ARPHA Preprints

PENSOFT

PREPRINT

Author-formatted, not peer-reviewed document posted on 20/04/2022

DOI: https://doi.org/10.3897/arphapreprints.e85293

Primula shimenensis (Primulaceae), a new species from karst caves in Hunan, China

Wei Zhang, Yu Zhang, Jian-wen Shao

ARPHA Preprints *Author-formatted, not peer-reviewed document posted on 20/04/2022* DOI: https://doi.org/10.3897/arphapreprints.e85293

Primula shimenensis (Primulaceae), a new species from karst caves in Hunan, China

Wei Zhang[1,2], Yu Zhang[1], Jian Wen Shao[1,2*]

1 College of Life Sciences, Anhui Normal University, Wuhu, Anhui 241000, China

2 Provincial Key Laboratory of Conservation and Utilization of Biological Resources, Wuhu, Anhui 241000, China

Corresponding author: Jian Wen Shao (shaojw@ahnu.edu.cn)

Abstract

Primua shimenensis W.Zhang & J.W.Shao, a new species from Hunan Province, China, is described. Its leaf morphology is similar to *P. merrilliana* complex and flower morphology similar to *P. cicutariifolia*, but it can be distinguished from the former by flower homostylous, corolla lobes cuneate-oblong and apex obviously emarginate, and can be differed from the latter by cotyledon triangular obovate, plants densely covered with glandular hairs, pinnae margin usually pinnatipartite, and special habitat (karst caves). The whole plastid genome of this new species is 151, 601–151, 630bp in length. Phylogenetic trees, based on the whole plastid genome sequences, revealed that the new species didn't genetically related to the above two morphologically similar species but closely related to *P. hubeiensis*. Currently, only three populations were discovered within a small distribution area, thus it is preliminarily considered as Vulnerable (VU) according to criteria of the IUCN Red List.

Keywords

Homostyly, section *Ranunculoides, P. merrilliana* complex, *P. cicutariifolia*, *P. hubeiensis*

Introduction

Primula L. is the largest genus in Primulaceae, comprises about 500 species worldwide. The genus is mainly distributed in temperate and alpine regions of the Northern Hemisphere, with only a few species in the Southern Hemisphere (Richards 2002). In China, there are approximately 300 native *Primula* species, and the modern distribution centers of this genus are located along both sides of the Himalayas to Yunnan and western Sichuan (Hu and Kelso 1996).

The sect. *Ranunculoides* C.M.Hu is a unique group in *Primula*, characterized by pinnately compound leaves, and calyx not inflated at the base (Hu 1990; Hu & Kelso 1996). This section now includes 8 recognized species, i.e., *P. merrilliana* complex (*P. merrilliana* Schltr., *P. qiupuensis* J.W.Shao, *P. zhexiensis* X.He & J.W.Shao, *P. wannanensis* X.He & J.W.Shao), *P. ranunculoides* F.H.Chen, *P. cicutariifolia* Pax, *P. jiugongshanensis* J.W.Shao and *P. hubeiensis* X.W.Li (Shao et al. 2012; He et al. 2017; Li et al. 2018; Xu et al. 2020; Zhang et al. 2020; He et al. 2021). They are all endemic to central or eastern China and often grow at the waterside or at the edge of broadleaf deciduous forests between the elevation of 50–1600 m (Shao et al. 2012; He et al. 2017;

Zhang et al. 2020; Zhang et al. 2021).

In March 2016, during our field expeditions in Shimen Village, Xinning County, Hunan Province, China, we encountered a suspicious species of sect. *Ranunculoides*. The plants were restricted to growing on the walls and ground near the entrance of karst caves, which are quite different from other known related species. After carefully morphological observations, together with evidence from molecular phylogenetic analyses based on chloroplast genome, this suspicious species was confirmed as a new species. Here, the investigation results are reported and the new species is named as *Primua shimenensis* W.Zhang & J.W.Shao and described.

Materials and methods

Sampling and morphological analyses

The studied populations were collected in Shimen Village (26°30'27.22"N, 110°40'56.82"E, Altitude: 468 m), Xinning County, Hunan Province, China. Voucher specimens were deposited at the herbarium of Anhui Normal University (ANUB). The morphological description of the new species was based on examination of fresh material and herbarium specimens. A total of 13 diagnostic characteristics of the new species were identified and compared to related species in the *Primula* sect. *Ranunculoides* (Shao et al. 2012; He et al. 2017; Li et al. 2018; Zhang et al. 2020; He et al. 2021).

Genome sequencing, assembly and annotation

Genomic DNA was extracted from dried leaves using a modified CTAB protocol (Doyle and Doyle 1987). The quality and concentration of DNA products were assessed via agarose gel electrophoresis and spectrophotometry, and qualified DNA sample was sent to BGI-Shenzhen (Shenzhen, China) for library construction and next-generation sequencing. Finally, we obtained c. 2 Gb of high-quality clean data, complete chloroplast genome was assembled using GetOrganelle described in Jin (2019), annotation was conducted with Plastid Genome Annotator (Qu et al. 2019), coupled with manual correction using Geneious v 9.1.4 (Kearse et al. 2012). The plastome of *P. hubeiensis* (Genbank accession number: MT268976) was used as reference genomes for annotation. The cp genome maps were drawn using OGDRAW (Greiner et al. 2019). All sequences generated in this study were submitted to the NCBI database, the accession numbers are ON208991 (*P. shimenensis*) and ON208990 (*P. shimenensis*), respectively.

