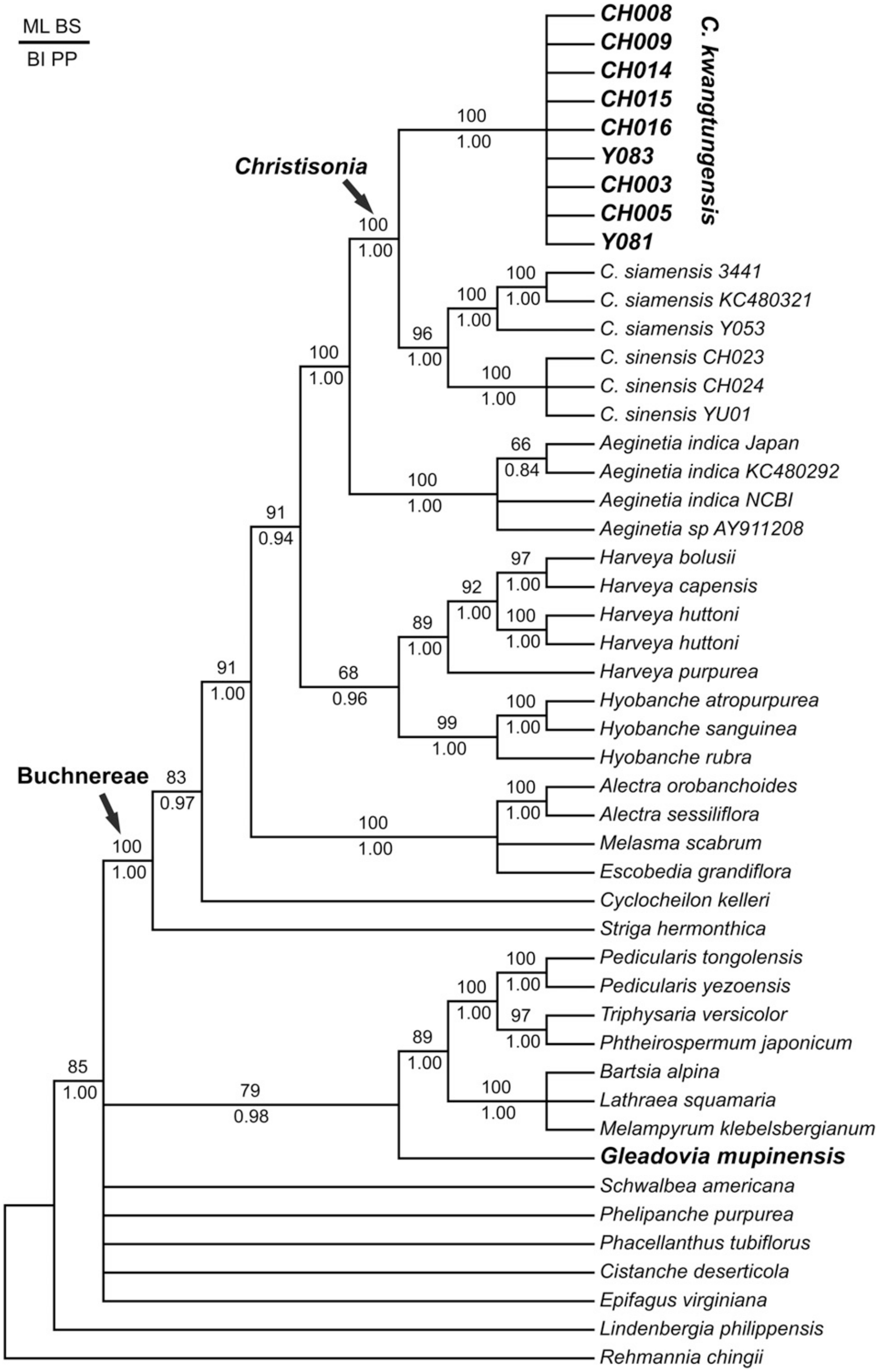

FIG. 1. Phylogeny inferred from nuclear ITS data. ML bootstrap values are presented above branches, and BI posterior probabilities are shown below branches.

by gene. Support values were estimated from 1000 bootstrap replicates. For both BI and ML analysis, *Rehmannia chingii* was used to root trees based on its phylogenetic position as sister to *Lindenbergia* + the clade of parasitic Orobanchaceae. All analyses were performed at the CIPRES Science Gateway (http://www.phylo.org).

