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Reappraisal of *Primula ranunculoides* (Primulaceae), an endangered species endemic to China, based on morphological, molecular genetic and reproductive characters

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The taxonomic status of *Primula ranunculoides* and other members of section *Ranunuculoides* is reappraised here based on data from morphological, reproductive and molecular characters. Multivariate analysis of morphological characters indicates that *P. ranunculoides* is a coherent species that can be distinguished from its sectional congeners *P. cicutariifolia* and *P. merrilliana* by the characters of simple kidney-shaped outer leaves and the unique clonal reproductive ability by which apices of the scape differentiate into bulblets at the late phases of flowering. Recognition of *P. ranunculoides* at the specific level is also supported by palynological characters, breeding system, cross pollination results and molecular phylogenetic analysis of nrDNA internal transcribed spacer sequences. A taxonomic revision of section *Ranunuculoides* is presented and a possible mechanism of speciation discussed. © 2012 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2012, **169**, 338–349.

ADDITIONAL KEYWORDS: nrDNA ITS – reproductive mode – section *Ranunculoides* – taxonomic revision – vegetative reproduction.

Primula L., the largest genus of Primulaceae, comprises > 400 species, and is mainly distributed in the temperate and high-mountain regions of the northern hemisphere, with only a few species scattered in the southern hemisphere. Its modern centre of distribution lies along both sides of the Himalayas (Yunnan Province and the west of Sichuan Province in China; Hu, 1990; Hu & Kelso, 1996; Richards, 2003). *Primula* has attracted wide attention among botanists and horticulturists because of its typically distylous (sometimes homostylous) flowers and high ornamental value (Richards, 1993, 2003). Multiple factors have contributed to speciation in the genus: these include recent adaptive radiation, hybridization, polyploidy, separation of gene pools from climatic and ecological shifts, and reproductive specialization related to the virtual obligate outcrossing associated with distyly and self-fertility associated with homostyly (Kelso, 1992; Zhang & Kadereit, 2004; Guggisberg *et al*., 2006; Mast, Kelso & Conti, 2006; Kelso, Beardsley & Weitemier, 2009). Taxonomic confusion in the genus has resulted from morphological variability with overlapping boundaries between species, narrow distributions in remote areas and insufficient or fragmentary specimens often serving as the only available material in some taxa. Thus, the original descriptions of many species are vague or inaccurate,

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and type specimens are lacking or fragmentary, with ambiguous assignment of names and incorrect usage. A recent comprehensive monograph of *Primula* (Richards, 2003) accepted 430 species names with > 600 synonyms; more than half of the species have been described repeatedly. As noted by Zhang & Kadereit (2004), integrative methods are needed to resolve some of the taxonomic issues in complex genera such as *Primula*.

The vast majority of *Primula* spp. are rosette-forming herbs with simple leaves (Richards, 2003). A few species native to China have pinnately compound leaves (Hu, 1990), the only three generally recognized species being *P. filchnerae* R.Knuth, *P. cicutariifolia* Pax and *P. merrilliana* Schltr., with leaf blades dissected to the mid-rib. *Primula cicutariifolia* and *P. merrilliana* are sister species and the only two members of section *Ranunculoides* C.M.Hu (formerly section *Pinnatae* R.Knuth), characterized by plants lacking pubescence and having a calyx not inflated at the base. *Primula filchnerae* is affiliated with section *Auganthus* (Link) Pax ex Balf.f., based on its shared characteristic pubescence and basally inflated calyx. These relationships are supported by molecular data (Hao, Hu & Lee, 2002; Yan *et al*., 2010). Apart from these three species, a fourth species with pinnately compound leaves, *P. ranunculoides* F.H.Chen, was described in 1948 and its holotype (*Yaoguo Xiong 1000*, collected from Yinglu village, Wuling county, Jiangxi province, in 1945) is kept in Lushan Botanic Garden herbarium (Chen, 1948). Chen (1951) provided supplementary information for this species. However, Hu (1990) considered that *P. ranunculoides* was similar in the shape of corollas and leaves to *P. cicutariifolia*, the holotype of which was collected from Hangzhou Lingying Temple near West Lake by Limpricht (about 550 km from where the holotype of *P. ranunculoides* was collected) in 1913. Therefore, the later published name, *P. ranunculoides*, was treated as a synonym of *P. cicutariifolia* (Hu, 1990). This treatment was followed in the vast majority of the literature (Hu & Kelso, 1996; Hao *et al*., 2002; Chen, 2009). However, Richards (2003) maintained *P. ranunculoides* as a separate species based on his perceived criteria of a perennial life-form in *P. ranunculoides* and distylous flowers > 15 mm in diameter, in contrast to *P. cicutariifolia* with homostylous flowers < 9 mm in diameter and an apparent annual growth habit. Evidence for these criteria remains lacking, we believe, as the habit type judged from herbarium materials, especially for gracile herbs, is not always convincing. For example, *P. merrilliana* was believed to be perennial (Hu, 1990; Hu & Kelso, 1996; Richards, 2003), but we found its habit to be biennial both in the wild and in the greenhouse. Furthermore, some *Primula* spp. can be both homostylous and distylous (Piper & Charlesworth, 1986). Therefore, the taxonomic status of *P. ranunculoides* remains unclear and suggests the need for a reappraisal.