Phylogenetic analyses

In order to know the phylogenetic relationship between this new species and other species in the section, we downloaded 5 accessions cp genome sequences of related species from the NCBI: *P. merrilliana* complex (MT268977), *P. cicutarrifoli*a (MT268974), *P. hubeiensis* (MT268976), *P. jiugongshanensis* (MT937162), *P. ranunculoides* (MT268978). All sequences were aligned with MAFFT v.7 (Katoh and Standley 2013) using the default settings and adjusted manually where necessary using MEGA 7.0.14 (Kumar et al. 2016). Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) methods with *P. flchnerae* (MK888698) as outgroup. The ML analysis was conducted using RAxML-HPC

BlackBox v.8.1.24 at the CIPRES Science Gateway website (Miller et al. 2010; Stamatakis et al. 2014) with 1000 bootstrap replicates, the (GTR) + G + I model was used in ML analyses. For the BI analysis, the best substitution model was determined according to Bayesian information criterion (BIC) with ModelFinder (Kalyaanamoorthy et al. 2017). The BI analysis was performed using MrBayes v3.2 (Ronquist et al. 2012). The Markov chain Monte Carlo (MCMC) algorithm was run for 10 million generations and the trees were sampled every 1000 generations. Convergence was determined by examining the average standard deviation of the split frequencies (< 0.01). The first 25% of the trees were discarded as a burn-in and the remaining trees were used to generate the consensus tree.

Results

Characteristics of the complete plastid genome

The length of complete plastid genome of *P. shimenensis* comprised 151,601–151,630 bp (Fig. 1). It possessed typical quadripartite structure: IRa, IRb, LSC and SSC, the characteristics and statistics of the plastid genome are summarized in Table 1.