Morphological Observations—Morphological data were collected and described here based on living plants in the field. Voucher specimens were collected and deposited at the herbarium of South China Agricultural University (CANT).

RESULTS AND DISCUSSION

Phylogenetic Analyses—The aligned ITS, *matK*, *rpl16*, and *trnL-L-F* matrixes were 696 bp, 1665 bp, 1552 bp, and 2290 bp, respectively. The ITS dataset contained 437 variable and 379 parsimony-informative sites, and the concatenated plastid dataset had 2723 variable and 1517 parsimony-informative sites (*matK*: 900 variable / 519 parsimony-informative sites; *rpl16*: 681 / 350; and *trnL-L-F*: 1141 / 647).

All phylogenetic analyses using ITS (Fig. 1), plastid (Fig. 2), and the total (Fig. 3) datasets recovered the monophyly of the tropical clade of Orobanchaceae, and strongly supported four holoparasitic genera (*Aeginetia*, *Christisonia*, *Harveya*, and *Hyobanche*) as monophyletic. In the holoparasitic lineage of the tropical Orobanchaceae, the Asian *Aeginetia* and *Christisonia* formed a clade. Individuals of the Danxia population of *G. kwangtungensis* were monophyletic, then as sister to *C. sinensis* + *C. siamensis* (Figs. 1, 2, 3). In contrast, *G. mupinensis* was recovered in the Orobancheae clade by both the plastid and total datasets, consistent with Fu et al. (2017). Therefore, *G. kwangtungensis* should be included in *Christisonia*, as a species separate from *C. sinensis*. Herein, we propose a new combination, *Christisonia kwangtungensis* (Hu) G.D.Tang, J.F. Liu & W.B. Yu.

Morphological Comparisons—*Christisonia kwangtungensis* is similar to *C. sinensis* in having a terete calyx and a greatly thickened connective appendage in the two lower anthers. However, C. *kwangtungensis* has a conspicuously (vs. inconspicuously) inflated corolla tube, which may be white, pink, or rose-red (vs. white), and clustered hairs in the throat of the corolla tube (vs. scattered) (Table 1; Figs. 4–6). The corolla tube of *C. kwatungensis* is also significantly shorter (3.3–4.0 cm)