In the present study, based on intensive fieldwork, studies of living collections and the relevant herbarium materials, analysis of molecular data from nrDNA internal transcribed spacer (ITS) sequences, and artificial pollination experiments under cultivation conditions, we appraise the taxonomic status of *P. ranunculoides* and delimit and classify members of *Primula* section *Ranunculoides*, a distinct section of the genus from eastern China.

MATERIAL AND METHODS

Population sampling and morphological observations

We found 15 populations from the known distribution area of section *Ranunculoides* (Table 1; Fig. 1), including populations YL and LYS, from which the holotypes of *P. ranunculoides* and *P. cicutariifolia* were originally collected, respectively. During March–April 2006, 6–15 flowering plants were randomly selected in each population for morphological observations and measurements, and one to three leaves per individual were sampled for DNA extraction (see details in Table 1). The following traits were observed or measured: longest leaf length, number of pinnae, pinnae shape, longest peduncle length, number of umbels, corolla diameter, width and shape of corolla lobes, and height of stigma and anther in the tube (i.e. distylous or homostylous). Pollen was collected from herbarium materials of the above flowering plants and photographed in the laboratory using a JSM-6300 scanning electron microscope to assess its morphology. During October–November 2008, 20–60 seedlings of each of three populations (SJ, TJQ and TLK), representing *P. ranunculoides*, *P. merrilliana* and *P. cicutariifolia*, respectively, were dug up for transplantation to the greenhouse for controlled artificial pollination experiments.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from 10–20 mg dried leaf tissue following the CTAB procedure (Doyle, 1991). Double-strand polymerase chain reaction (PCR) was used to amplify the entire ITS region, using universal primers ITS5 and ITS4 (White *et al*., 1990). The amplifications were performed in 30-μL reaction mixtures containing 25–30 ng genomic DNA, 1× PCR reaction buffer (Mg^{2+}-free), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each forward and reverse primer and 1.0 U of *Taq* polymerase. After an initial denaturation at 94 °C for 5 min, amplification

Table 1. Locations of the investigated populations

Population code	Origin	N_1	N_2	N_3
Primula ranunculoides				
YL	Yinlu village, Wuning county, Jiangxi province, 29°00′N, 114°43′E, 419 m	*c*. 200	9	3
SJ1	Huangjin village, Tongshan county, Hubei province, 29°24′N, 114°27′E, 212 m	*c*. 700	7	2
SJ2	Huangjin village, Tongshan county, Hubei province, 29°23′N, 114°27′E, 206 m	*c*. 900	11	2
SJ3	Huangjin village, Tongshan county, Hubei province, 29°24′N, 114°27′E, 232 m.	*c*. 200	7	2
Primula merrilliana				
LD	Liudu village, Shitai county, Anhui province, 30°19′N, 117°51′E, 171 m	*c*. 950	15	1
PLD	Penlai village, Shitai county, Anhui province, 30°14′N, 117°03′E, 100 m	>1500	15	1
QYS	Qiyan mountain, Xiuling county, Anhui province, 29°49′N, 118°02′E, 165 m	*c*. 400	15	1
GC	Goucun village, Taiping county, Anhui province, 30°07′N, 117°57′E, 255 m	*c*. 300	10	1
THF	Taohuafen mountain, Taiping county, Anhui province, 30°06′N, 118°09′E, 712 m	*c*. 200	8	1
TJQ	Tanjiaqao village, Taiping county, Anhui province, 30°06′N, 118°10′E, 303 m	>3000	15	1
Primula cicutariifolia				
DMD	Damao island, Zhoushang city, Zhejiang province, 29°57′N, 122°03′E, 21 m	*c*. 400	15	3
PK	Panken valley, Panan county, Zhejiang province, 28°59′N, 120°32′E, 829 m	*c*. 200	11	2
LYS	Failaifeng mountain at Linying Tample, Hangzhou city, Zhejiang province, 30°15′N, 120°06′E, 35 m	*c*. 100	6	2
TDK	Toudao valley of Longwang mountain, Anjie county, Zhejiang province, 30°24′N, 119°25′E, 708 m	*c*. 150	10	2
TLK	Tiaoling valley, Jingxian county, Anhui province, 30°31′N, 118°36′E, 407 m.	>1000	15	3

