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Paphiopedilum notatisepalum, a new species of slipper orchid (Cypripedioideae, Orchidaceae) from China based on morphological and DNA evidence

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Abstract

A new species of *Paphiopedilum* (Orchidaceae) from Yunnan, China, is described and illustrated based on morphological and molecular evidence. Morphological comparisons indicate that the new species *P. notatisepalum* is highly similar to *P. henryanum*, from which it differs by its leaves with large yellow spots, shorter scape, larger flower, ovate, white sepals and petals that are pale purple-red with large purple spots and yellow-white margins. Molecular analyses of combined nuclear and plastid datasets (nrITS and *matK*) indicate that *P. notatisepalum* is sister to *P. barbigerum*, which has a green leaves and pale yellow-green sepals and petals. The morphological and molecular evidence support the hypothesis that *P. notatisepalum* is a new species.

Key words: Asian orchids, orchid phylogenetics, Venus slipper, reticulate evolution, lady's slipper orchids

Paphiopedilum Pfitzer (1886: 11), the largest genus of the subfamily Cypripedioideae (slipper orchids) comprising 96 accepted species (data collected from KBG, 01/2014), is native to the subtropical and tropical regions of Southeast Asia and is an ideal group for investigating the evolution of island species diversity (Liu *et al*, 2009). Monophyly of the genus as it is currently circumscribed has been strongly supported by previous studies (Cox *et al*. 1997; Chochai *et al*. 2012; Guo *et al*. 2012). Chochai *et al*. (2012) studied phylogenetic relationships in the genus with nuclear ribosomal spacers (nrITS) and four plastid DNA regions and obtained good resolution. However, nrITS and plastid DNA trees showed some discordance in topology, and Guo *et al.* (2015) found some evidence that reticulation exists in this genus.

During botanical trips to Malipo in Yunnan, China, in 2015 and 2016 several populations of *Paphiopedilum* were found in a broad-leaved forest on several limestone mountains. The dorsal sepal has recurved margins, leaves of these plants have large yellow spots, sepals are white and pale purple-red petals have large purple spots, somewhat similar to *P. henryanum* Braem (1987: 4). Although the new entity has obvious differences from species similar in morphology, whether there is a similar difference at the molecular level as estimated by phylogenetic markers was considered worthy of study.

Material and methods

Macromorphology observation:—Specimens were deposited in the National Orchid Conservation Center herbarium (NOCC). Measurements, colours and other details given in the description are based on the examination of living material. The floral structure of the fully opened flowers was observed using a Leica M205A microscope (Leica Microsystems Ltd., Heerbrugg, Germany).

Sampling:—Fresh leaves of this putatively new entity were collected. All sampled plants were transplanted to the National Orchid Conservation Center of China (Shenzhen, China). The sequences of some allied species and outgroup species were available from GenBank. Sample information and vouchers are provided in Table 1.

TABLE 1. Species and DNA regions sequenced for analysis, as well as GenBank accession numbers. A dash (–) indicates missing data, an asterisk (*) denotes sequences obtained in this study, and the remaining sequences are from GenBank.