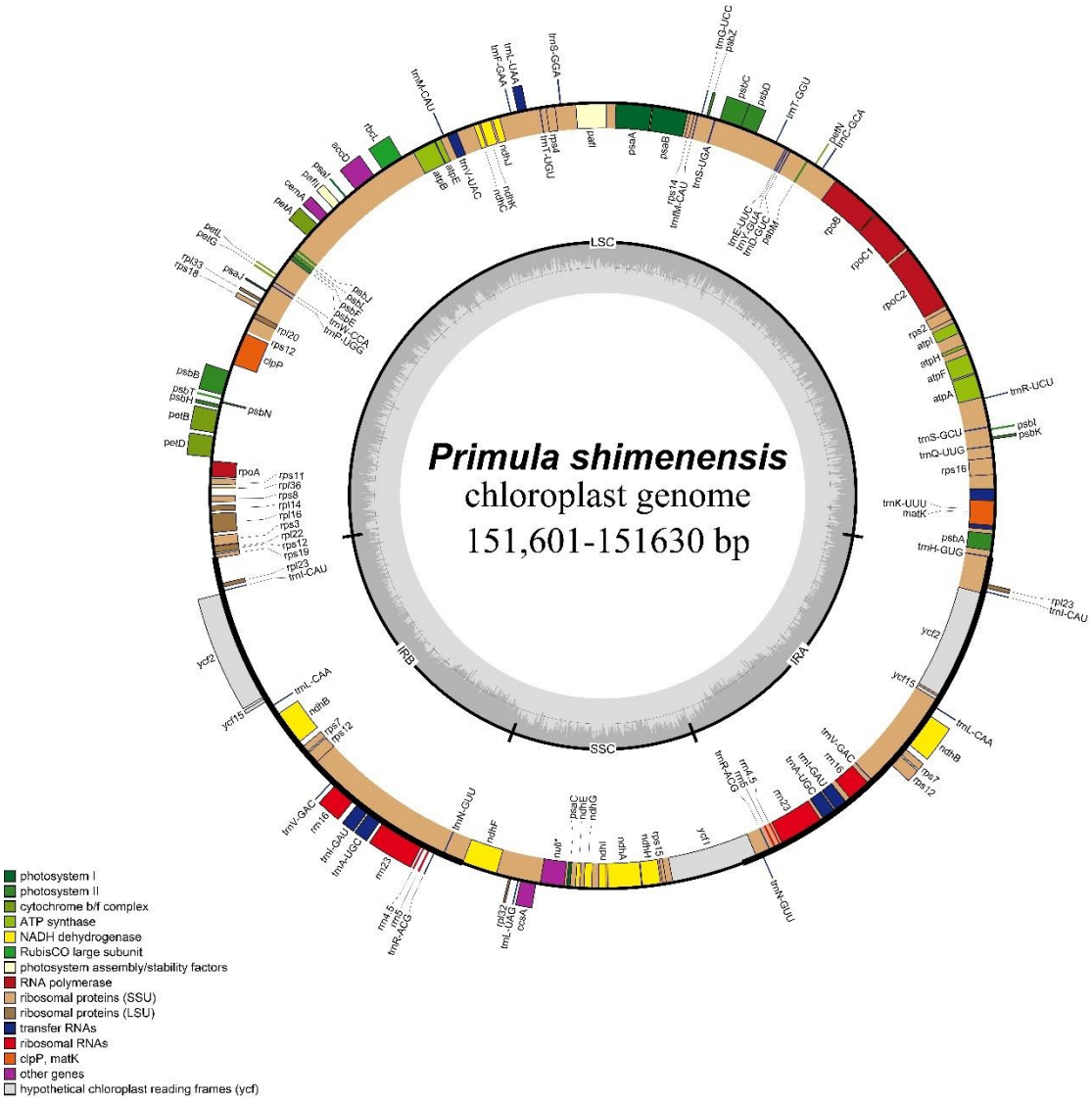

Figure 1. Plastid genome map of *P. shimenensis* sp. nov.

Table 1. Basic characteristics of cp genomes of *Primula shimenensis* sp. nov..

Characteristic	*Primula shimenensis*
Total length (bp)	151,601–151,630
GC%	36.8%–36.8%
LSC length (bp)	83,421–83,466
SSC length (bp)	17,583–17,599
IR length (bp)	25,292
Total genes	113
Protein-coding genes	80
rRNA genes	4
tRNA genes	29

Molecular phylogenetic relationship

Phylogenetic relationships among the species of the sect. *Ranunculoides,* including this new species, were constructed based on the whole plastid genome using ML and BI analyses. The results showed that *P. merrilliana* complex, *P. cicutariifolia* and *P. jiugongshanensis* clustered one clade, and the other three species (*P. shimenensis P. hubeiensis* and *P. ranunculoides*) clustered another clade (Fig. 2). *P. shimenensis* is a sister species of *P. hubeiensis,* and their individuals were respectively grouged into a monophyly with high support (posterior probability (PP) = 1, bootstrap support (BS) = 100%) (Fig. 2).

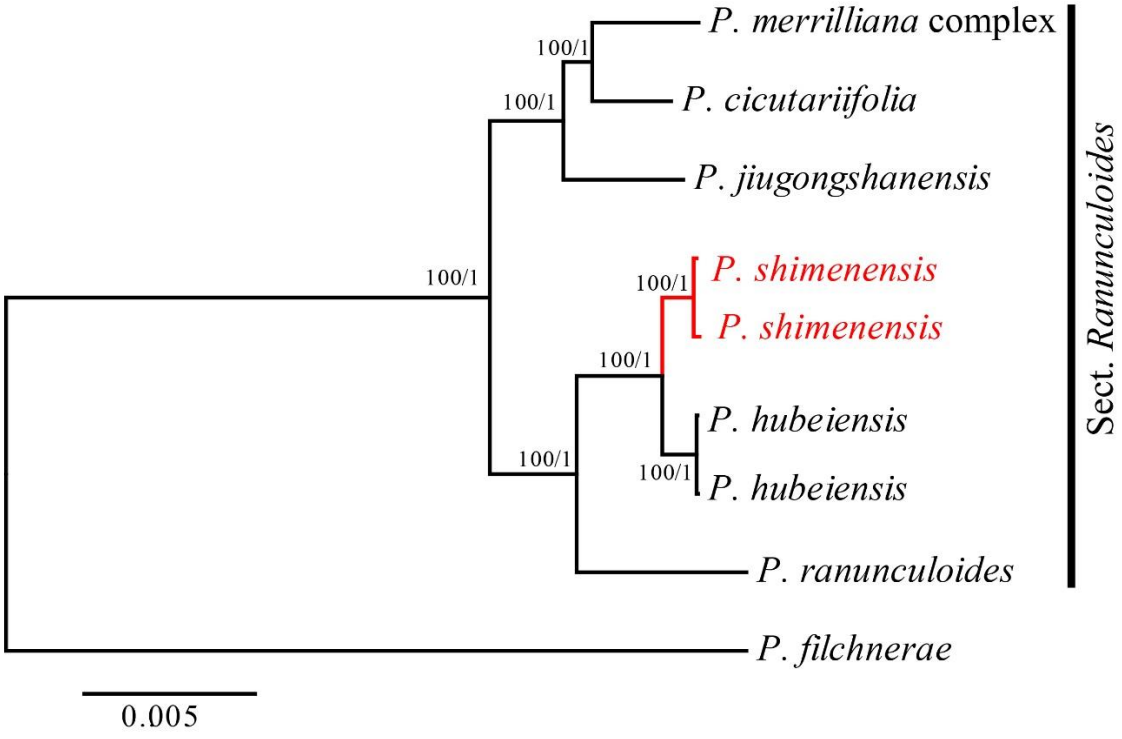

Figure 2. Phylogenetic relationships of *P. shimemensis* sp. nov. and related species inferred from ML and BI analyses based on the whole plastid genome. Numbers on the branches indicate the bootstrap support of the ML and the posterior probability of BI analyses.

