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Paphiopedilum erythroanthum, a new species of slipper orchid (Cypripedioideae, Orchidaceae) from China based on morphological and molecular data

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Abstract

A new species of *Paphiopedilum* (Orchidaceae) from Yunnan, China is described and illustrated based on examining morphological and molecular evidence. Morphological comparisons indicate that the new species *P. erythroanthum* is somewhat similar to *P. henryanum*, from which it differs by its shorter scape, larger and purple-red flower without purple spots, an obovate dorsal sepal, narrowly oblong petals with recurved apex and a cordate staminode. Molecular analyses of combined nuclear and plastid datasets (ITS and *matK*) indicate that *P. erythroanthum* is sister to *P. notatisepalum*, which has leaves with large yellow spots, a shorter scape, larger flower, white sepals and petals with large purple spots. The morphological and molecular evidence support the hypothesis that *P. erythroanthum* is a new species.

Keywords: Chinese orchids, orchid phylogenetics, Venus slipper, reticulate evolution

Introduction

The largest genus of the subfamily Cypripedioideae (slipper orchids), *Paphiopedilum* Pfitzer (1896:11), is native to the subtropical and tropical regions of Southeast Asia, comprising 96 accepted species (data collected from KBG, 01/2014), and it is an ideal group for investigating the evolution of island species diversity (Liu *et al*. 2009). The current circumscription has been strongly supported in previous studies (Cox *et al*. 1997, Chochai *et al*. 2012, Guo *et al*. 2012). Guo *et al.* (2015) found ITS and plastid DNA results showed discordance and stated that it is likely that reticulate evolution exists in this genus.

During a botanical trip to Malipo, Yunnan (China) in 2018, several populations of *Paphiopedilum* were found in the broad-leaved forests of several limestone mountains. The new entity is somewhat similar morphologically to *P. henryanum* Braem (1987: 4), but the new entity has deep red sepals and petals and a pink lip without purple spots.

Material and methods

Macromorphology:—Gross morphological data were obtained during fieldwork, and specimens were deposited in the herbarium of the Fujian Agriculture and Forestry University. Measurements, shapes, colours and other details given in the description are based on the examination of living material. Floral structure of the fully opened flowers was observed using a Novel NDZ-606 microscope (Ningbo Yongxin Optics Co., Ltd. Ningbo, China).

Sampling:—All sampled plants were transplanted to the Haixia Orchid Conservation Center of China (Fuzhou, China). The sequences of some allied and outgroup species were download from GenBank (Table 1).

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

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solutions (>0.7 mol/L NaCl), CTAB forms complexes with proteins and polysaccharides but does not precipitate nucleic acids. The organic acid is extracted to remove impurities such as proteins, polysaccharides, and phenols, and then ethanol is used precipitate the nucleic acids.

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

.....continued on the next page

TABLE 1. (Continued**)**

Species	ITS	*matK*
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
Paphiopedilum erythroanthum Z.J.Liu, X. Y. Liao & S.R.Lan	MK895546*	MK935187*

Sequence comparisons and phylogenetic analysis:—Amplification, sequencing and data analysis were performed according to Zhang *et al.* (2013). Two markers, nrDNA ITS 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 ITS, *matK* and all data combined. Data analyses including the maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) methods were performed as described by Li *et al.* (2016).

Results

Morphological comparison:—A detailed morphological comparison between the new species and other similar species in *Paphiopedilum* (Table 2) indicates that new species, *P. erythroanthum*, is similar to *P. henryanum*, from which it differs by its deep-red sepals and petals, pale purple lip, no purple spots, an obovate dorsal sepal, narrowly oblong petals with recurved and apically shortly acuminate and cordate staminode. 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 654 bp, of which 96 were variable and 97 were potentially parsimony-informative. The length of the *matK* matrix was 1039bp, of which 51 were variable and 42 were potentially parsimony-informative. For the two regions combined, the aligned matrix was 1786 characters, of which 147 (8.23%) were variable and 139 (7.78%) were potentially parsimony-informative (Table 3).

The ML tree based on the combined data is presented, and to this we added internal support from the ML, MP and Bayesian analyses (BB_{ML}, BB_{MP} and PP, respectively; Figure 1). The overall phylogenetic relationships indicated in this study are consistent with those in Chochai *et al.* (2012) and Guo *et al.* (2015). The newly identified species, *P. erythroanthum*, is a sister to *P. notatisepalum* Z.J.Liu, M.Wang & S.R.Lan (2017:161) in the analyses of nrITS and combined nrITS/*matK*. Support for the position of the new species is low, and phylogenetic placements were not resolved in *matK*. Given the isolated position of *P. erythroanthum* and the morphological differences, it is appropriate that it be treated as a new species.

