

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

Wei Zhang^{1,2}, Yu Zhang¹, 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)

Academic editor: Avelinah Julius | Received 12 April 2022 | Accepted 30 May 2022 | Published 13 June 2022

Citation: Zhang W, Zhang Y, Shao JW (2022) *Primula xinningensis* (Primulaceae), a new species from karst caves in Hunan, China. *PhytoKeys* 199: 155–166. <https://doi.org/10.3897/phytokeys.199.85231>

Abstract

Primula xinningensis Wei Zhang bis & J.W.Shao, a new species from Hunan Province, China, is described. Its leaf morphology is similar to the *P. merrilliana* complex and flower morphology similar to *P. cicutariifolia*, but it can be distinguished from the former by the black pollen sac, corolla lobes apex obviously emarginate and can be differed from the latter by cotyledon triangular obovate, plants densely covered with glandular hairs and special habitat (karst caves). The whole plastid genome of this new species is 151, 601–151, 630 bp in length. Based on the whole plastid genome sequences, phylogenetic trees revealed that the new species did not genetically relate to the above two mentioned morphologically similar species, but it was 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, *P. cicutariifolia*, *P. hubeiensis*, *P. merrilliana* complex, section *Ranunculoides*

Introduction

Primula L. is the largest genus in Primulaceae and comprises about 500 species world-wide. The genus is mainly distributed in temperate and alpine regions of the Northern Hemisphere, with only a few species in the Southern Hemisphere, i.e. Africa, tropical Asia and South America (Richards 2002). There are approximately 300 native *Primula* species in China and the modern distribution centres 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*, characterised by pinnately compound leaves and calyx not inflated at the base (Hu 1990; Hu and Kelso 1996). This section now includes *P. ranunculoides* F.H.Chen, *P. cicutariifolia* Pax, *P. jiugongshanensis* J.W.Shao, *P. hubeiensis* X.W.Li and *P. merrilliana* complex (Shao et al. 2012; He et al. 2017; Li et al. 2018; He et al. 2021). They are all endemic to central or eastern China and often grow at the waterside or the edge of broadleaf deciduous forests between 50 and 1600 m (Shao et al. 2012; He et al. 2017; 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 to karst caves and are quite different from other known related species. After careful morphological observations, together with evidence from molecular phylogenetic analyses, based on the chloroplast genome, this suspicious species was confirmed as a new species. Here, the investigation results are reported, the new species is named as *Primula xinningensis* Wei Zhang bis & J.W.Shao and is described.

Materials and methods

Sampling and morphological analyses

The studied specimens 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 10 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; 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 the qualified DNA sample was sent to BGI-Shenzhen (Shenzhen, China) for library construction and next-generation sequencing. Finally, we obtained ca. 2 Gb of high-quality clean data, the complete chloroplast genome was assembled using GetOrganelle described in Jin et al. (2019) and the 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 the 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. xinningensis*), **ON208990** (*P. xinningensis*) and **ON221323** (*P. hubeiensis*), respectively.

Phylogenetic analyses

In order to determine the phylogenetic relationship of the new species, we downloaded 28 accessions cp genome sequences of *primula* from the NCBI (Fig. 2). 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 *Androsace paxiana* and *Lysimachia congestiflora* as outgroups (Xu et al. 2020). The ML analysis was conducted using RAXML-HPC BlackBox v.8.1.24 at the CIPRES Science Gateway website (Miller et al. 2010; Stamatakis 2014) with 1000 bootstrap replicates, the (GTR) + G + I model being 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 v.3.2 (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 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. xinningensis* 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 summarised in Table 1.