Species	ITS	*matK*
Sect. *Parvisepalum*		
Paphiopedilum armeniacum S.C.Chen & F.Y.Liu	EF156086	JQ660906
Paphiopedilum hangianum Perner & O.Gruss	JX088558	KP311998
Sect. *Concoloria*		
Paphiopedilum bellatulum (Rchb.f.) Stein	JX088553	JN181448
Paphiopedilum niveum (Rchb.f.) Stein	JQ660879	KC692139
Sect. *Cochlopetalum*		
Paphiopedilum glaucophyllum J.J.Sm.	JQ929321	AY557205
Paphiopedilum liemianum (Fowlie) K.Karas. & K.Saito	JQ929333	JQ929385
Paphiopedilum primulinum M.W.Wood & P.Taylor	JQ929342	JN181451
Paphiopedilum victoria-mariae (Sander ex Mast.) Rolfe	EF156156	KP312031
Paphiopedilum victoria-regina (Sander) M.W.Wood	AY643441	JQ660893
Sect. *Pardalopetalum*		
Paphiopedilum haynaldianum (Rchb.f.) Stein	JQ929325	JQ929379
Paphiopedilum parishii (Rchb.f.) Stein	JQ929340	JQ660891
Sect. *Coryopedilum*		
Paphiopedilum adductum Asher	JQ929305	JQ182191
Paphiopedilum gigantifolium Braem, M.L.Baker & C.O.Baker	EF156103	KP312011
Paphiopedilum glanduliferum (Blume) Stein	JQ929319	JQ660887
Paphiopedilum kolopakingii Fowlie	JQ929331	JQ929383
Paphiopedilum philippinense (Rchb.f.) Stein	JQ929341	JQ929393
Paphiopedilum praestans (Rchb.f.) Pfitzer	AY643452	JQ660886
Paphiopedilum randsii Fowlie	EF156132	JQ929396
Paphiopedilum rothschildianum (Rchb.f.) Stein	JQ660865	JQ660888
Paphiopedilum stonei (Hook.) Stein	EF156146	JQ660889
Sect. *Paphiopedilum*		
Paphiopedilum barbigerum Tang & F.T.Wang	AY643442	KP312035
Paphiopedilum charlesworthii (Rolfe) Pfitzer	JX088552	JQ929365
Paphiopedilum druryi (Bedd.) Stein	JQ929316	JQ660894
Paphiopedilum exul (Ridl.) Rolfe	EF156101	JQ929371
Paphiopedilum guangdongense Z.J.Liu & L.J.Chen	FJ899752	KP312085
Paphiopedilum gratrixianum Rolfe	FJ899753	JQ929377
Paphiopedilum henryanum Braem	JX088551	KP312047
Paphiopedilum hirsutissimum (Lindl. ex Hook.) Stein	HQ998459	JN181449
Paphiopedilum insigne (Wall. ex Lindl.) Pfitzer	JQ660874	JQ660898
Paphiopedilum spicerianum (Rchb.f.) Pfitzer	JQ929347	JQ929399
Paphiopedilum stenolomum Z.J.Liu, O.Gruss & L.J.Chen	-	KP312090
Paphiopedilum tigrinum Koop. & N.Haseg.	JQ929351	KP312055
Paphiopedilum tranlienianum O.Gruss & Perner	EF156151	KP312056
Paphiopedilum villosum (Lindl.) Stein	JQ660875	JQ660899
Sect. *Barbata*		
Paphiopedilum acmodontum M.W.Wood	EF156081	KP312058
Paphiopedilum appletonianum (Gower) Rolfe	EF156084	KC692116
Paphiopedilum argus (Rchb.f.) Stein	AJ564363	JQ660896
Paphiopedilum barbatum (Lindl.) Pfitzer	JQ660872	KC692121
Paphiopedilum bullenianum (Rchb.f.) Pfitzer	KC692109	KC692141
Paphiopedilum callosum (Rchb.f.) Stein	JQ929308	KC692131
Paphiopedilum ciliolare (Rchb.f.) Stein	JQ929311	GU120221
Paphiopedilum hennisianum (M.W.Wood) Fowlie	JQ929326	JQ929380
Paphiopedilum javanicum (Reinw. ex Lindl.) Pfitzer	EF156120	GU120220
Paphiopedilum lawrenceanum (Rchb.f.) Pfitzer	EF156122	JQ929384
Paphiopedilum purpuratum (Lindl.) Stein	AJ564364	KP312072
Paphiopedilum schoseri Braem & H.Mohr	AY643462	GU120214
Paphiopedilum sugiyamanum Cavestro	GU120205	GU120215
Paphiopedilum sukhakulii Schoser & Senghas	JQ929349	JQ929401
Paphiopedilum superbiens (Rchb.f.) Stein	EF156148	JQ660897
Paphiopedilum tonsum (Rchb.f.) Stein	EF156150	GU120216
Paphiopedilum venustum (Wall. ex Sims) Pfitzer	HQ998472	HQ998513
Paphiopedilum violascens Schltr.	EF156160	JQ929406
Paphiopedilum wardii Summerh.	JX088546	JN181450
Paphiopedilum notatisepalum Z.J.Liu, M.Wang & S.R.Lan	KY662379*	KY662380*

DNA extraction:—For each sample, genomic DNA was isolated from 0.2 g silica gel-dried or 0.4 g fresh leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) with 4% CTAB instead of 2%. Leaf tissue was ground in liquid nitrogen before adding the CTAB buffer.