TABLE 2. Comparison of diagnostic characteristics for *P. erythroanthum*, *P. notatifolium, P. henryanum, P. barbigerum and P. coccineum.*

Characteristics	***P. erythroanthum***	***P. notatifolium***	***P. henryanum***	***P. barbigerum***	***P. coccineum***
Leaf colour	Pale green	Green with large yellow spots	Deep green with narrow yellow-white margins	Deep green	Deep green
Scape	12–13 cm long, green, densely purple-pubescent	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	12–16 cm long, brownish-green, shortly brown to dull yellowish pubescent
Flower diameter	7.2–7.5 cm	7.2–7.5 cm	6.0–7.0 cm	6.0–7.0 cm	4.0–6.0 cm
Dorsal sepal	Obovate, 2.3–2.7 × 3.5–3.8 cm, deeply red with narrow yellow-white margins	Ovate, 2.3–2.7 × 3.5–3.8 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	Suborbicular, 2.8–3.8 × 3.6–3.8 cm, white with purple-brown centrally at basal half
Synsepal	Narrowly ovate-elliptic, 0.8–1.0 × 1.5–1.7 cm, deeply red	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	Elliptic, 2.4–3.2 × 1.5–2.1 cm, pale yellow-green
Petals	Narrowly oblong, 4.0–4.3 × 0.6–0.7 cm, deep red, recurved and short-acuminate	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-margined, obscurely trideuticulate at apex	Narrowly oblong, 3.0–4.0 × 1.4–2.0 cm, pale brown with deeper veins, undulate margins	Narrowly oblong, 2.7–3.5× 1.4–1.9 cm, pale brown with deeper veins, with narrow yellow-green margins
Lip	Pouch ellipsoid, 3.2–3.5 × 1.7–2.0 cm, pale purple	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	Ellipsoid-ovoid, 2.2–2.4 × 1.6–2.0 cm, pale purple
Staminode	Cordate, 6–7 × 5–6 mm	Obovate, 8–9 × 6–7 mm	Obcordate, 7–9 × 7–8 mm	Obovate-obcordate, 7–10 × 8–10 mm	Obovate-obcordate, 6–9 × 8–9 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	54	654	96	97 (14.83)	263	0.83	0.90
matK	54	1039	51	42 (4.04)	111	0.87	0.90
Combined	54	1786	147	139 (7.78)	385	0.70	0.89