N_1, number of flowering individuals; N_2, number of samples for morphological characters measured; N_3, number of samples for ITS sequenced.

was performed for 35 cycles (30 s at 94 °C, 45 s at 57 °C and 90 s at 72 °C) followed by 8 min at 72 °C. The amplified product was purified and then sequenced using the ABI-PRISM3730 automated DNA Sequencer.

Artificial pollination experiments

To assess the compatibility of intra- and inter-species crosses, we used individuals from the sampled populations (SJ, TJQ and TLK). The flowers of *P. ranunculoides* (SJ) and *P. merrilliana* (TJQ) are distylous and those of *P. cicutariifolia* are homostylous. Twenty-one pollination treatments were conducted during March–April 2009 (details in Table 2). To ensure that pollen on recipient stigmas came only from the experimental donor flowers, all flowers of *P. ranunculoides* and *P. merrilliana* were carefully emasculated before pollination. The anther and the stigma of the homostylous *P. cicutariifolia* are the same height and flowers complete self-fertilization early in anthesis. Because the bud is so small (< 1 mm in diameter), emasculation prior to anthesis was impossible without damage to the stigmatic surface, and for this species we were unable to examine cross pollination using other pollen donors.

Data analysis

A data matrix using eight quantitative characters (length of leaf, scape, stalk and tube, the number of pinnae and umbels, corolla diameter and lobe width) was made for each population with each individual as an operational taxonomic unit (OTU). Principal coordinate analysis (PCA) was conducted using MVSP-Version 3.1 (Kovach, 1999).

All DNA sequences were aligned using CLUSTAL X software (Thompson *et al*., 1997) with default parameters and manually checked. Based on the phylogenetic tree of this genus constructed by Mast *et al*. (2001), we retrieved some ITS sequences from GenBank for some other species (detail in Fig. 5) to root the tree. With *Androsace paxiana* R.Knuth (GenBank accession number AF323705) as outgroup,

Figure 1. The distribution of *Primula* section *Ranunculoides*, showing the geographical position of the populations investigated in this study. (△) *Primula ranunculoides*; (○) *P. merrillana*; (▲) *P. cicutariifolia*. Population codes as in Table 1.

the phylogenetic trees were constructed using the neighbor joining (NJ), maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian analysis (B) methods. The neighbor joining tree with Kimura two-parameter distance (Kimura, 1980) was constructed in MEGA 4 (Tamura *et al.*, 2007) and bootstrapped (1000 iterations) to assign confidence levels to each branch. MP and ML trees were constructed by PAUP 4.0b10 (Swofford, 2002). MP analyses used branch-and-bound or heuristic searches with 100 random addition sequence replicates, a maximum of 1000 trees saved per round, tree bisection–reconnection (TBR) branch swapping and MULTREES on. All character states were treated as unordered and equally weighted, with gaps as missing data. The bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates. The evolutionary models for ML and Bayesian analyses were optimized by using Akaike's Information Criterion (AIC) using ModelTest 3.06 (Posada & Crandall, 1998), and the GTR+G model was suggested as the best. For the ML analyses, optimal gene trees were found via branch-and-bound or heuristic searches with 1000 replicates of random sequence addition, TBR branch swapping and MULTREES on, and clade robustness was estimated by bootstrap analysis of 1000 replicates. Bayesian analysis resulted in a consensus tree using the Markov Chain Monte Carlo (MCMC) method by MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Two separate analyses were conducted, each consisting of four chains, and random starting trees. The chains were run for 8000 000 generations with trees being sampled every 100 generations, the stationary tree structure was determined visually, burn-in trees were discarded and the remaining trees were used to estimate Bayesian posterior probabilities.