Morphological comparison

In morphology, this new species is very similar to *P. merrilliana* complex in leaf pinnae shape and degree of division, and similar to *P. cicutariifolia* in floral characters, but can be easily distinguish from the former by flower homostylous, corolla lobes cuneate-oblong and apex obviously emarginate, and can be differed from the later by cotyledon triangular obovate, pinna margin usually pinnatipartite, and plants densely covered with glandular hairs (Table 2, Figs 3–4). Although in phylogenetic relationship, *P. shimemensis* closely related to *P. hubeiensis,* there were obvious morphological differences between them in pinna division pattern (segments margin entire vs segments margin serrate), the length of glandular hair (0.07–0.42 mm vs 0.76-0.88 mm) and flowers size and type (homostylous and corolla diameter 8–12 mm vs distyly and corolla diameter 13–18 mm) (Table 2, Figs 3–4). Detailed morphological comparisons between the new species and other related species in sect. *Ranunculoides* are summarised in Table 2.

ARPHA Preprints Author-formatted, not peer-reviewed document posted on 20/04/2022 DOI: https://doi.org/10.3897/arphapreprints.e85293

Table 2. Morphological characters comparison between *P. shimenensis* sp. nov. and its related species.

Characters	***P. shimenensis***	***P. hubeiensis***	***P. ranunculoides***	***P. jiugongshanensis***	***P. cicutariifolia***	***P. merrilliana* complex**
Floral morph	Homostylous	Distylous	Distylous	Distylous	Homostylous	Distylous or homostylous
Umbel layers	1	1–2	2–3	1–3	1	1–3
Corolla diameter	8–12 mm	13–18 mm	14–19 mm	13–17 mm	6–10 mm	9–19 mm
Corolla lobes	Apex conspicuously emarginate	Apex conspicuously emarginate	Apex conspicuously emarginate	Apex conspicuously emarginate	Apex conspicuously emarginate	Apex rounded
Scape length	0.8–2 cm	3.5–9.6 cm	4–9 cm	3–9 cm	1–3 cm	1.3–9 cm
Reproductive modes	Sexual	Sexual	Sexual and asexual	Sexual	Sexual	Sexual
Pollens	Pantoporate	Pantoporate	Stephanocolpate	Pantoporate	Pantoporate	Pantoporate or stephanocolpate
Pollen sac	Black	Yellow	Yellow	Yellow	Yellow	Yellow
Cotyledon	Triangular obovate	Ovate	Ovate and kidney-shaped	Ovate	Ovate	Ovate
Older Leaves	Pinnatisect, with 11–19 pinnae, the terminal pinna similar to others, 3-lobed or parted	Pinnatisect, with 13–19 pinnae, the terminal pinna similar to others, 3-lobed or parted, margin coarsely dentate	Pinnatisect, with 3–9 pinnae, the terminal pinna kidney or elliptic, oberviously larger than others, repand to parted	Pinnatisectwith 7–19 pinnae, the terminal pinna similar to others, usually 3-lobed	Pinnatisect, with 7–17 pinnae, the terminal pinna similar to others, 3-lobed	Pinnatisect, with 11–21 pinnae, the terminal pinna similar to others, 3-lobed or parted
Glandular hairs	0.07–0.42 mm	0.76–0.88 mm	without	without	without	without
Distribution	Hunan	Hubei	Hunan, Hubei, Jiangxi	Hubei	Anhui, Zhejiang	Anhui, Zhejiang
Habitat	Karst caves	Shady damp rock crevices	Stream sides or under broadleaf deciduous forests of northern slopes	Under broadleaf deciduous forests of northern slope	Stream sides or under broadleaf deciduous forests of northern slopes	Stream sides or under broadleaf deciduous forests of northern slopes

ARPHA Preprints *Author-formatted, not peer-reviewed document posted on 20/04/2022* DOI: https://doi.org/10.3897/arphapreprints.e85293