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

| Characteristic | <i>Primula xinningensis</i> |
|----------------------|-----------------------------|
| 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 |

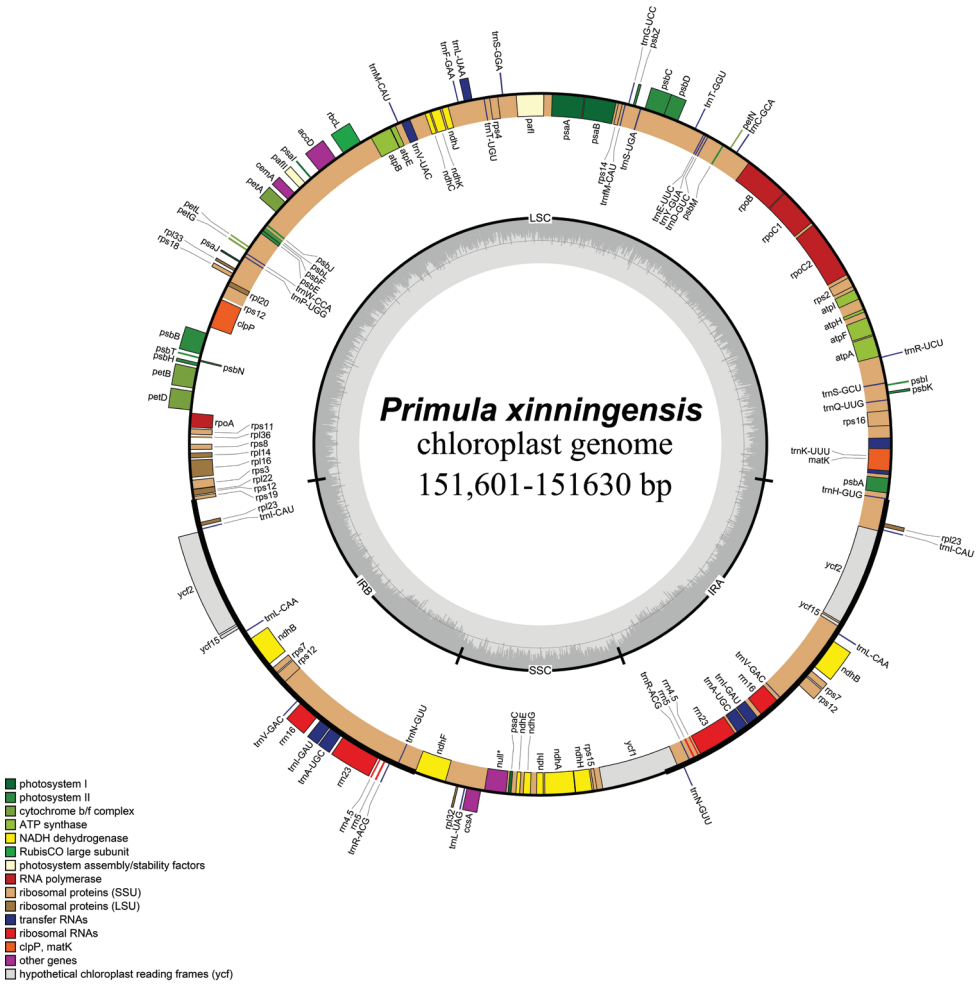


Figure 1. Plastid genome map of *P. xinningensis* sp. nov.