Sequence comparisons and phylogenetic analysis:—Amplification, sequencing and data analysis were performed according to Zhang *et al.* (2013). Two markers (nrITS DNA and plastid *matK*) were used in this study. Primer information is listed in Li *et al.* (2014). Sequences for the newly identified species have been deposited in GenBank (Table 1). The matrices include nrITS alone, *matK* alone and all data combined. Data analyses including maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) methods were performed as previously described by Li *et al.* (2016).

Results

Morphological analysis:—A detailed comparison between the new species and other similar species in *Paphiopedilum* was made (Table 2). Morphological comparisons indicate that new species *P. notatisepalum* is most similar to *P. henryanum*, from which it differs by its leaves with large yellow spots, shorter scape, larger flower, ovate white sepals, and light purple-red petals with large purple spots and yellow-white margins (Fig. 1). There are also features that distinguish the new species from all other described *Paphiopedilum* species.

Phylogenetic analysis:—The length of the nrITS aligned matrix was 777 bp, of which 106 were variable and 99 were potentially parsimony-informative. For *matK*, the aligned matrix was 814 bp, of which 39 (4.79%) were variable and 33 (4.10%) were potentially parsimony-informative. Detailed information for each dataset is provided in Table 3.

The illustrated tree (Fig. 2) is the ML tree but it displays the results of the analyses of the combined data from the ML, MP and Bayesian analyses (BB_{ML}, BB_{MP} and PP, respectively). The general phylogenetic relationships indicated in this study are consistent with those in Chochai *et al.* (2012) and Guo *et al.* (2015). The newly identified species, *Paphiopedilum notatisepalum*, is sister to *P. barbigerum* Tang & Wang (1940: 23) in the analyses of nrITS and combined nrITS plus *matK* (Fig. 2), which has a green leaves and pale yellow-green sepals and petals. Given the isolated position of *P. notatisepalum* and the morphological differences, it is appropriate that it be treated as a new species. Support for the position of the new species is weak, and phylogenetic placements were not resolved in *matK*, so it cannot be excluded that it is instead sister to *P. henryanum*.

TABLE 2. Comparison of diagnostic characteristics for *P. notatisepalum*, *P. henryanum* and *P. barbigerum*.

Characteristics	*P. notatisepalum*	*P. henryanum*	*P. barbigerum*
Leaf color	Green with large yellow spots	Deep green with narrow yellow-white margins	Deep green
Scape	8–10 cm long, green, densely purple-pubescent	14–17 cm long, green to brownish-green, with brown-purple-pubescent	10–16 cm long, brownish-green, densely covered with purple-brown hairs
Flower	7.5–8.2 cm across	6.0–7.0 cm across	6.0–7.0 cm across
Dorsal sepal	Ovate, 3.5–3.8 × 2.3–2.7 cm, white with large purple spots	Suborbicular-ovate, 3.0–3.5 × 3.2–3.8 cm, pale yellow-green, with large purple-brow spots	Suborbicular, 2.8–3.8 × 3.6–3.8 cm, white with green-brown centrally at basal half
Synsepal	Narrowly ovate-elliptic, 3.0–3.3 × 1.7–2.0 cm, white with purple spots	Elliptic-ovate, 2.5–3.0 × 1.4–1.8 cm, pale-yellow-green	Elliptic, 2.5–3.3 × 1.4–2.0 cm, pale yellow-green
Petal	Narrowly obovate-oblong, 4.0–4.3 × 1.4–1.6 cm, light purple-red with large purple spots, undulate- and recurved margins	Narrowly obovate, 3.2–3.8 × 1.4–1.8 cm, pink-purple, with purple spots in basal half, undulate margins	Narrowly oblong, 3.0–4.0 × 1.4–2.0 cm, pale brown with deeper veins, undulate margins
Lip	Pouch ellipsoid, 3.5–4.0 × 1.9–2.2 cm, pale purple-red	Pouch ellipsoid, 2.3–2.8 × 2.2–2.5 cm, pink-purple	Ellipsoid-ovoid, 2.0–2.5 × 1.5–2.0 cm, pale brown
Staminode	Obovate, 8–9 × 6–7 mm	Obcordate, 7–9 × 7–8 mm	Obovate-obcordate, 7–10 × 8–10 mm

TABLE 3. Statistics from the analyses.