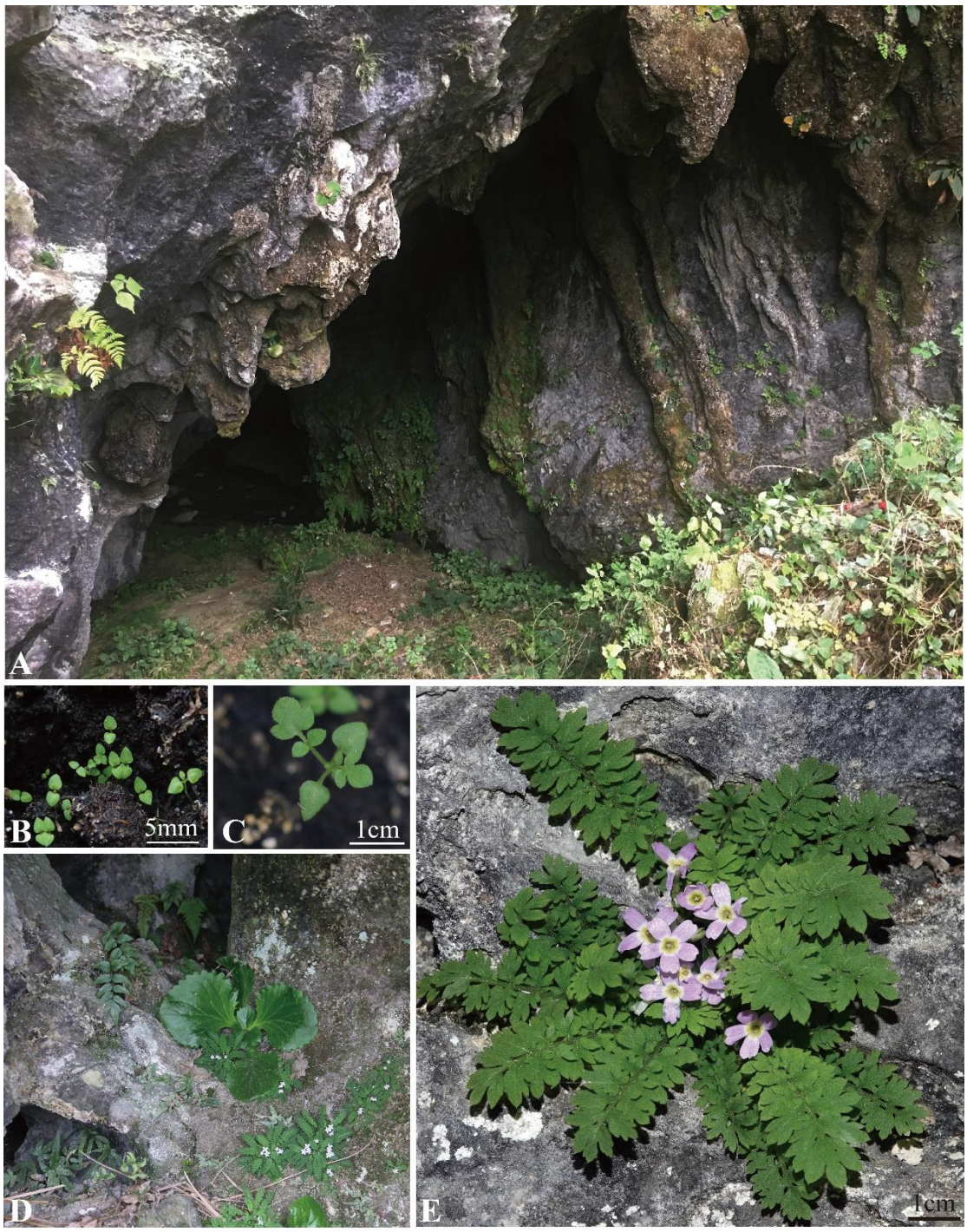

Figure 3. Living images of *P. shimenensis* sp. nov. **A, D** habitat **B, C** plant in seedling **E** plant in flowering.