Molecular phylogenetic relationship

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

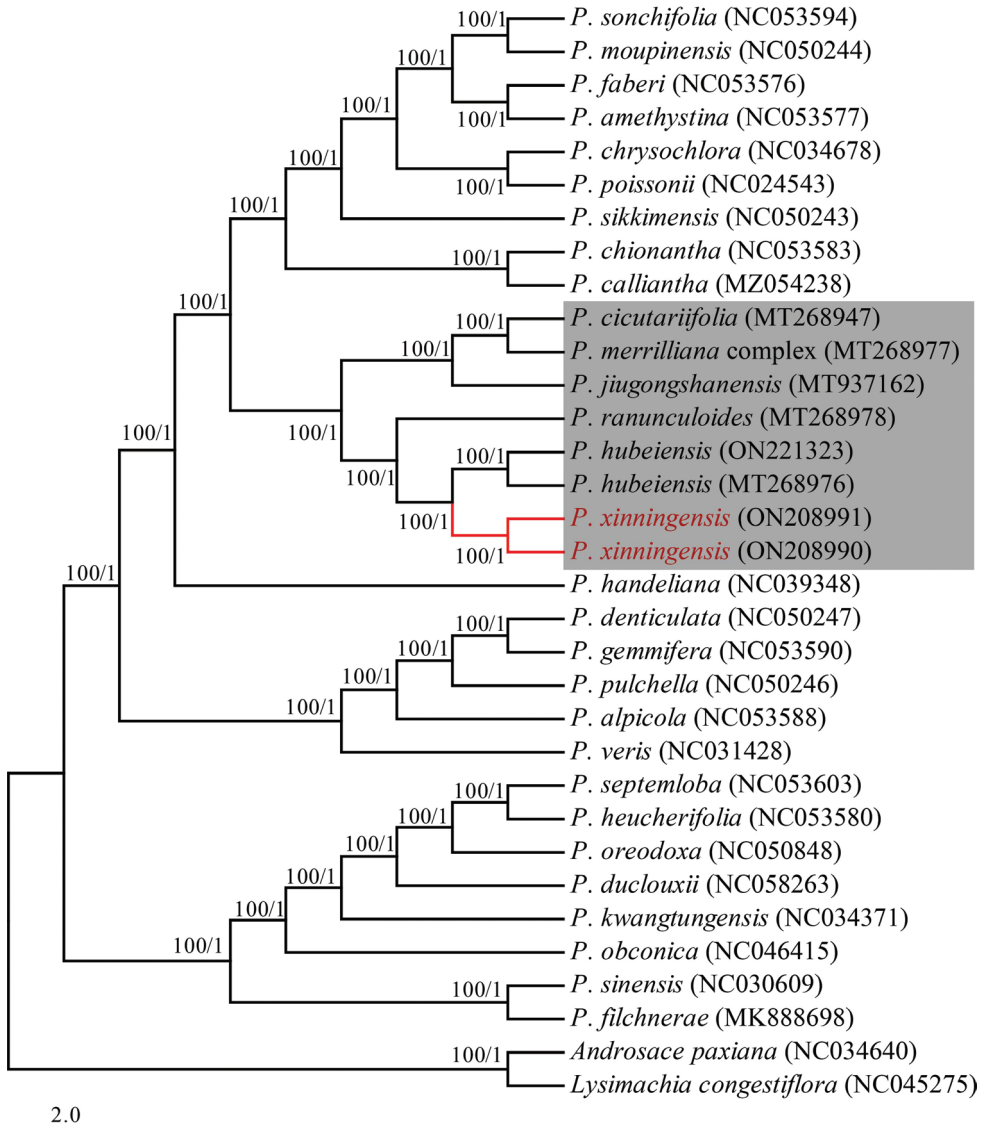


Figure 2. Phylogenetic relationships of *P. xinningensis* sp. nov. and related species inferred from ML and BI analyses, based on the whole plastid genome. Numbers on the branches indicate the bootstrap support of the ML and the posterior probability of BI analyses. NCBI accession numbers were shown in the parentheses.

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 distinguished from the former by the black pollen sac, corolla lobes apex obviously

Table 2. Morphological and ecological features comparison between *P. xinningensis* sp. nov. and its related species.

| Features | <i>P. xinningensis</i> | <i>P. hubeiensis</i> | <i>P. cicutariifolia</i> | <i>P. merrilliana</i> complex |
|------------------|---|--|--|---|
| Floral morph | Homostylous | Distylous | Homostylous | Distylous or homostylous |
| Umbel layers | 1 | 1–2 | 1 | 1–3 |
| Corolla diameter | 8–12 mm | 13–18 mm | 6–10 mm | 9–19 mm |
| Corolla lobes | Apex conspicuously emarginate | Apex conspicuously emarginate | Apex conspicuously emarginate | Apex rounded |
| Scape length | 0.8–2 cm | 3.5–9.6 cm | 1–3 cm | 1.3–9 cm |
| Pollens | Pantoporate | Pantoporate | Pantoporate | Pantoporate or stephanocolpate |
| Pollen sac | Black | Yellow | Yellow | Yellow |
| Cotyledon | Triangular obovate | 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 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 | Leaves and scape densely covered with glandular hairs (0.07–0.42 mm) | Leaves and scape densely covered with glandular hairs (0.76–0.88 mm) | without | without |
| Distribution | Hunan | Hubei | Anhui, Zhejiang | Anhui, Zhejiang |
| Habitat | Karst caves | Shady damp rock crevices | Stream sides or under broadleaf deciduous forests of northern slopes | Stream sides or under broadleaf deciduous forests of northern slopes |