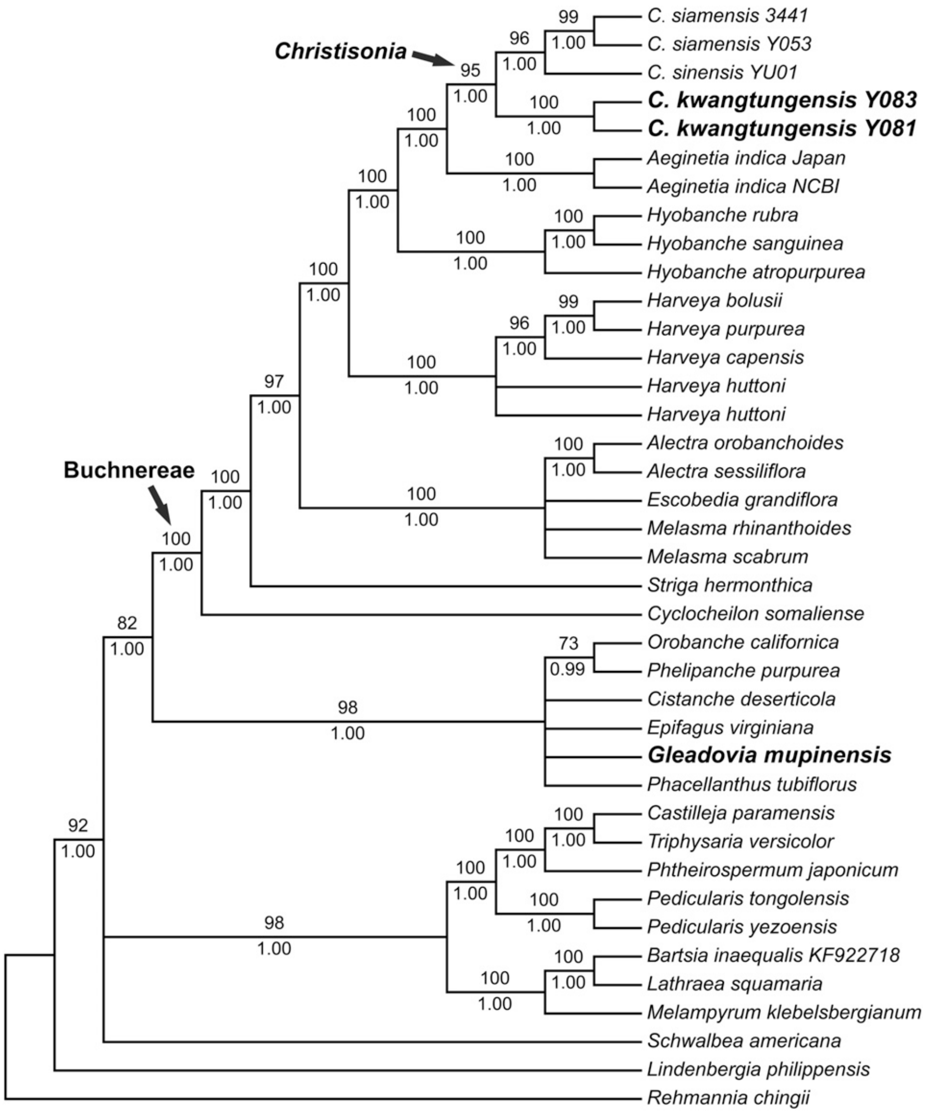

FIG. 2. Phylogeny inferred from the concatenated plastid data, including three plastid genes, *matK*, *rpl16*, and *trnL-L-F*. ML bootstrap values are presented above branches, and BI posterior probabilities are shown below branches.

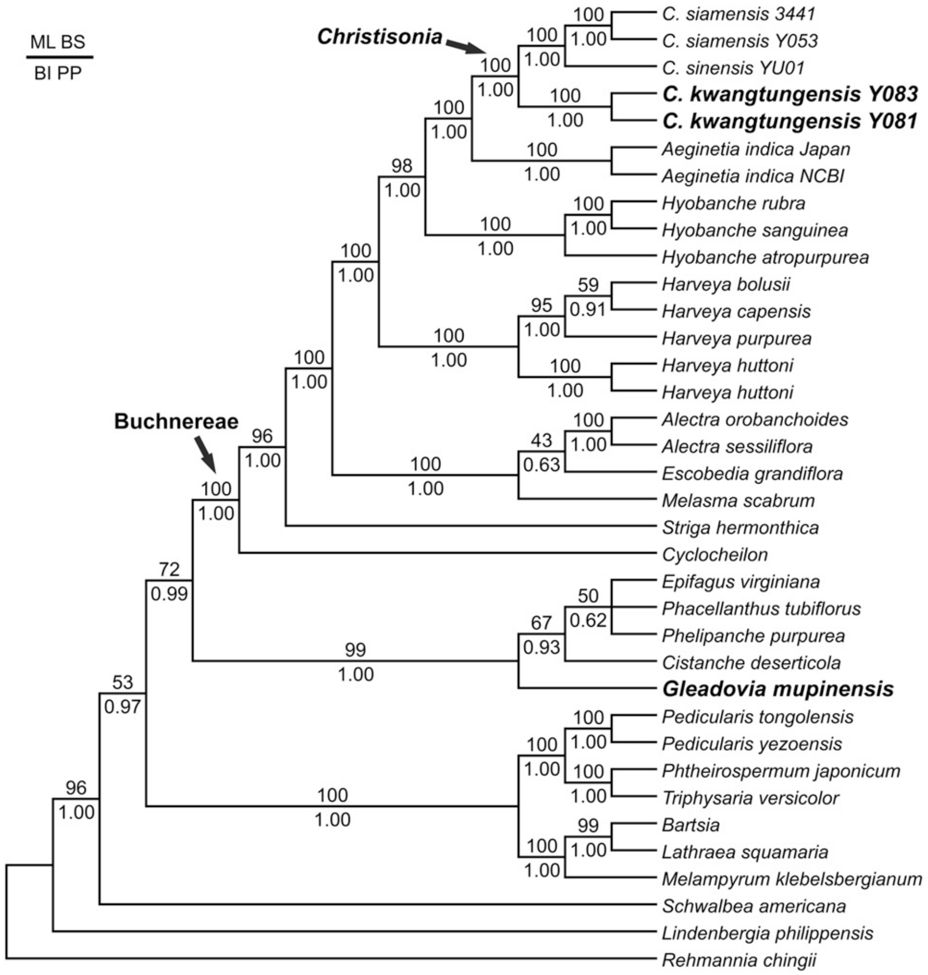

FIG. 3. Phylogeny inferred from the total dataset, including ITS and three plastid genes. ML bootstrap values are presented above branches, and BI posterior probabilities are shown under branches.