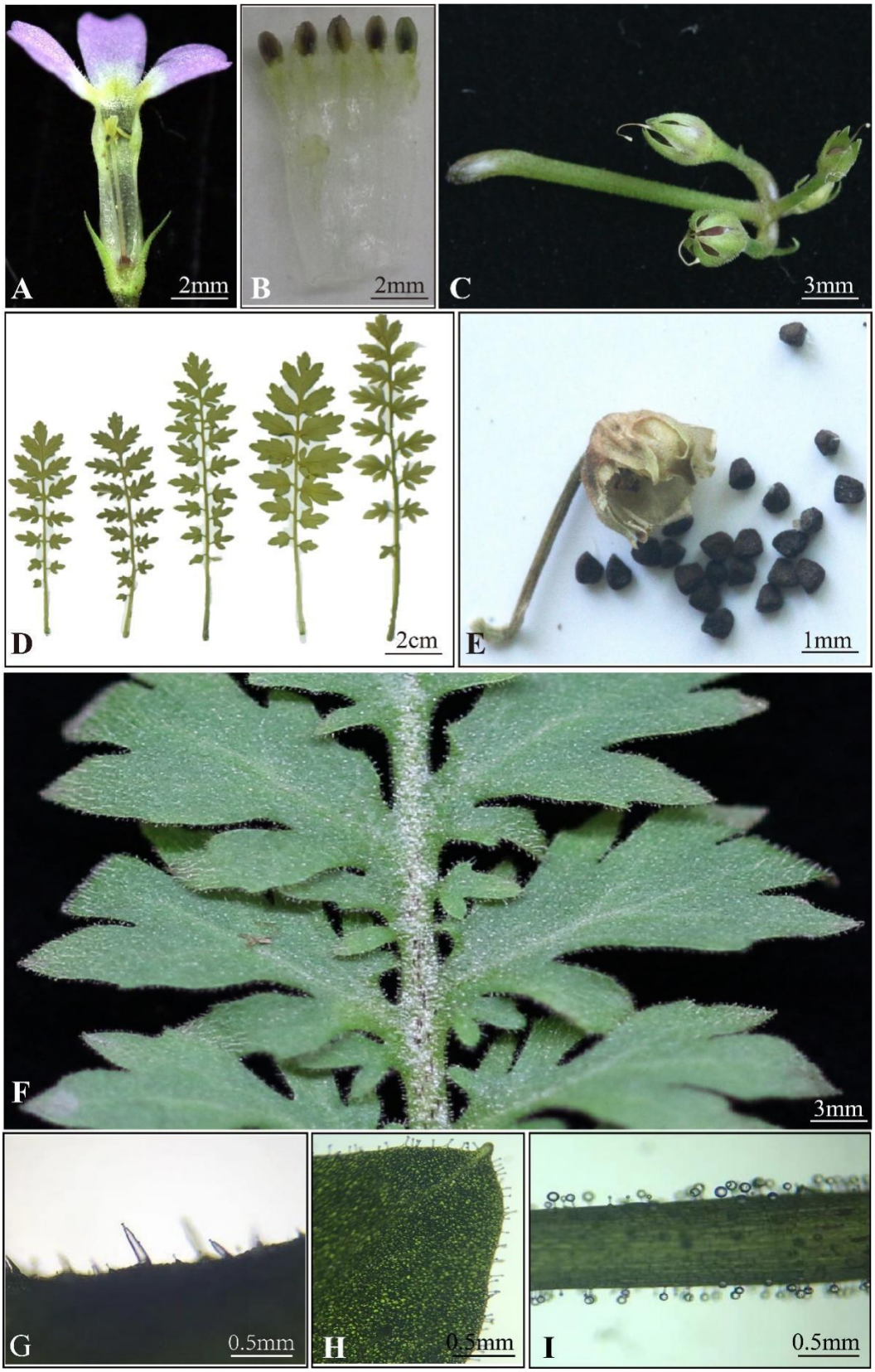

Figure 4. Morphological characters of *P. shimenensis* sp. nov. **A, B** longitudinally dissected of floral tube **C** infructescence **D, F** leaves morphology **E** opened capsule and seeds **G** leaf surface glandular hairs **H** leaf margin glandular hairs **I** rachis glandular hairs.