emarginate and can be differed from the latter by cotyledon triangular obovate, pinna margin usually pinnatipartite and plants densely covered with glandular hairs (Table 2, Figs 3, 4). Although, in the phylogenetic relationship, *P. xinningensis* is 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. *Ranunculooides* are summarised in the following key:

Key to the species of sect. *Ranunculooides*

- 1 Corolla lobe apices rounded..... *P. merrilliana* complex
- Corolla lobe apices obviously emarginated..... 2
- 2 Compound leaves with 3–9 pinnae; scape apices differentiating to bulblets late in flowering..... *P. ranunculooides*
- Compound leaves with 7–21 pinnae; scape apices lacking bulblets 3
- 3 Plants densely covered with glandular hairs..... 4
- Plants glabrous..... 5

- ## Taxonomic treatment

Figs 3–5

Conservation status. Vulnerable (VU D1 and D2). This species is endemic to China, Hunan Province, Xinning County, Shimen Village. We only found three populations,

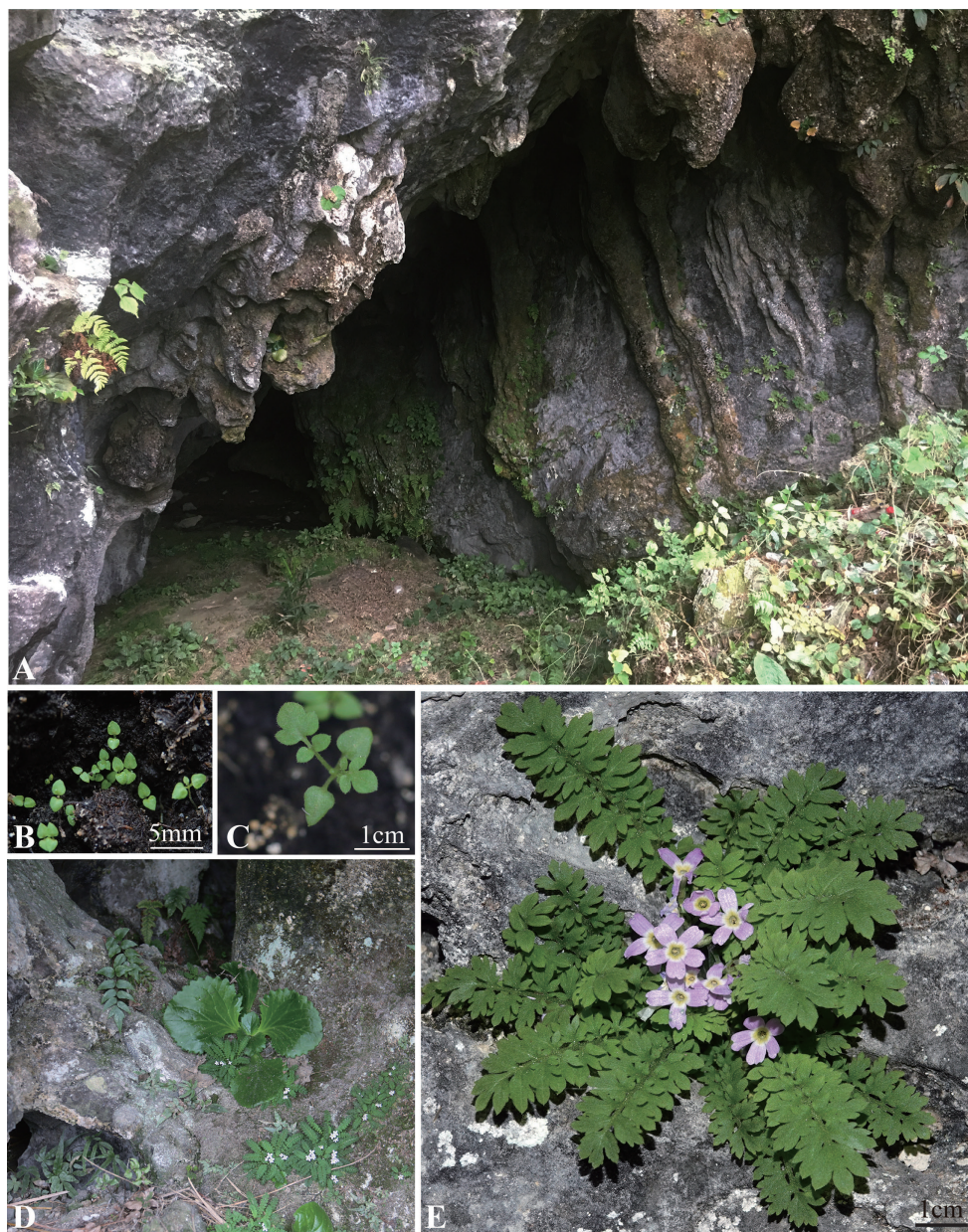


Figure 3. Living images of *P. xinningensis* sp. nov. **A, D** habitat **B, C** plant in seedling **E** plant in flowering.

all of them near the entrance to 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 km² and the known Area of Occupancy (AOO) is less than 0.5 km². Therefore, the conservation status of this new species is evaluated as ‘Vulnerable’ (VU) as it meets criterion D1 and D2, according to the IUCN Red List Categories and Criteria (IUCN 2019). In addition, *P. shimemensis* is a homostylous