than that of either *C. sinensis* (5.5–7.0 cm) or *C. siamensis* (4.5–6.0 cm). Additionally, the corolla of *C. kwatungensis* differs in color from that of *C. siamensis* (bluish-violet). Therefore, *C. kwangtungensis* is morphologically distinct from either *C. sinensis* or *C. siamensis*.

Taxonomic Revision of Christisonia in China—Although *C. hookeri* is the only species listed in *Flora of China* (Zhang and Tzvelev 1998), *C. siamensis* has been found in Mengla, southern Yunnan Province (Tan 2013). Additionally, Parnell et al. (2014) and van der Ent and Wong (2015) suggested that the Chinese material of *C. hookeri* should be *C. scortechinii* Prain. Based on comparisons of morphological descriptions and photographs, we found that the plants in Thailand and Malaysia were different from plants in southern China and Taiwan Island. It is clear that *C. scortechinii* is a tropical species, whereas the Chinese plants are in temperate and subtropical areas. So far, *C. hookeri* and *C. sinensis* are the only two species of *Christisonia* recorded in the temperate area from Sikkim Himalaya to south

TABLE 1. Morphological comparisons of key features among *C. kwangtungensis*, *C. sinensis* and *C. siamensis*.

Characters	*C. kwangtungensis*	*C. siamensis*	*C. sinensis*
Calyx color	White or purple	White	White
Calyx lobes/teeth	3–4	(2–)3(–4)	(2–)3–4(–5)
Calyx length	1.8–2.2 cm	1.5–2.5 cm	2–3 cm
Calyx: corolla length ratio (fresh material)	< 0.5	< 0.5	0.5
Corolla color	White or rose-red	Bluish to violet	White
Corolla tube	Conspicuously inflated	Conspicuously inflated	Not conspicuously inflated
Corolla tube length	3.3–4.0 cm	4.5–6.0 cm	5.5–7.0 cm
Hairs within the throat of corolla tube	Densely clustered at the throat	Unknown	Scattered
Known distribution	Guangdong	Yunnan (and India, Thailand, and Laos)	Chongqing, Fujian, Guizhou, Guangxi, Hainan, Sichuan, and Yunnan
Habitat	Bamboo forest at 90–120 m	Bamboo forest or tropical forest at 300–800 m	Bamboo forest at 750–2700 m