DNA region	No. of taxa	Aligned length	No. variable characters	No. informative characters (%)	Tree length	Consistency index	Retention index
ITS	53	777	106	99 (12.74)	275	0.83	0.90
matK	54	814	39	33 (4.10)	83	0.90	0.95
Combined	54	1591	145	132 (8.30)	370	0.82	0.90

FIGURE 1. *Paphiopedilum notatisepalum* and its allies. A. *Paphiopedilum notatisepalum*. B. *Paphiopedilum barbigerum*. C. *Paphiopedilum henryanum*.

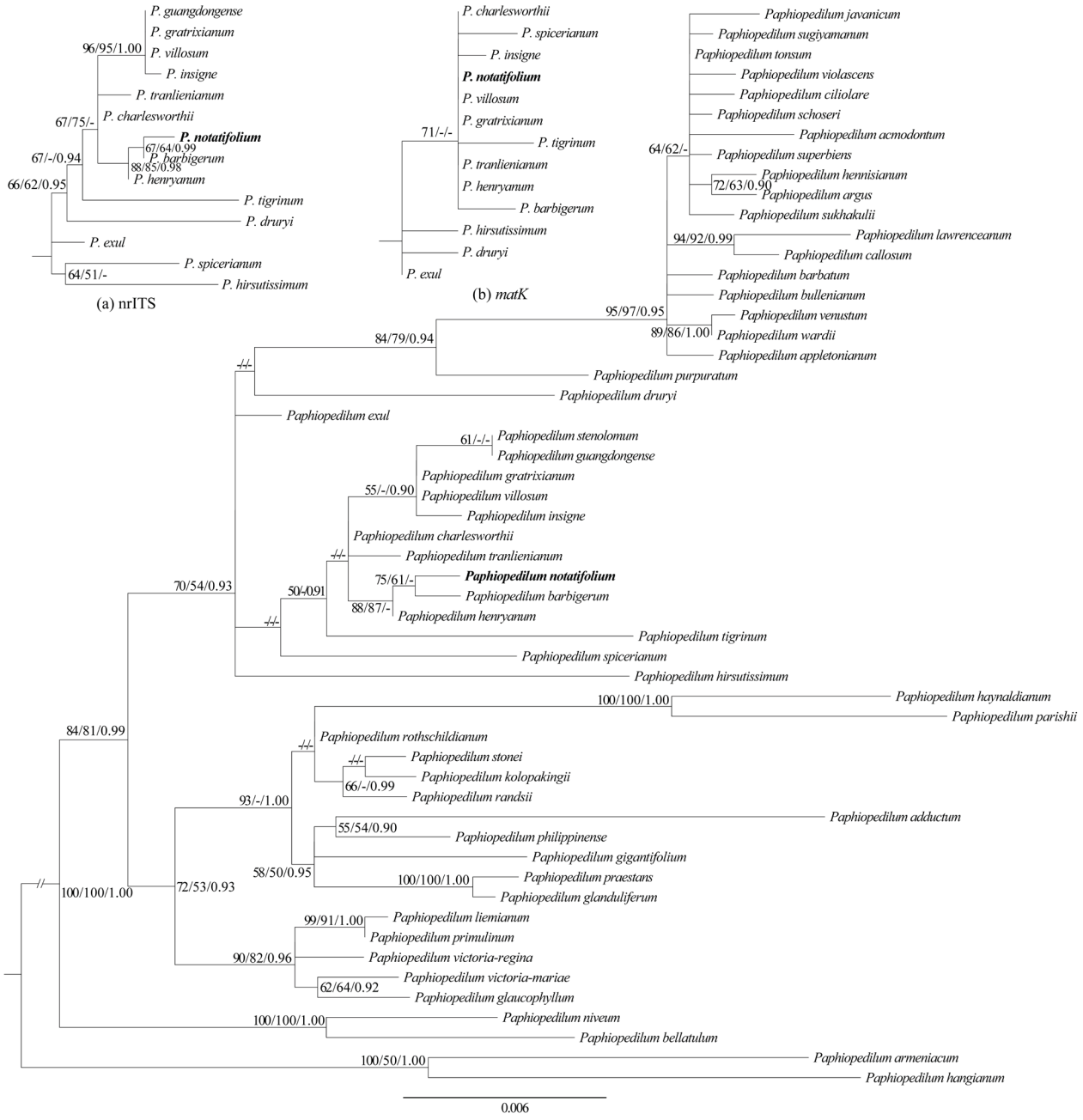

FIGURE 2. Phylogenetic tree for selected species of *Paphiopedilum* constructed with a combined matrix of nrITS and plastid *matK*. Analysis of subgen. *Paphiopedilum* based on separate nrITS (a) and plastid *matK* (b) data are shown in the top left corner. The ML tree is illustrated. Numbers near the nodes are bootstrap percentages and Bayesian posterior probabilities (BS_{ML}, BS_{MP}, PP). A dash (–) indicates a node has less than 50 percent bootstrap or 0.90 Bayesian posterior probability.