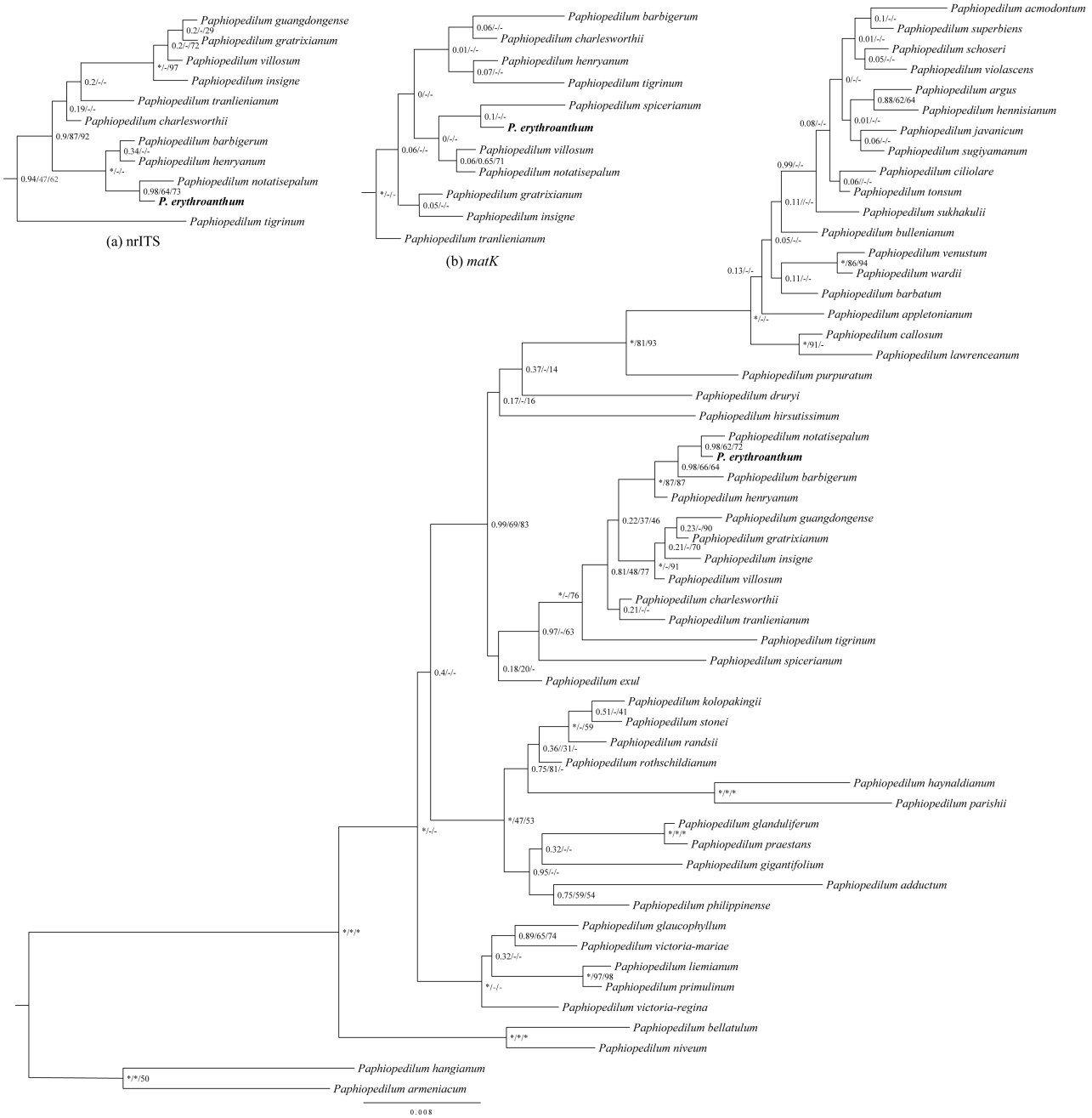

FIGURE 1. Phylogenetic tree for selected *Paphiopedilum* reconstructed with a combined matrix of nrITS and plastid *matK*. Analysis of selected *Paphiopedilum* based on separate nrITS (a) and plastid *matK* (b) data are shown in the top left corner. Numbers near the nodes are bootstrap percentages and Bayesian posterior probabilities (BS_{ML}, BS_{MP}, PP). "*" indicates that the node has BP 100 or PP 1.00. "-" indicates that the node is incongruent between the topology of the Bayesian tree and the MP/ML trees.

Discussion

Paphiopedilum erythroanthum belongs to *P.* 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. erythroanthum* is different from all other species in this genus in that the sepals and petals are deep red, which corresponds to other characters including the deep red sepals and petals, pale purple lip with no purple spots, an obovate dorsal sepal, narrowly oblong petals that are recurved and shortly acuminate apically and cordate staminode. In the phylogenetic analysis, the new species is sister to *P. notatisepalum* with a strong support in the analysis of nrITS alone and combined nrITS plus *matK*, although phylogenetic its position was not resolved with *matK*. *Paphiopedilum notatisepalum* has leaves with large yellow spots, a shorter scape, a larger flower, white sepals and petals with large purple spots. It is also possible that *P. erythroanthum* could be a natural hybrid species between some of the closely related species in this group, *P. barbigerum, P. notatifolium, P. coccineum* Perner & Herrmann (2000: 623) and *P. henryanum*, although they differ in morphology (Table 2), a subject that needs further study. In particular, a population genetic study of this putative species complex needs to be undertaken because the use of phylogenetic markers with a single individual of each species could provide misleading results.

Taxonomic treatment

Paphiopedilum erythroanthum Z.J.Liu, X.Y.Liao & S.R.Lan, *sp. nov* (红花兜兰) (Figs 2, 3A)

Type:—CHINA. Yunnan (云南): Malipo (麻栗坡), in crevices of shady cliffs or rocky of limestone at an elevation of 1300 m, 28 Jul. 2018, *Liao F002* (holotype: FAFU).