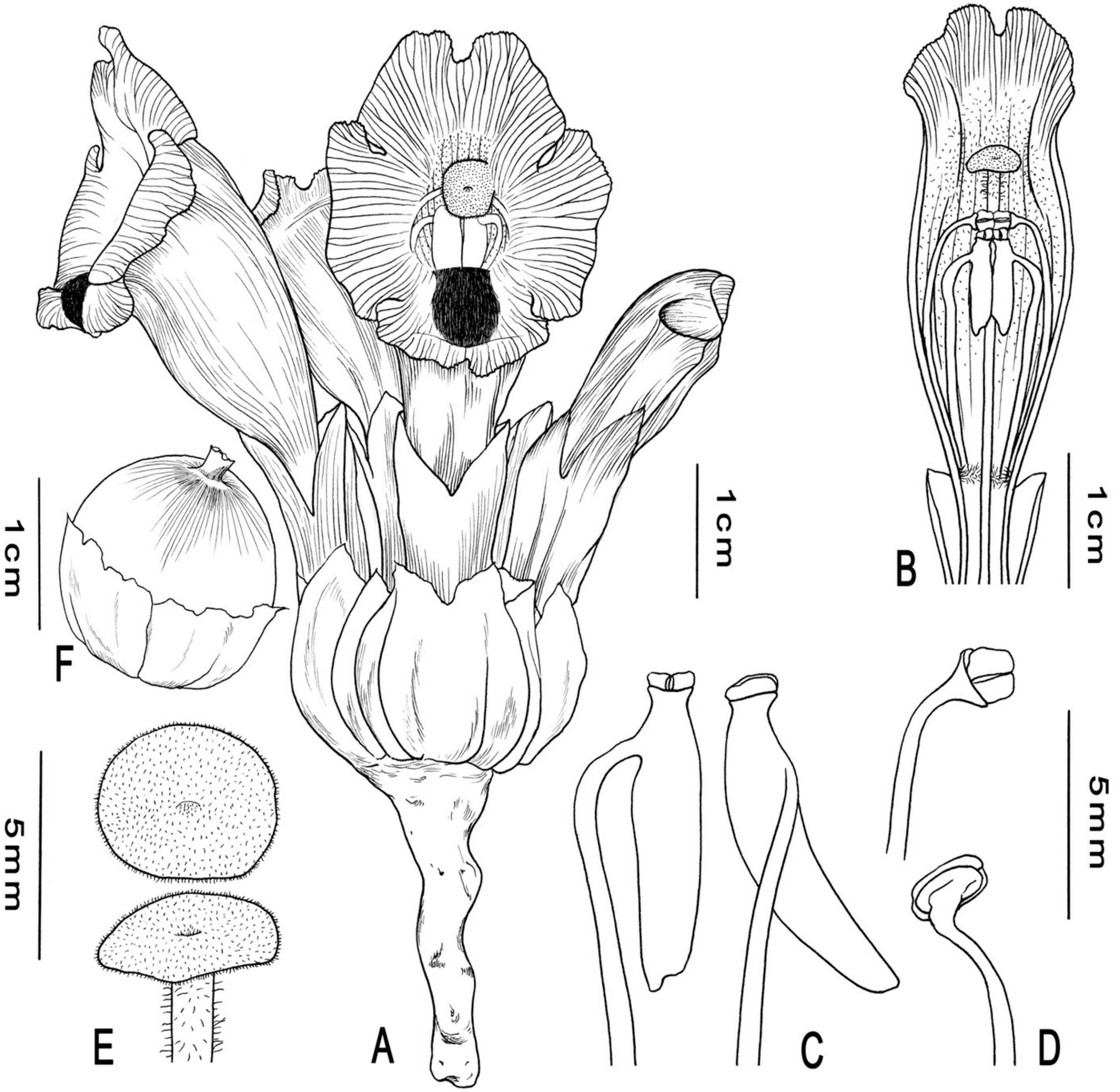

FIG. 4. Illustration of *Christisonia kwangtungensis*. A. Flowering plant. B. Longitudinal section of the flower. C. Short stamens and anthers. D. Long stamens and anthers. E. Stigma. F. Capsule with persistent calyx and style.

China (Beck-Mannagetta 1930). In *Christisonia*, *C. hookeri* is the only species described as having a bilobate stigma (see Beck-Mannagetta 1930). After checking the type materials of *C. hookeri* conserved at Royal Botanic Gardens, Kew (K), we found that the open stigmas were folded, which might have misled Hooker and Thomson (1884), who treated the visible part of the stigma as an orbicular lobe. During revision of Chinese *Christisonia*, Zhang (1986) treated *C. sinensis* as synonym of *C. hookeri*. Besides the shape of the stigma, however, van der Ent and Wong (2015) indicated that *C. sinensis* can be distinguished from *C. hookeri* by having a white calyx (vs. violet), a pure white corolla without violet seams, and short acute projection of the anther appendage. Because we had no fresh materials of *C. hookeri* from the Sikkim Himalaya, we conservatively retained the name *C. sinensis* for Chinese plants excluding those of the Danxia population, which we regarded as *C. kwatungensis* (≡ *G.* kwatungensis) herein.

According to molecular analyses and morphological comparisons, we have demonstrated that *C. kwangtungensis* should be considered a separate species. Therefore, we recognized three species in China: *C. kwangtungensis, C. siamensis*, and *C. sinensis*. Of them, *C. sinensis* has the widest distribution, ranging from southwest to east in China, including two new records in Fujian (Chen et al. 2017) and Zhejiang (Xie et al. 2017); *C. kwangtungensis* is endemic to Danxia, Guandong, and *C. siamensis* is only found in Mengla, Yunnan.