Discussion

Paphiopedilum notatisepalum belongs to subg. *Paphiopedilum* sect. *Paphiopedilum* based on its helmet-shaped lip, single-flowered inflorescence and uniformly green leaves occasionally with deep green venation adaxially. However, *P. notatisepalum* is different from all species in this genus in that the leaves have large yellow spots, which corresponds with other characters including a larger flower with white sepal and pink petals with large and small purple-red spots. The characters of the new species are stable in the all individuals from three populations at different sites, 20 individuals of which were transplanted in The National Orchid Conservation Center of China (NOCC). In particular, the yellow spots remain in the plants in cultivation, indicating that these are not due to lack of minerals and do have a genetic basis. The new species is described based on these 20 individuals and illustrated from on the holotype. In

the phylogenetic analysis, the new species is sister to *P. barbigerum* with a only weak support in the analysis of nrITS and combined nrITS plus *matK*, and the phylogenetic placements were not resolved in *matK*. It is also possible that *P. notatisepalum* could be a natural hybrid species between *P. barbigerum* and *P. henryanum*, a subject that needs further study. In particular, a population genetic study of this putative species complex needs to be undertaken. Use of phylogenetic markers with a single individual of each species could provide misleading results.

Taxonomic treatment

Paphiopedilum notatisepalum Z. J. Liu, M. Wang & S. R. Lan *sp. nov* (Figs 3, 4) (紫斑兜兰)

Type:—CHINA. Yunnan（云南）: Malipo（麻栗坡）, in crevices of shady cliffs or rocks of limestone, 1300 m, 8 Oct. 2016, *Liu 9349* (holotype: NOCC).