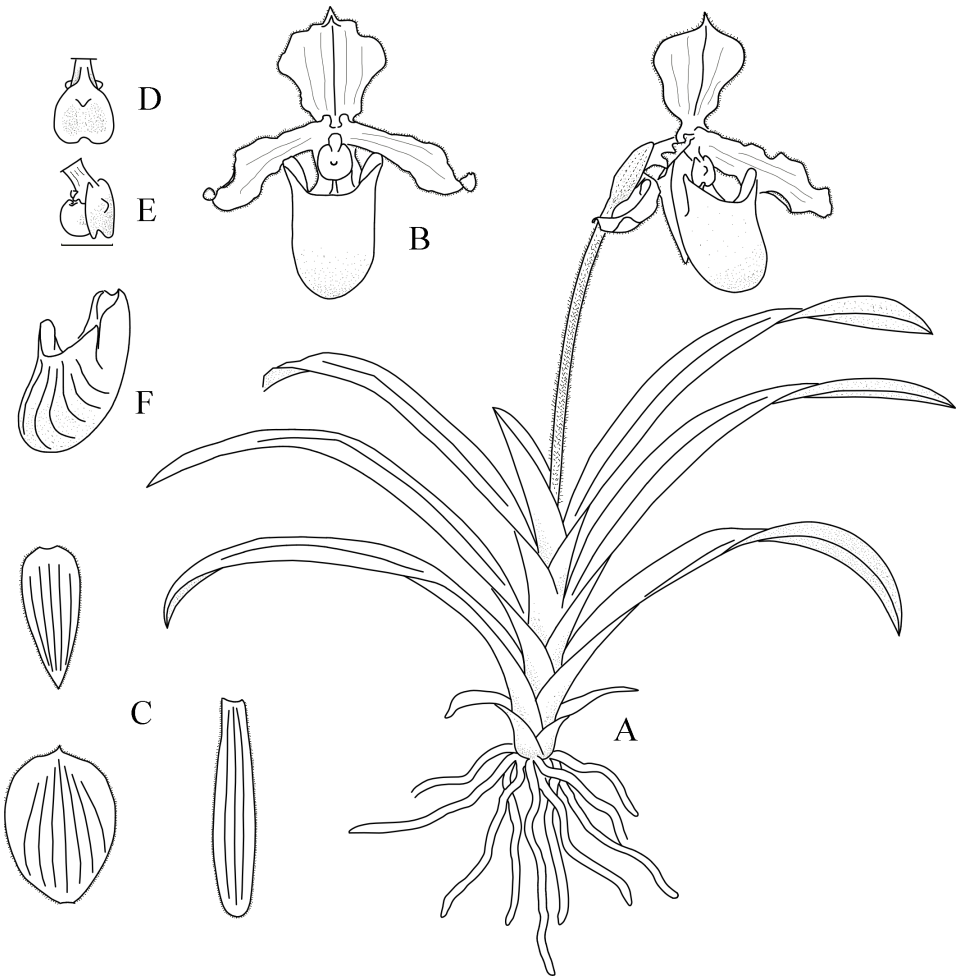

FIGURE 2. *Paphiopedilum erythroanthum* Z.J.Liu, X.Y.Liao & S.R.Lan. A. Flowering plant. B. Flower, front view. C. Dorsal sepal, petal, and synsepal, front view. D. Staminode, front view. E. Column, side view. F. Lip, side view. Drawn by X.Y. Liao from *Liao F002* (holotype: FAFU).

FIGURE 3. *Paphiopedilum erythroanthum* and morphologically similar species. A. *Paphiopedilum erythroanthum*. B. *Paphiopedilum notatisepalum*. C. *Paphiopedilum henryanum*. D. *Paphiopedilum barbigerum*.

This new species is similar to *Paphiopedilum notatisepalum*, from which it differs in its larger plants with green leaves and longer scape, smaller and purple-red flower without purple spots, obovate dorsal sepal, narrowly oblong petals with a recurved apex and cordate staminode.

Lithophytic herbs. Leaves 5–7, narrowly oblong, 10–20 × 1.7–2.0 cm, apex unequally bilobulate and minutely tridenticulate, deep green, with purple basal markings. Inflorescnece arching, 12–13 cm long, green, densely purple-pubescent, terminally 1-flowered; floral bract ovate, pale green, 3.0–3.5 ×1.5–2.0 cm; pedicel and ovary 3.3–4.0 cm long, green densely purple-pubescent; flower 7.2–7.5 cm across; dorsal sepal deep red with yellow-white margins; synsepal deep red, petals deep red; lip pale purple with yellow-white margins; staminode pale purple, with a yellow-green umbo; dorsal sepal obovate, 2.4–2.7 × 3.5–3.8 cm, minutely ciliate, margins recurved at middle ; synsepal narrowly ovate-elliptic, 0.8–1.0 × 1.5–1.7 cm, minutely ciliate, apex bilobulate; petals narrowly oblong, 4.0–4.3 × 0.6–0.7 cm, recurved and short-acuminate apically, minutely ciliate, with long hairs at base; lip helmet-shaped; pouch ellipsoid, 3.2–3.5 × 1.7–2.0 cm, mouth slightly auriculate on both sides; staminode cordate, 6–7× 5–6 mm wide, papillose and with a small umbo adaxially, apex emarginate.

Flowering:—July to September.

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

Etymology:—Referring to the red flower, from the Greek *erythros* (any red) and *anthos* (flower).

Conservation status:—According to the IUCN criteria (2012), *P. erythroanthum* should be considered critically endangered.

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