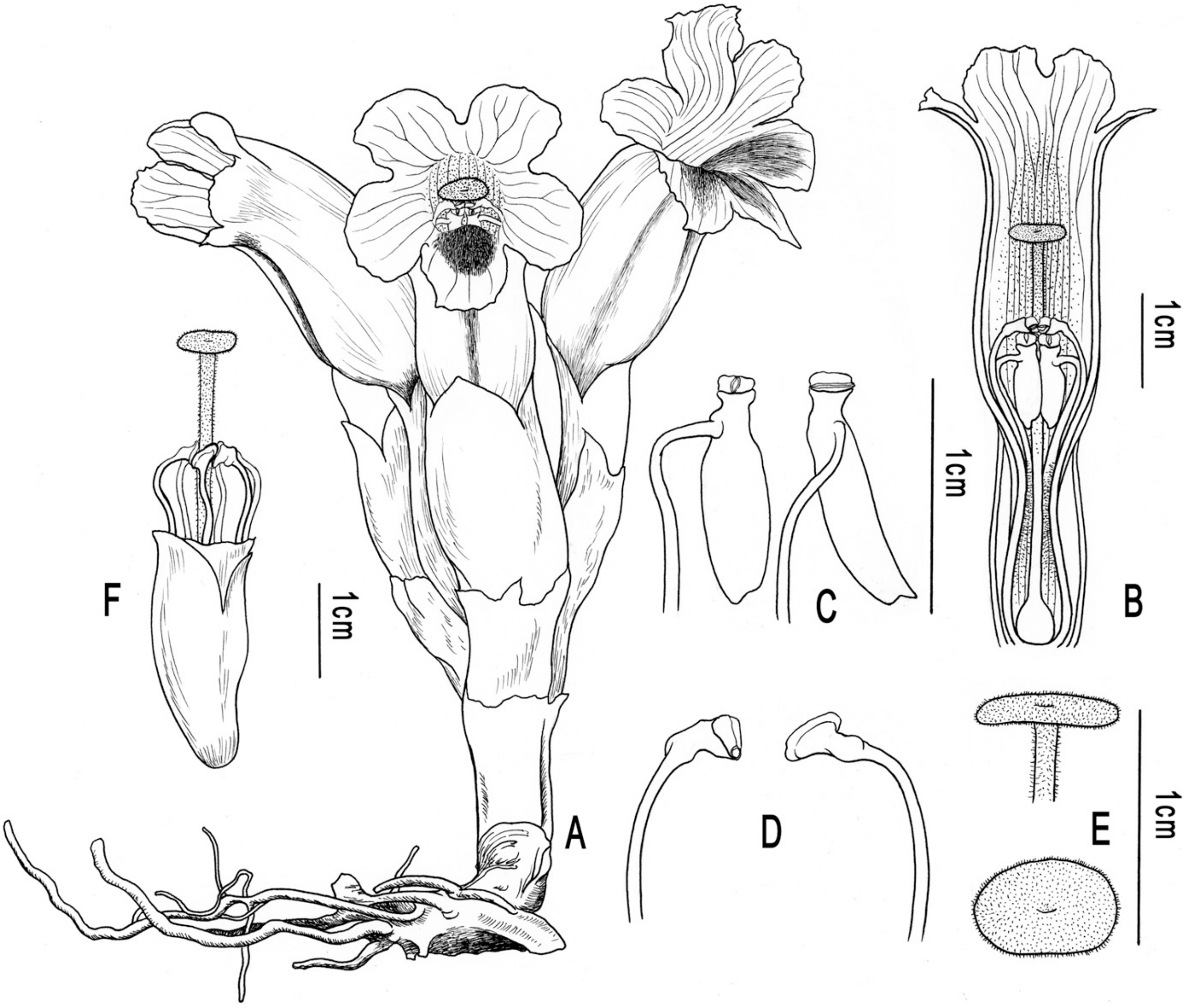

FIG. 5. Illustration of *Christisonia sinensis*. A. Flowering plant. B. Longitudinal section of the flower. C. Short stamens and anthers. D. Long stamens and anthers. E. Stigma. F. Dissected flower showing isomorphic anthers, as found in a few specimens.

TAXONOMIC TREATMENT

Christisonia kwangtungensis (Hu) G.D. Tang, J.F. Liu & W.B. Yu, comb. nov. (广东假野菰). *Gleadovia kwangtungensis* Hu in Sunyatsenia 1939: 4(1–2): 7–9, f. 1. TYPE: CHINA. Guangdong, foot-hills of Danxia mountain, in bamboo forest. July 24, 1930, *S. P. Ko. 50660*, (holotype: IBSC, 0004800!).