RESULTS

Morphological characters

PCA for the eight quantitative characters revealed that the 169 samples cluster into three groups (Fig. 2). Group A includes 57 individuals collected from the TLK, TDK, PK, LYS and DMD populations, group B contains 78 individuals sampled from the PLD, QYS, LD, GC, THF and TJQ populations, and Group C comprised 34 individuals from the YL, SJ1, SJ2 and SJ3 populations. Group A (encompassing material attributed to *P. cicutariifolia*) was clearly separated from the other two groups, although there were a few individuals (< 5%) that fell between group B and group C. Field morphological observations and herbarium study also supported these results (Figs 3, 4). Plants coming from populations TLK, TDK, PK, LYS and DMD are characterized by one to three

Table 2. Numbers of seeds produced by artificial pollination within and between populations SJ2, TJQ and TLK

		Pollen donor				
		P. ranunculoides (SJ2)		*P. merrilliana* (TJQ)		
Pollen recipient		Pin	Thrum	Pin	Thrum	*P. cicutariifolia* (TLK)
P. ranunculoides (SJ2)	Pin	0 ($N = 43$)	22.06 ± 3.30 ($N = 17$)	0 ($N = 46$)	0 ($N = 33$)	0 ($N = 28$)
	Thrum	21.20 ± 2.35 ($N = 20$)	0 ($N = 43$)	0 ($N = 33$)	0 ($N = 39$)	0 ($N = 20$)
P. merrilliana (TJQ)	Pin	0 ($N = 30$)	0 ($N = 30$)	19.44 ± 5.56 ($N = 10$)	45.44 ± 2.29 ($N = 21$)	0 ($N = 30$)
	Thrum	0 ($N = 30$)	0 ($N = 30$)	50.40 ± 1.60 ($N = 10$)	41.50 ± 4.64 ($N = 12$)	0 ($N = 30$)
P. cicutariifolia (TLK)		–	–	–	–	15.34 ± 0.75 ($N = 30$)

simple, ovate, outer leaves that are withered at anthesis, inner compound leaves with 7–17 pinnae, homostylous flowers, a corolla diameter of 6–11 (mean 8.85) mm with lobes cuneate-oblong, 2–3 mm in width, the apex slightly emarginate; pollen is pantoporate. Individuals from populations PLD, QYS, LD, GC, THF and TJQ (encompassing material attributable to *P. merrilliana*) can be identified by one to three ovate, simple outer leaves withered at anthesis, inner leaves with 13–21 pinnae, flowers distylous with corolla diameter of 11–19 (mean 15.64) mm, lobes elliptical to oval, about 5 mm in width, and a rounded apex; pollen is also pantoporate. Individuals from populations YL, SJ1, SJ2 and SJ3 (representing material attributed to *P. ranunculoides*) are represented by two to six simple kidney-shaped outer leaves, usually withered at anthesis, inner leaves with three to nine pinnae, distylous flowers, with a corolla diameter of 14–19 (mean 16.96) mm, lobes cuneate-oblong, about 5 mm in width, and apex obviously emarginate; most notably, the scape apex differentiated into bulblets during late flowering, and the pollen is stephanocolpate. Furthermore, based on the observations of plants (from populations SJ1, TLK and TJQ) transplanted in the greenhouse for the following three years, we found that these characters remain constant without variance in cultivated conditions.

nrDNA ITS sequence characters

The trees generated with the NJ (shown in Fig. 5), ML, MP and Bayesian methods all had the same major topology. The 27 samples representing 15 populations clustered into three groups (Clades A, B and C; Fig. 5) with robust support. Consistent with the clusters revealed by the morphological characters (Fig. 2), the nine individuals from populations YL, SJ1, SJ2 and SJ3 form one group (Clade C; Fig. 5), the six individuals from populations PLD, QYS, LD, GC, THF and TJQ grouped in another (Clade B; Fig. 5) and the 12 individuals from populations TLK, TDK, PK, LYS and DMD compose another independent group (Clade A; Fig. 5).

Reproductive characters and artificial pollination

The results of artificial pollination experiments (Table 2) and field observations show that the reproductive mode of plants in populations YL, SJ1, SJ2 and SJ3 (*P. ranunculoides*) is similar. Their flowers are distylous and, as expected for a distylous species, seed set occurred only from legitimate inter-morph pollination; little to no seed set occurred from intramorph and self pollination (Table 2). Strikingly, their