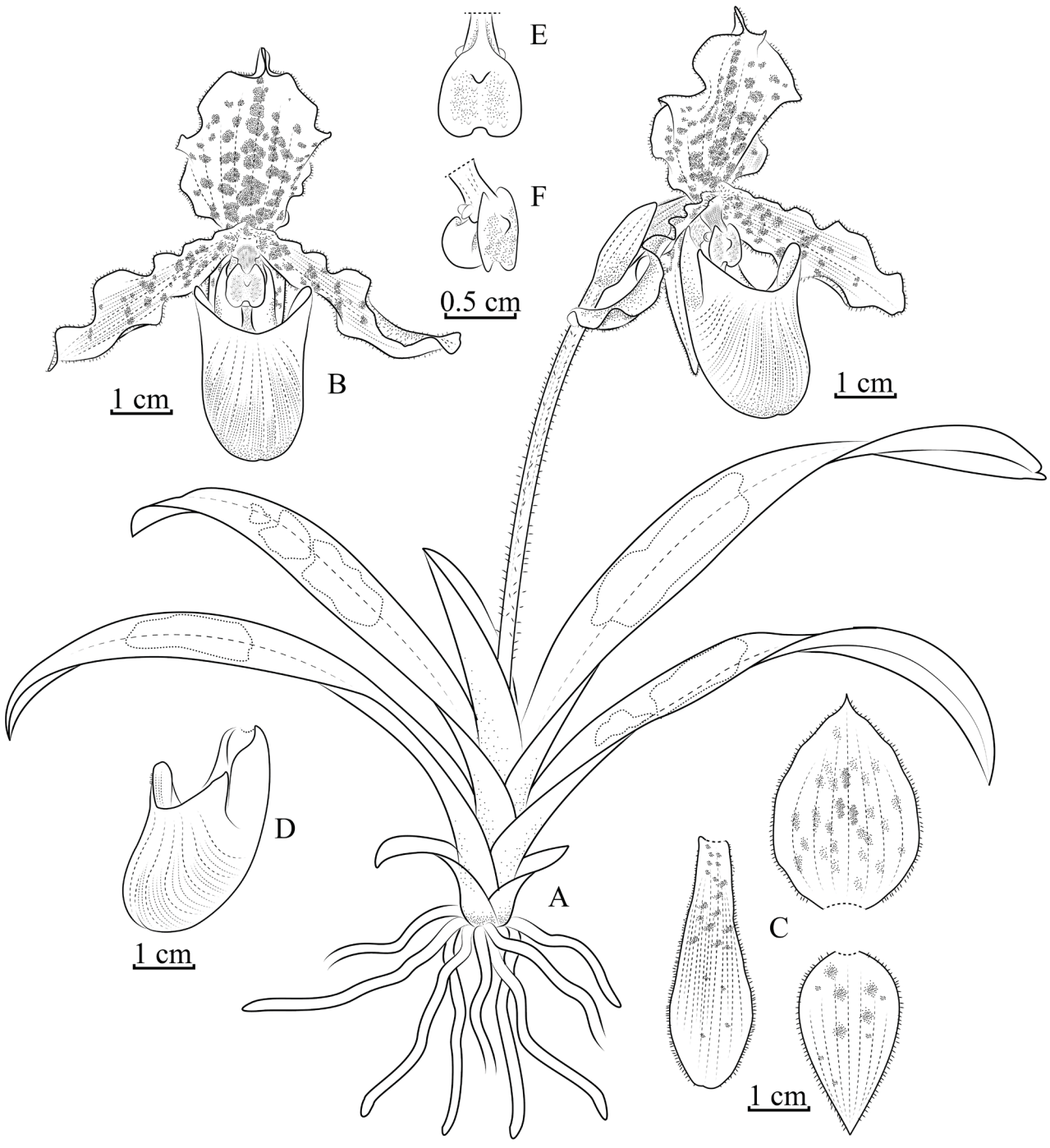

FIGURE 3. *Paphiopedilum notatisepalum* Z.J.Liu, M.Wang & S.R.Lan. A. Flowering plant. B. Flower, front view. C. Dorsal sepal, petal, and synsepal. D. Lip, side view. E. Staminode, front view. F. Column, side view. Drawn by L. J. Chen from *Liu 9349* (holotype).

FIGURE 4. *Paphiopedilum notatisepalum* Z.J.Liu, M.Wang & S.R.Lan. A. Flowering plant. B. Flower, front view. C. Flower, back view. D. Staminode, front view. E., F, Flowers, side view.

This new species is morphologically similar to *Paphiopedilum henryanum*, from which it differs by the smaller plants with large yellow spots on the leaves, shorter scape, larger flower, white and narrower sepal and light purple-red petals with larger purple spots and yellow-white margins.

Lithophytic herbs. Leaves 3–5, narrowly oblong, 8.0–17.0 × 1.2–2.0 cm, apex unequally bilobulate, minutely tridenticulate, deep green, with large yellow spots and purple markings at the base. Inflorescence arching, 8–10 cm long, green, densely purple-pubescent, terminally 1-flowered; floral bract ovate, pale green, with small purple spots, 3.0–3.5 ×1.5–2.0 cm; pedicel and ovary 3.3–4.0 cm long, green, densely purple-pubescent; flower 7.5–8.2 cm in diameter; dorsal sepal white with large purple spots; synsepal white with purple spots; petals pale purple-red with large purple spots and yellow-white margins; lip pale purple-red with yellow-white margins; staminode pale purple with brownish-yellow margins and a yellow umbo; dorsal sepal ovate, 3.5–3.8 ×2.4–2.7 cm margins minutely ciliate, recurved; synsepal narrowly ovate-elliptic, 3.0–3.3 × 1.7–2.0 cm, minutely ciliate, apex bilobulate; petals narrowly obovate-oblong, 4.0–4.3 × 1.4–1.6 cm long, margins undulate and recurved, obscurely tridenticulate apically, minutely ciliate, with long hairs at base; lip helmet-shaped; pouch ellipsoid, 3.5–4.0 × 1.9–2.2 cm, opening slightly auriculate on both sides; staminode obovate, 8–9 × 6–7 mm, papillose, with a small umbo adaxially, apex emarginate.

Flowering period:—September–November.

Distribution and habitat:—In crevices of shady cliffs or rocks in evergreen broad-leaved forests in limestone areas in southeastern Yunnan, China.

Etymology:—The species name *notatisepalum* refers to purple-maroon-spotted sepals, from the Greek *notati* (spot) and *sepalum* (sepal).

Conservation status:—Using the World Conservation Union Red List Categories and Criteria (IUCN, 2012), *P. notatisepalum* should be treated as critically endangered.

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