Root parasitic herb; stem fleshy, 1.5–2.2 cm long, 6 mm in diam; scales lanceolate, 4 mm long, caducous; flowers sessile, fasciculate; bracteoles spathulate, broad, emarginate at apex, glabrous, one side sometimes completely adnate to the calyx; calyx tubular-campanulate, irregularly 4-lobed above the middle, glabrous, 1.8–2.2 cm long, lobes dentate, often degraded; corolla bilabiate, rose-red, pink, or rarely white, pilose inside, tube slightly incurved, inflated at the throat, 3.3–4.0 cm long, corolla lobes imbricate; upper-lip slightly cucullate, 9–12 mm long, 12–15 mm broad, 2-lobed at apex; lower-lip 10–12 mm long, 12–15 mm broad, 3-lobed, lobes erect-spreading, obovate-oblong, obtuse at apex, mid-lobe much smaller, with a big yellow stripe extending to the middle of corolla tube; stamens 4, didynamous, the base of the 2 fertile anther-cells not mucronate and slightly divaricate, connectives scarcely produced; ovary ovoid, glabrous; style incurved, 3.2–3.8 cm long, glabrous below, pilose above; stigma peltate, 3 mm in diam, white, sometimes purple at the margin, densely pilose; capsules rounded, glabrous, purple stripes on the surface, styles persistent when ripe. Figures 4, 6.

Distribution and Phenology—*Christisonia kwangtungensis* grows in wet areas under the bamboo forests between 90 m and 120 m on Danxia mountain, Shaoguan, Guangdong Province, China. Flowering is in July and August, and fruiting is in August and September.

ACKNOWLEDGMENTS

The research was partially supported by a grant from the Natural Science Foundation of Guangdong Province, China to G.-D.T. (#2014A030310465), the National Science Foundation (US) award numbers 1119746 and 1119801, and the Project of the Department of Forest of Guangdong Province (Yu-Cai-Nong [2017] No. 83). We thank Dong-Ping Zhao for providing photos for Fig. 6; Li-Bing Zhang for helpful comments;

FIG. 6. A–F. *C. kwangtungensis*. A. Field specimen with rose-red corolla. B. Lateral view of flower. C. Front view of four heteromorphic anthers and stigma. D. Lateral view of four heteromorphic anthers and stigma. E. Hairs clustered within the throat of the corolla. F. Capsules. G–J. *C. sinensis*. G. Field specimen. H. Calyx and corolla. I. Longitudinal section of flower showing inconspicuously inflated corolla tube, four heteromorphic anthers and stigma. J. Scattered hairs within the throat of corolla tube.

Xiao-Ying Luo, Sheng-Zhe Jian, and Zai-Xiong Chen from the World Geopark of Danxia Mountain for field assistance. We also are grateful to two anonymous reviewers who helped improve the manuscript.

AUTHOR CONTRIBUTIONS

G-DT and W-BY conceived the study. G-DT, J-FL, LH, C-MZ, and LHL collected the morphological data. GDT provided the species description. L-HL drew the illustrations. CPR and W-BY collected DNA sequences and did the phylogenetic analyses. G-DT, CPR, and WBY wrote and revised the manuscript. All the authors approved the final version.

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APPENDIX 1. Summary of taxon sampling for this study. Species names and authorities are followed by voucher information except when all sequence data have been obtained from NCBI. Genes sampled are ITS, *matK*, *rpl16*, and the *trnL-F* spacer. For species with multiple accessions (*A. indica*, *C. kwatungensis*, *C. sinensis*, *C. siamensis*, *Ha. huttonii*), colormorph and population data have been included where available.