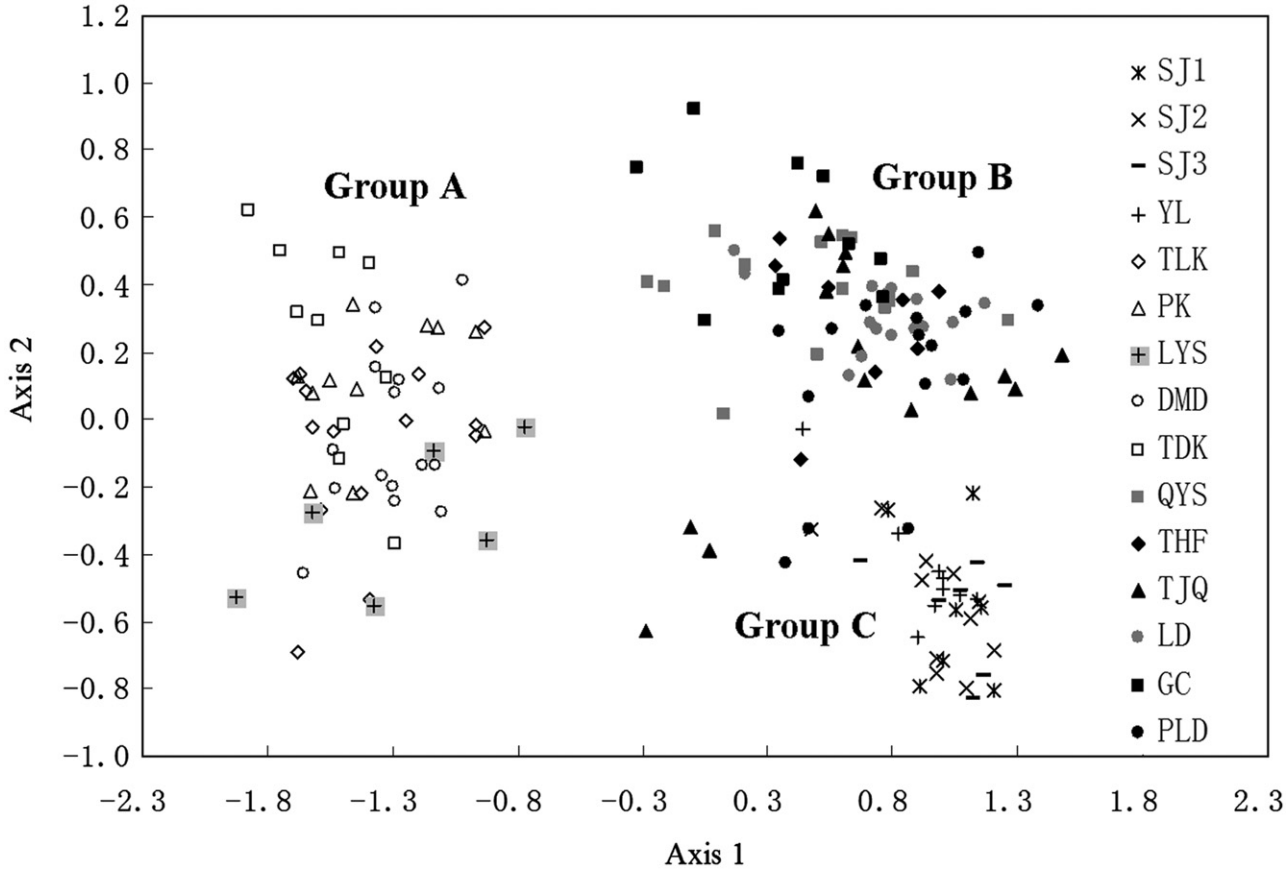

Figure 2. PCA based on eight quantitative characters. Axis 1 represents 56.15% of the total variation and axis 2 represents 15.21% of the variation. Population codes as in Table 1.

scape apices differentiate into bulblets rather than flowers during late stages of flowering. These bulblets can live through summer (June–September) by lying on the surface of wet ground or rock, or hiding in the moss or screes, then develop to new plantlets in October–November. Thus, the plants in populations YL, SJ1, SJ2 and SJ3 have both sexual and asexual reproductive modes. In contrast, plants in the other populations exhibit only a sexual mode of reproduction. Flowers of populations TLK, TDK, PK, LYS and DMD (*P. cicutariifolia*) are consistently homostylous and complete self fertilization at the beginning of blooming, whereas flowers of populations PLD, QYS, LD, GC, THF and TJQ (*P. merrilliana*) are distylous and seed set has previously been shown to require pollinator service (Shao *et al*., 2008). However, artificial pollination experiments on the TJQ population indicate that, although highest seed set occurs from inter-morph pollination, intra-morph and self pollination can also produce some seed. This species, or at least this population, appears to be primarily outcrossed, with some additional capacity for self and intra-morph fertility. The artificial pollination experiments also indicate that hybridization between populations SJ1, TLK and TJQ (representing *P. ranunculoides*, *P. cicutariifolia* and *P. merrilliana*, respectively) did not result in viable seeds, and provide support for the conclusion that there is reproductive isolation between the taxa in these populations.