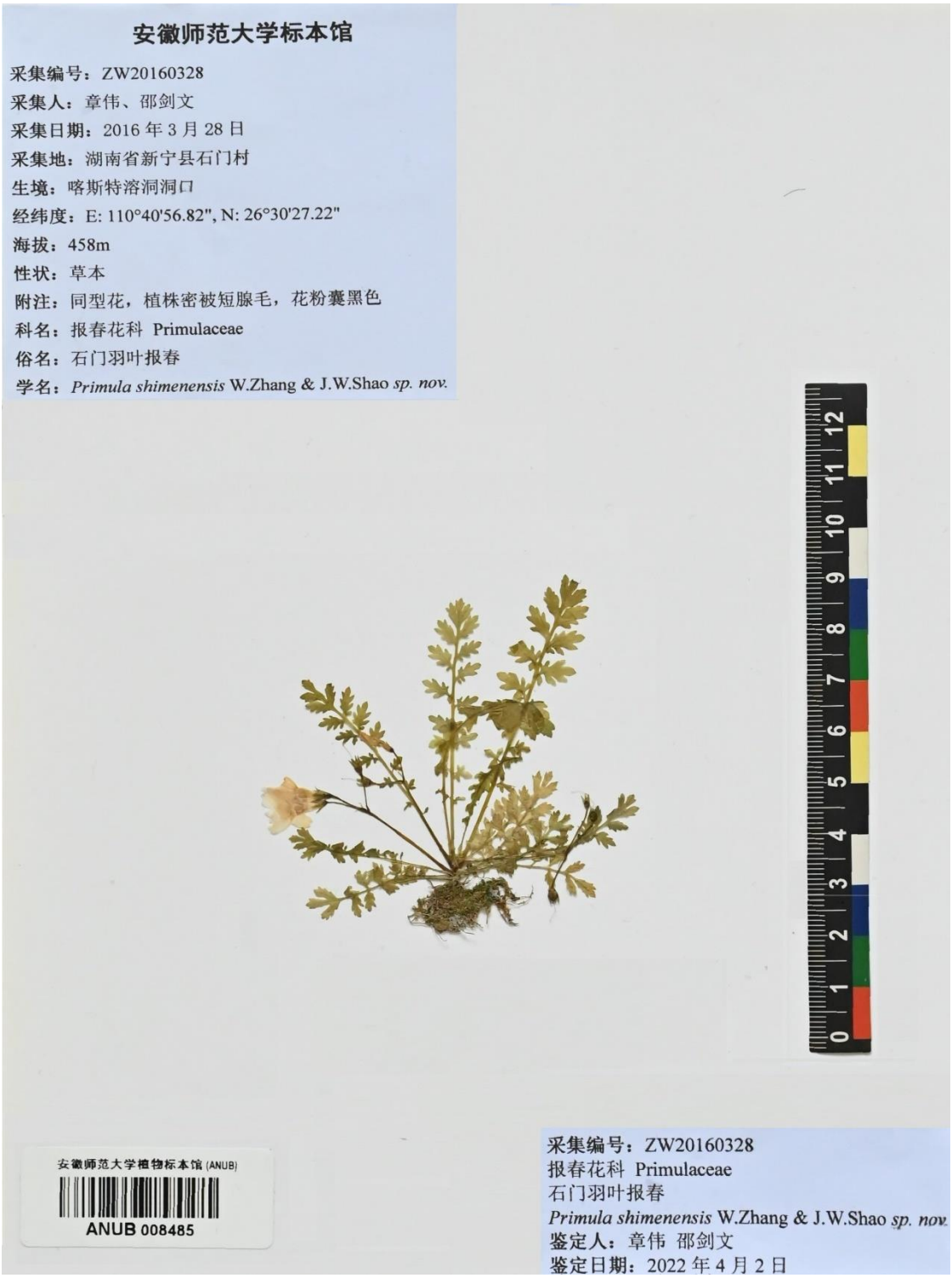

Figure 5. Holotype of *P. shimemensis* W.Zhang & J.W.Shao, sp. nov.

ARPHA Preprints *Author-formatted, not peer-reviewed document posted on 20/04/2022* DOI: https://doi.org/10.3897/arphapreprints.e85293

Taxonomic treatment

***Primula shimemensis* W.Zhang & J.W.Shao, sp. nov.**

Figs 3–5

Type. China. Hunan, Xinning County, Shimen village, 26°30'27.22"N, 110°40'56.82"E, atl. 458 m, 28 Mar 2016, *Wei.Zhang & Jian.Wen.Shao* ZW20160328 (holotype: ANUB!; isotypes: ANUB!, PE!) (Fig 5).

Diagnosis. Flowers long homostyled, corolla lobe apices conspicuously emarginate, pollen sac black, leaves and scape densely covered with short glandular hairs.

Description. Herb biennial, dwarf, densely covered with short glandular hairs. Leaves 9–25 in an open rosette; petiole 1.0–2.0 cm long; leaf blade pinnatisect, 4.0–8.0 cm long, 1.0–2.0 cm wide; pinnae 5–9 pairs, elliptic, margin usually pinnatifid, segments 3–5, apex mucronulate. Scapes 1–8 in each plant, 0.8–2.0 (–2.5) cm tall, carrying 1 umbel, usually 3 flowers per umbel; bracts linear lanceolate, 2–3 mm long. Pedicel slender, 0.3–0.8 cm. Flowers long homostylous. Calyx narrowly campanulate, 1.0–3.0 mm long, split to the middle; lobes lanceolate, apex acuminate, not outward curvature. Corolla pale red, flowers limb 8.0–12.0 mm across, lobes obovate, ca. 2.5 mm wide, apex conspicuously emarginate, corolla tube 5.8-7.0 mm, both stamens and stigma at the mouth of the corolla tube, pollen sac black. Capsule subglobose, 1.0–3.0 mm in diam, dehiscing by valves.

Phenology. Flowering from March to April, fruiting from April to May.

Chinese name. Shí-mén-yǔ-yè-bào-chūn (石门羽叶报春).

Etymology. The specifc epithet '*shimenensis*' refers to the locality, Shimen village, Xinning City, Hunan, China.

Distribution and Ecology. *P. shimemensis* is known only from Shimen village, Xinning City, Hunan, China. Growing on the walls or ground of karst caves, at an elevation of 385–487 m. The main associated species were *Primulina latinervis* (W.T.Wang) Mich. Möller & A. Weber. (Gesneriaceae), *Cyrtomium fortunei* J.Sm. (Dryopteridaceae) and *Pteris multifida* Poir. (Pteridaceae).

Conservation status.

This species is endemic to China, Hunan Province, Xinning County, Shimen village. We only found three populations, all of them near the entrance of the karst caves and each with about 100-250 individuals. The surrounding area is cultivated field with strong human activities. The Extent of Occurrence (EOO) is less than 10 km^2 and the known Area of Occupancy (AOO) is less than 0.5 km^2. The conservation status can be evaluated as Vulnerable (VU) D2, based on the IUCN Red List Categories and Criteria (IUCN 2019). In addition, *P. shimemensis* is a homostylous species. Although *Primula* containing ca. 500 species, there are only ca. 45 species have monomorphic populations (Mast et al. 2006). Therefore, the recognization of this new species increases the homostylous species diversity in *Primula* and can provide valuable material for studying the evolution and maintenance mechanism of distylous flowers.

Additional specimen examined.

China. Hunan: Xinning County, Wanfeng forest farm, atl. 450 m, 22 Apr 1995, *Lin Bo Luo* 00205565 (PE); Xinning County, Wanfeng forest farm, atl. 450 m, 22 Apr 1995, *Lin Bo Luo* 00353811 (IBK); Xinning County, Shuimiao town, Jiangmu village, atl. 347 m, 19 Feb 2014, *Xun Lin Yu & Hui Zhou* 028303 (CSFI); Xinning County, Shuimiao town, Jiangmu village, atl. 347 m, 19 Feb 2014, *Xun Lin Yu & Hui Zhou* 028304 (CSFI).