Aeginetia indica L., Japan SRA DRX076502: MK356329*, MK358418*, MK358425*, MK358435*; NCBI: KC480292, —, —, —; 339, NCBI: AY596819, —, EU253639, EU572720. *Aeginetia* sp.: AY911208, —, —, —. *Alectra orobanchoides* Benth.: EU253620, AF489960, EU253652, EU256660. *Alectra sessiliflora* (Vahl) Kuntze: EU253614, JX091308, EU253646, EU256654. *Bartsia alpina* L.:FJ790046, —, —, —. *Bartsia inaequalis* Benth.:—, KF922718, KF922718, KF922718. *Castilleja paramensis* F. González & Pabón-Mora:—, KT959111, KT959111, KT959111. *Christisonia kwangtungensis* (Hu) G.D.Tang, J.F.Liu & W.B.Yu, pink flower CH008, G.-D. Tang & YWB2013142-8 (CANT): MK356333*, —, —, —;pink flower CH009, G.-D. Tang & YWB2013142-9 (CANT): MK356334*, —, —, —; red flower CH014, G.-D. Tang & YWB2013142-3 (CANT): MK356335*, —, —, —; red flower CH015, G.-D. Tang & YWB2013142-4 (CANT): MK356336*, —, —, —;red flower CH016, G.-D. Tang & YWB2013142-5 (CANT): MK356337*, —, —, —; red flower Y083, G.-D. Tang & YWB2013142-2 (CANT): MK356338*, MK358420*, MK358427*, MK358437*; white flower CH003, G.-D. Tang & YWB2013141-3 (CANT): MK356339*, —, —, —; white flower CH005, G.-D. Tang & YWB2013142-5 (CANT): MK356340*, —, —, —; white flower Y081, G.-D. Tang & YWB2013141-1 (CANT): MK356341*, MK358421*, MK358428*, MK358438*. *Christisonia sinensis* G.Beck, CH023, W.-B. Yu YWB2013060-4 (KUN): MK356330*, —, —, —; CH024, W.-B. Yu YWB2013060-5 (KUN): MK356331*, —, —, —; YU01, W.-B. Yu YWB2013060 (KUN): MK356332*, MK358419*, MK358426*, MK358436*. *Christisonia siamensis* Craib, Middleton 3441 (GH): EU253610, —, EU253640, —; NCBI: KC480321, —, —, —; Y053, W.-B. Yu et al. s.n. (HITBC): MK356342*, MK358422*, MK358429*, —. *Cistanche deserticola* Ma: AB217873, KC128846, KC128846, KC128846. *Cyclocheilon kelleri* Engl.: EU259256, —, —, —. *Cyclocheilon somaliense* Oliv.:—, AJ429336, EU259218, EU264183. *Epifagus virginiana* (L.) W.P.C. Barton: AY209290, EPFCPCG, EPFCPCG, —. *Escobedia grandiflora* (L. f.) Kuntze: EU259277, —, EU253641, EU256650. *Gleadovia mupinensis* H.H. Hu, W.-B. Yu et al. YWB201506130 (HITBC): KY706614, KY706624, MK358430*, MK358439*. *Harveya bolusii* Kuntze: AY911227, AF051983, EU253637, EU256647. *Harveya capensis* Hook.: AF120224, AF489961, EU253636, EU256648. *Harveya huttoni* Hiern, McMaster 2403-1 (OS,NBG): EU253606, —, EU253635, EU256646; NCBI: KC480343, JX091323, —, —. *Harveya purpurea* Harv.: KC480344, AF051984, —, EU264202. *Hyobanche atropurpurea* Bolus: KC480346, AF051986, EU253632, EU256643. *Hyobanche rubra* N.E. Br.: KC480348, JX091324, EU259246, EU264207. *Hyobanche sanguinea* L.: KC480349, AF051987, EU253633, EU256644. *Lathraea squamaria* L.: JF900500, KM652488, KM652488, KM652488. *Lindenbergia philippensis* (Cham. & Schltdl.) Benth.: KC480290, HG530133, HG530133, HG530133.

Appendix 2. Primer information for PCR amplification and sequencing.

ITS (Gruenstaeudl et al. 2009): 18sF (ACCGATTGAATGGTCCGGTGAAGTGTTCG); 26sR (CTGAGGACGCTTCTCCAGACTACAATTCG). ***matK*** (Hollingsworth et al. 2009): 3F_KIM (CGTACAGTACTTTTGTGTTTACGAG); 1R_KIM (AATATCCAAATACCAAATCC). ***rpl16*** (Jordan et al. 1996): rpL16F71(GCTATGCTTAGTGTGTGACTCGTTG); rpL16R1516 (CCCTTCATTCTTCCTCTATGTTG). ***trnL-F*** (Taberlet et al. 1991): trnc (CGAAATCGGTAGACGCTACG); trnf (ATI'TGAACTGGTGACACGAG).