DISCUSSION

Although there are many different criteria for delimiting species and they do not always coincide, more and more taxonomists advocate that the more lines of evidence that can be brought to bear on the question of species delimitation, the better (Sites & Marshall, 2004; Dayrat, 2005; De Queiroz, 2005, 2007). Taxonomists are unanimous in treating two entities as two different species when clear morphological distinction, reproductive isolation and reciprocal monophyly exist (Dayrat, 2005; De Queiroz, 2007). On these criteria, we strongly argue that *P. ranunculoides* should be treated as a separate species rather than as a synonym of *P. cicutariifolia*. According to our study, *P. ranunculoides* (from populations YL, SJ1, SJ2 and SJ3) can be distinguished from *P. cicutariifolia* (from

Figure 3. Morphology of the three studied species. A–D, *Primula ranunculoides*. A, morphology in the wild. B, specimen, showing the morphology in the early flowering stage, arrow pointing to the bulblets; b, juvenile plant, showing the outer kidney-shaped simple leaves. C, specimen, showing the morphology in the late flowering stage, arrow showing the apex of scapes differentiated into bulblets. D, leaf morphology; d, bulblet morphology under SEM. E, G, *Primula merrilliana*. E, morphology in the wild; e, juvenile plant, showing the outer oval simple leaves. G, leaf morphology. F, H, *Primula cicutariifolia*. F, morphology in the wild; f, juvenile plant, showing the outer oval simple leaves. H, leaf morphology. Scale bars = 2 cm, except in d = 2 mm.

Figure 4. Corolla and pollen morphology of the three studied species. A, B, F, G, *Primula ranunculoides*; C, D, H, I, *Primula merrilliana*; E, J, *Primula cicutariifolia*. A–E, corolla morphology; scale bars = 6 mm. F–J, pollen morphology under SEM; scale bars = 4 μm. A, C, F, I, thrum flower. B, D, G, H, pin flower. E, J, homostylous flower.