Key to the species of sect. *Ranunculoides*

1 Corolla lobe apices rounded……..…. *P. merrilliana* complex

– Corolla lobe apices obviously emarginated…….…….....….2

2 Compound leaves with 3–9 pinnae; scape apices differenting to bulblets late in flowering…….…….....…..*P. ranunculoides*

– Compound leaves with 7–21 pinnae; scape apices lacking bulblets…….....3

3 Plants densely covered with glandular hairs…….....4

– Plants glabrous…….....5

4 Cotyledon triangular obovate, pinna segments margin entire, flower homostylous, corolla diameter 8–12 mm…….....….....….....*P. shimenensis*

– Cotyledon ovate, pinna segments margin serrate, flower distylous, corolla diameter 13–18 mm,……..….....*P. hubeiensis*

5 Flower homostylous, umbels solitary, limb ca. 6–10 mm across.....*P. cicutariifolia*

– Flower distylous, umbels usually 2, limb ca.11–19 mm across....*P. jiugongshanensis*

Acknowledgements

This work was supported by National Natural Science Foundation of China (No.32070370) and China's National Basic Science and Technology Program (2019FY101810)

References

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19(1): 11–15.

Greiner S, Lehwark P, Bock R (2019) OrganellarGenomeDRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. Nucleic Acids Research 47(1): 59–64. https://doi.org/10.1093/nar/gkz238

Hu CM (1990) *Primula*. In: Chen FH, Hu CM (Eds) Flora Reipublicae Popularis Sinicae, Chapter 59. Science Press, Beijing, 1–245.

Hu CM, Kelso S (1996) Primulaceae. In: Wu ZY, Raven PH (Eds) Flora of China. Science Press & Missouri Botanical Garden Press, Beijing & St. Louis.

He X, Cao JJ, Zhang W, Li YQ, Zhang C, Li XH, Shao JW (2021) Integrative taxonomy of herbaceous plants with narrow fragmented distributions: a case study on *Primula merrilliana* species complex. Journal of Systematics and Evolution. https://doi.org/10.1111/jse.12726

He X, Song LY, Wu YF, Liu J, Shao JW (2017) *Primula jiugongshanensis* sp. nov. (Primulaceae) from China, based on morphological and molecular evidence. Nordic Journal of Botany 35(3): 328–333. https://doi.org/10.1111/njb.01471

IUCN (2019) Guidelines for Using the IUCN Red List Categories and Criteria. Version 14. Prepared by the Standards and Petitions Committee. http://www.iucnredlist.org/documents/RedListGuidelines.pdf [accessed 4 Sep 2019]

Jin JJ, Yu WB, Yang JB, Song Y, Li DZ (2019) GetOrganelle: a fast and versatile toolkit for accurate de novo assembly of organelle genomes. Genome Biology 21(1): 241. https://doi.org/10.1186/s13059-020-02154-5

Kalyaanamoorthy S, Minh BQ, Wong T, von Haeseler A, Jermiin LS (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. Nature methods, 14(6): 587–589. https://doi.org/10.1038/nmeth.4285

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Tierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics (Oxford, England) 28(12): 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution. 33:1870–1874. https://doi.org/10.1093/molbev/msw054

Li XW, Bao DC, Huang HD, Xie JF (2018) *Primula hubeiensis* (primulaceae), a new species from central china. Novon A Journal for Botanical Nomenclature 25(2): 162–165. https://doi.org/10.3417/2016032

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE) 1–8. https://doi.org/10.1109/GCE.2010.5676129

Mast AR, Kelso S, Conti E (2006) Are any primroses (*Primula*) primitively monomorphic? New Phytologist 171(3): 605–616. https://doi.org/10.1111/j.14 69-8137.2006.01700.x

Qu XJ, Moore MJ, Li DZ, Yi TS (2019) PGA: a software package for rapid, accurate, and flexible batch annotation of plastomes. Plant Methods 15: 50. https://doi.org/10.1186/s13007-019-0435-7

Richards A (2002) Primula (2nd edn). B.T. Batsford, London.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539-542. https://doi.org/10.1093/sysbio/sys029

Shao JW, Wu YF, Kan XZ, Liang TJ, Zhang XP (2012) Reappraisal of *Primula ranunculoide*s, (Primulaceae), an endangered species endemic to China, based on morphological, molecular genetic and reproductive characters. Botanical Journal of the Linnean Society 169(2): 338–349. https://doi.org/10.1111/j.1095-8 339.2 012.01228.x

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313. https://do i.org/

10.1093/ bioinformatics/btu033

Xu W, Xia BS , Li XW (2020) The complete chloroplast genome sequences of five pinnate-leaved *Primula* species and phylogenetic analyses. Scientific Reports 10(1): 20782. https://doi.org/10.1038/s41598-020-77661-3

Zhang W, Hu YF, He X, Zhou W and Shao JW (2021) Evolution of autonomous selfing in marginal habitats: spatiotemporal variation in the floral traits of the distylous *Primula wannanensis*. Frontiers in Plant Science 12:781281. https://doi.org/10 .3389/fpls.2021.781281

Zhang C, Zhang JY, Chen FW, Chen, WH, Chen DS, Shao, JW (2020) *Primula qiupuensis*, a new species in primulaceae from china. Phytotaxa, 441(2):176–182. https://doi.org/10.11646/phytotaxa.441.2.4