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MOLECULAR AND MORPHOLOGICAL EVIDENCE FOR NATURAL HYBRIDIZATION BETWEEN *PRIMULA SECUNDIFLORA* FRANCHET AND *P. POISSONII* FRANCHET (PRIMULACEAE)

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Natural hybridization occurs commonly in plants and blurs their interspecific delimitation. It is unclear whether spontaneous hybridization occurs in natural populations of *Primula* in China. In this study we examined natural hybridization between *P. secundiflora* and *P. poissonii* based on morphological and molecular analyses of individuals with intermediate morphology. Most morphological characters of these individuals were found to be intermediate between the putative parental species, but plant height and corolla tube length were significantly transgressive. Molecular analyses based on nuclear internal transcribed spacer (ITS) additivity and two types of cloned sequences (each corresponding respectively to one of two parental species) clearly suggested that these individuals result from hybridization between *P. secundiflora* and *P. poissonii*. We further used a maternally inherited chloroplast DNA fragment (*rpl*16 intron) to trace the maternal composition of the hybrids. Among 26 hybrids, ten (two thrums, eight pins) had the *P. secundiflora* haplotype, while 16 (ten thrums, six pins) possessed the *P. poissonii* haplotype. These results suggest that both parents served as the mother donors of the hybrids. Reciprocal hybridization between the two species seems to be symmetrical rather than unidirectional.

Key words: Hybridization, *Primula secundiflora*, *P. poissonii*, ITS, *rpl*16 intron.

INTRODUCTION

Natural hybridization, the production of viable offspring from interspecific mating, is a relatively common feature of vascular plant species and has been demonstrated to play an important role in plant evolution and diversification (Anderson, 1949; Stebbins, 1959; Arnold, 1992; Rieseberg and Wendel, 1993). Clear documentation and illustration of hybridization patterns can provide very useful insights for delimitation of interspecific boundaries, detection of reproductive barriers, and determining how a taxon has diversified (Ellstrand et al., 1996; Rieseberg and Carney, 1998). The frequencies and directions of hybridization remain unclear for most genera, although a few authors have asserted that occasional hybridization between recognizable species is the rule in flowering plants (Stebbins, 1950).

Primula L. is one of the species-rich genera of west China and adjacent regions, with more than 75% of the total number of species (~425) distributed in this region (Hu, 1994; Richards, 2002). Interspecific hybridization has never been recorded in this distribution center of the genus (Hu, 1994; Richards, 2002), but hybridization events are assumed to be common in this genus in other regions (Richards, 2002). In sect. *Primula* three species (*P. elatior*, *P. veris*, and *P. vulgaris*) are isolated by ecological differentiation but interspecific hybridization occurred frequently in some mixed natural populations in England (Clifford 1958; Woodell, 1960; Valentine, 1961; Woodell, 1965; Woodell, 1969; Stace, 1975). In sect. *Aleuritia* it was confirmed that tetraploid *P. egaliksensis* (2n = 40) originated from interspecific hybridization between the diploids *P. mistassinica* (2n = 18) and

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Fig. 1. Specimens of *Primula secundiflora*, putative hybrid, and *P. poissonii*. (**a**) Inflorescences of *P. secundiflora* (1–2), putative hybrid (3–4), and *P. poissonii* (5–6), (**b**) Pin and (**c**) thrum flowers of *P. secundiflora*, putative hybrid, and *P. poissonii* (from left to right).