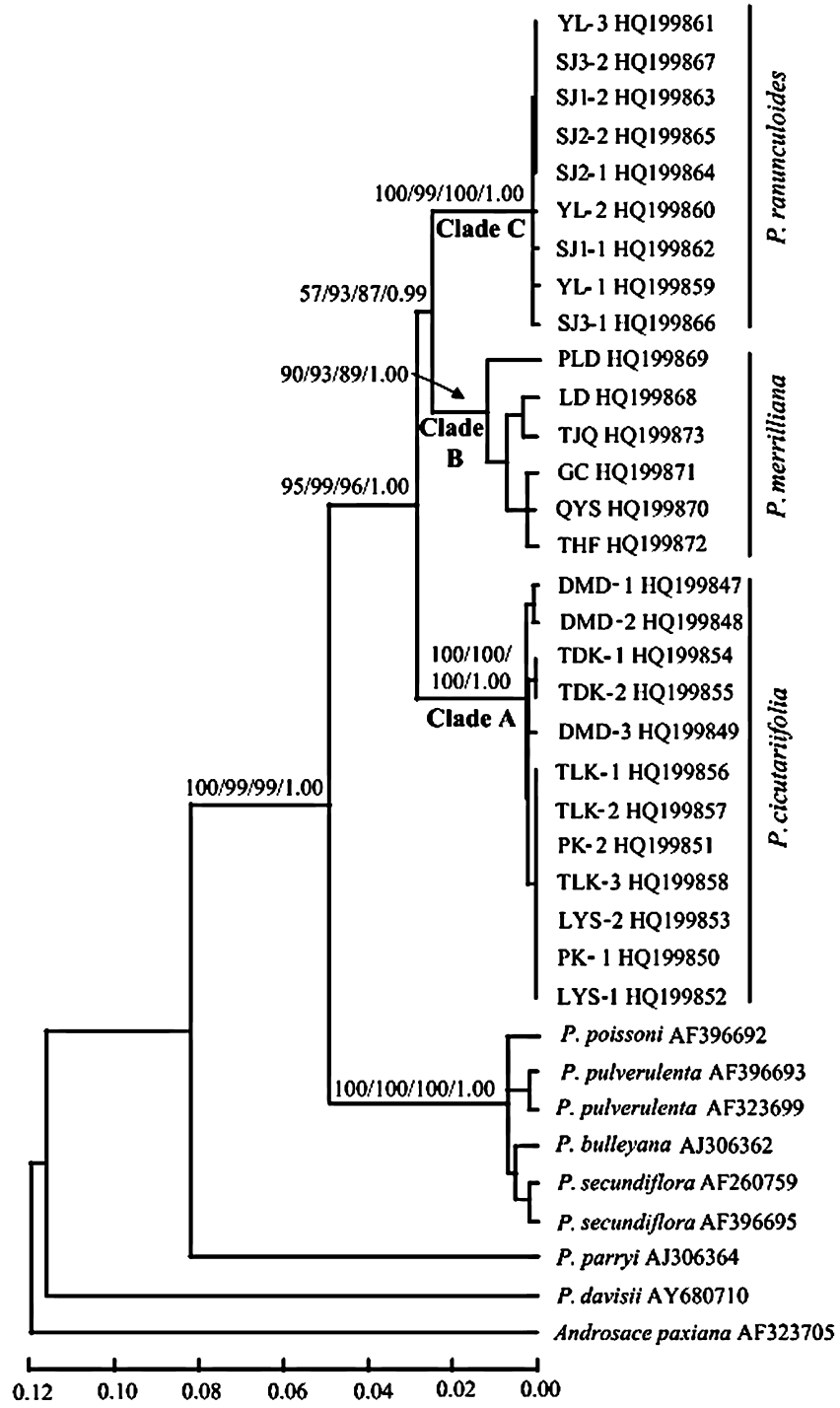

Figure 5. Neighbor joining tree based on nrDNA ITS sequences for 27 samples from 15 populations of the three studied species. Numbers above each branch indicate NJ bootstrap support, ML bootstrap support, MP bootstrap support and Bayesian posterior probabilities. The sequences with GenBank accession numbers HQ199847–HQ199873 were sequenced in this study, and the others were retrieved directly from GenBank. Population codes as in Table 1.

populations TLK, TDK, PK, LYS and DMD) by having simple kidney-shaped outer leaves, compound inner leaves with two to four pairs of lobes, larger flowers, stephanocolpate pollen and scapes that differentiate into bulblets rather than flowers. Phylogenetic trees based on nrDNA ITS sequences using multiple analyses show that *P. ranunculoides* and *P. cicutariifolia* are reciprocally monophyletic, and artificial pollination between them failed to produce progeny. Both *P. ranunculoides* and *P. cicutariifolia* are also distinguished from *P. merrilliana* by morphological, reproductive and genetic characters. These factors led us to conclude that *P. ranunculoides*, originally described by Chen in 1948, is a distinct species, and that *Primula* section *Ranunculoides* should include three species: *P. ranunculoides*, *P. merrilliana* and *P. cicutariifolia*. The differences between them are shown by the following key:

1. Outer simple leaves kidney-shaped, inner compound leaves with three to nine pinnae, scape apices differentiated to bulblets late in flowering. .....*P. ranunculoides*
1. Outer simple leaves ovate, inner compound leaves with seven to 21 pinnae, scape apices lacking bulblets.....2
2. Odd-pinnate compound leaf with 13–21 pinnae, umbels usually two or three, flower corolla lobes elliptical to oval and apex rounded..... .....*P. merrilliana*
2. Odd-pinnate compound leaf with 3–17 pinnae, umbels solitary, corolla lobes cuneate-oblong and apex obviously emarginate .....*P. cicutariifolia*

Primula ranunculoides F.H.Chen, Notes Roy. Bot. Gard. Edinburgh. 20: 120, pl. 259. 1948. TYPE: China Jiangxi, Wuning, *Y.G. Xiong 1000* (holotype, LBG!).

Primula merrilliana Schltr., Repert. Spec. Nov. Regni Veg. 19: 384. 1924. TYPE: China Anhui, *K.K. Tsoong 3290* (holotype, B; isotype, PE!).

Primula cicutariifolia Pax, Jahresber. Schles. Gen. Vaterl. Cult. xciii 1. Abt. 2, Zool.-Bot. 1. 1916. TYPE: China Zhejiang, Hangzhou Lingying Temple, *H.W. Limpricht 822* (holotype, B).

= *Primula erodioides* Schltr., Repert. Spec. Nov. Regni Veg. 19: 384. 1924. TYPE: China Anhui, *K.K. Tsoong 3285* (holotype, B; isotype, PE!).