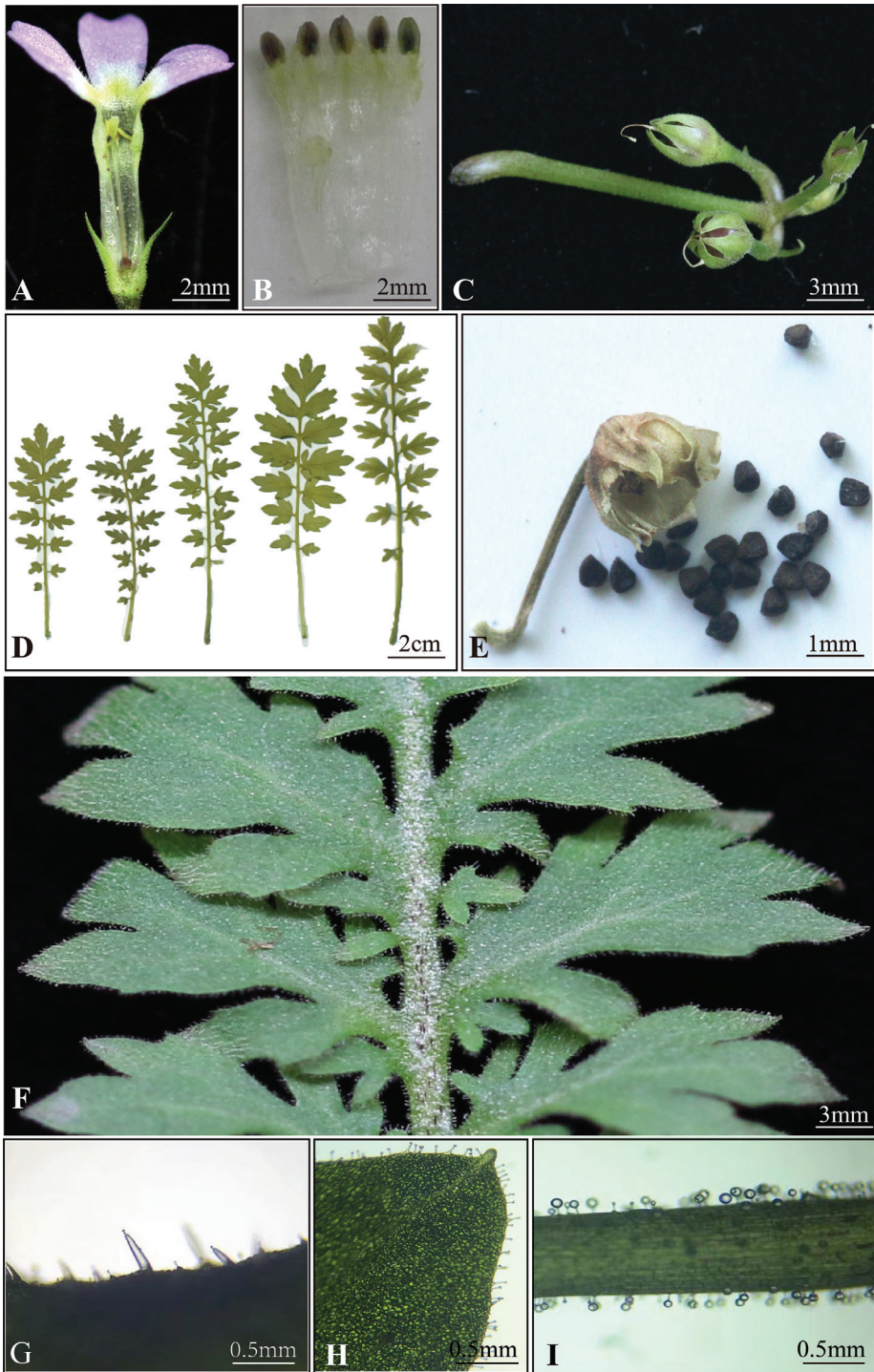


Figure 4. Morphological characters of *P. xinlingensis* sp. nov. **A, B** longitudinally dissected of floral tube **C** infructescence **D, F** leaves morphology **E** opened capsule and seeds **G** leaf surface glandular hairs **H** leaf margin glandular hairs **I** rachis glandular hairs.



Figure 5. Holotype of *P. xinningensis* Wei Zhang bis & J.W.Shao, *sp. nov.*

species. Although *Primula* containing ca. 500 species, there are only ca. 45 species having monomorphic populations (Mast et al. 2006). Therefore, the recognition 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, alt. 450 m, 22 Apr 1995, *Lin Bo Luo* 00205565 (PE); Xinning County, Wanfeng Forest Farm, alt. 450 m, 22 Apr 1995, *Lin Bo Luo* 00353811 (IBK); Xinning County, Shuimiao Town, Jiangmu Village, alt. 347 m, 19 Feb 2014, *Xun Lin Yu & Hui Zhou* 028303 (CSFI); Xinning County, Shuimiao Town, Jiangmu Village, alt. 347 m, 19 Feb 2014, *Xun Lin Yu & Hui Zhou* 028304 (CSFI).

Acknowledgements

This work was supported by National Natural Science Foundation of China (No.32070370) and China's National Basic Science and Technology Program (2019FY101810). The authors also thank the reviewers for their constructive comments to improve this manuscript.

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>
- 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>
- 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* (early view). <https://doi.org/10.1111/jse.12726>
- 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.
- 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): e241. <https://doi.org/10.1186/s13059-020-02154-5>
- Kalyaanamoorthy S, Minh BQ, Wong T, von Haeseler A, Jermini 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(7): 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>
- Mast AR, Kelso S, Conti E (2006) Are any primroses (*Primula*) primitively monomorphic? *The New Phytologist* 171(3): 605–616. <https://doi.org/10.1111/j.1469-8137.2006.01700.x>
- 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>
- 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(1): e50. <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 ranunculoides*, (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.1095-8339.2012.01228.x>
- Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* (Oxford, England) 30(9): 1312–1313. <https://doi.org/10.1093/bioinformatics/btu033>
- Xu WB, Xia BS, Li XW (2020) The complete chloroplast genome sequences of five pinnate-leaved *Primula* species and phylogenetic analyses. *Scientific Reports* 10(1): e20782. <https://doi.org/10.1038/s41598-020-77661-3>
- Zhang W, Hu YF, He X, Zhou W, 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: e781281. <https://doi.org/10.3389/fpls.2021.781281>