= *Primula ranunculoides* F.H.Chen. var. *minor* F.H.Chen, Acta Phytotax. Sin. 1: 178. 1951. TYPE: China Zhejiang, Hangzhou, *C.X. Zhong s.n.* (lectotype, here designated, LBG!)

These three species are all endemic to eastern China and their distribution boundaries do not overlap. *Primula ranunculoides* is now restricted to the common boundary area of Hubei, Jiangxi and Hunan provinces, *P. merrilliana* to southern Anhui province and *P. cicutariifolia* to eastern Anhui and Zhejiang provinces (Fig. 1). Their habitats are similar and they often grow at the waterside or at the edge of broadleaf deciduous forests between 50 and 900 m above sea level. *Primula ranunculoides* is of great conservation concern, with the size of its distribution areas now restricted to < 500 km^2 and its habitats has been severely damaged due to road reconstruction; about 40% of individuals have disappeared in recent years. According to the IUCN Red List Categories and Criteria (IUCN, 2001), *P. ranunculoides* should be an endangered species as it meets criterion B2b(iii)c(ii), deserving much more consideration for protection. Its endangered status has been obscured by the prior incorrect inclusion under the name *P. cicutariifolia*.

Our results indicate that the members of section *Ranunculoides*, with stephanocolpate or pantoporate pollen type and pinnately compound leaves, are remarkably distinct from other members of the genus (Richards, 2003), suggesting that it is an isolated group in *Primula*. Analyses using plastid DNA sequences previously revealed some alignment of section *Ranunculoides* with subgenus *Aleuritia* Spach (Mast *et al*., 2001, 2006; Yan *et al*., 2010), whereas the inflorescences resemble those of section *Auganthus* (Richards, 2003). Richards (2003) suggested it may appropriate to erect a new subgenus *Pinnatae* for this section.

Reproductive specialization is one of the strongest factors accounting for the diversity of *Primula* spp. (Kelso, 1992; Wedderburn & Richards, 1992; Guggisberg *et al*., 2006; Mast & Conti, 2006; Mast *et al*., 2006). Our results also confirm this assessment. Although section *Ranunculoides* only comprises three species, their reproductive characters are quite different. *Primula ranunculoides* has heterostylous flowers with an apparently strictly self-incompatible breeding system (effective pollinators unknown), although they can utilize asexual modes via bulblets. Species with some ability for clonal reproduction via sympodial rhizomes are common in *Primula*, but the capacity to produce clonal bulblets is described here for the first time in Primulaceae, although this capacity has occasionally occurred in Liliaceae and Polygonaceae (Price & Marshall, 1999). The other two members of section *Ranuculoides* are strictly sexual: *P. merrilliana* has heterostylous flowers with partial self-compatibility and pollination in the wild depends on pollinators, *Bombylius major* and/or *Anastoechus chinensis* (Shao *et al*., 2008). In *P. cicutariifolia* the flowers are homostylous, and proximity of the sexual organs results in high levels of self fertilization early in anthesis.

The question has arisen of why the reproductive system of these three closely related species changed so tremendously. It is well known that south-western China is the modern centre of *Primula* (Richards, 2003; Huang, 2011). Eastern China, where only

section *Ranunculoides* and rarely known other *Primula* spp. occur, is the Asian edge of the distribution of the genus. The 'abundant center model' (Hengeveld & Haeck, 1982; Jacquemyn, Brys & Hermy, 2002) suggests that fewer mates and pollinators are available at the edge than at the centre of the range of a species, so in this context, these peripheral species may have adapted to pollinator scarcity through the evolution of selfing modes. It is generally assumed that the ancestral state in *Primula* is heterostylous, as all cases of homostyly in *Primula* appear to be derived from distyly (Mast *et al*., 2006). Under such ecological conditions, selection for reproductive assurance may have favoured the establishment of clonal reproduction, partly self-compatible or homostylous mutants that were (or partly) independent of both mate density and pollinator activity (Stebbins, 1957; Fausto, Eckhart & Geber, 2001; Kalisz, Vogler & Hanley, 2004). Furthermore, the extreme physiographic heterogeneity of temperate eastern Asia, in conjunction with climate and sea-level change, has shaped species barriers and enhanced vicariance in isolated populations and provided abundant opportunities for evolutionary radiation through allopatric speciation (Qian & Ricklefs, 2000). Therefore, those populations with compensatory survival factors, such as clonal reproduction ability, self-compatibility and homostylous flowers, may have survived and expanded in succession, to evolve into species with the different reproductive modes seen today.

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