P. nutans (2n = 22) and occupied the circum-arctic gap between the parental species (Kelso, 1991; Kelso, 1992; Guggisberg et al., 2008). Although no hybrids have been documented in western China, a few individuals with intermediate morphology were difficult to assign to any recognized species during examination of Chinese specimens. For example, specimens we collected from the Zhongdian Plateau (Shangri-La, Yunnan) have intermediate morphology between *P. secundiflora* and *P. poissonii* (Fig. 1). These two species belong to sect. *Prolifera*, and are perennial herbs with little vegetative reproduction. They are widely distributed in the eastern Qinghai-Tibetan Plateau, and their distributions overlap in Shangri-La, MuLi and KangDing, with *P. secundiflora* northward to Qinghai and westward to Tibet, and *P. poissonii* southward to Kunming (Hu, 1990; Richards, 2002). *P. secundiflora* is distributed at 3000–4800 m a.s.l. and *P. poissonii* is distributed at 2500–3400 m. They have the same number of chromosomes (2n = 22) (Zhu et. al., 2001) and similar vegetative organs morphology, with evergreen rosettes of smooth ascending oblanceolate leaves, but their reproductive parts morphologies differ distinctly. The corolla of *P. secundiflora* is bell-shaped and exannulate, but that of *P. poissonii* is flat-faced with a conspicuous golden eye (Fig. 1). Specimens collected at a few sites on the Zhongdian Plateau where the two species co-occur show intermediate morphology. It has eluded us whether these peculiar individuals represent the offspring of natural hybridizations between those species.

ITS and chloroplast sequences have been widely used to study intraspecific variation, interspecific divergence and systematics in the genus *Primula* (Mast et al., 2001; Zhu et al., 2002; Mast et al., 2006; Guggisberg et al., 2006; Wang et al., 2008). Here we used these two types of sequences to verify the hybrid origin of these intermediate individuals. First we used the entire nrDNA ITS region (ITS1-5.8S-ITS2) to examine interspecific divergence between the putative parental species and to assess the hybrid origin of the intermediate individuals. This nuclear fragment is inherited from both parents and the hybrids usually show nucleotide additivity at sites diverging between the parents (Sang et al., 1995; Noyes, 2006; Li et al., 2007; Qiu et al., 2008; Zhang et al., 2008). Second, we used the chloroplast DNA fragment of the *rpl*16 intron to identify the maternal origin of each hybrid and estimate the direction of hybridization, because the chloroplast genome is maternally inherited in most angiosperms (Harris and Ingram, 1991; Olmstead and Palmer, 1994; Mogensen, 1996).

The two studied species are typical distylous plants (Hu, 1990; Richard, 2002). In a population there are two floral morphs (anthers and stigmas positioned at reciprocal heights; Webb and Lloyd, 1986). This reciprocal herkogamy is often accompanied by a self-incompatibility system that prevents self and intramorph fertilization (for review: Barrett and Cruzan, 1994), and such a breeding system has been confirmed in both *P. secundiflora* and *P. poissonii* (Wedderburn and Richards, 1990), so the putative interspecific hybridization should occur between reciprocal morphs of the two species. Overall, our objectives were (1) to verify natural hybridization between *P. secundiflora* and *P. poissonii* through morphological and molecular analyses; and (2) to trace the direction of hybridization between the two species, if natural hybridization did occur.

MATERIALS AND METHODS

SAMPLING STRATEGY

The study site is located in the Napahai Nature Reserve (Shangri-La County, Yunnan Province, China; 27°54'06"N, 99°37'11"E; altitude 3300 m), where *P. secundiflora* and *P. poissonii* occur together. We counted the individuals in 15 small random plots (2×2 m): 365 for *P. secundiflora* and 222 for *P. poissonii*. A total of 26 putative hybrids (14 pins, 12 thrums) with intermediate morphology were found within this area (50×300 m). We further selected 30 typical individuals of each morph for *P. secundiflora* and *P. poissonii* for morphological analyses. After making morphological measurements, the leaves of each individual (~1 g fresh weight) were dried for DNA analyses. All 26 putative hybrids and 10 individuals (5 pins, 5 thrums) of each parental species were used for molecular analyses. All voucher specimens are deposited at the Xishuangbanna Tropical Botanical Garden.

MORPHOLOGICAL ANALYSIS

Eight morphological traits were examined and/or measured: plant height (maximum height from base of stem), flower color, inflorescence architecture (number of whorls), the absence or presence of farina on the stem, eye of flower, corolla tube length, stigma height, and anther height. For measurement of flowers we usually chose the largest flower of each selected individual. The number of putative hybrids was less than 30 for both morphs, so we harvested 2–4 flowers from each putative hybrid. Finally, 30 flowers of each morph in each of the three groups were recorded following the standard of Kálmán et al. (2007) using digital-slide calipers. SPSS for Windows ver. 11 (SPSS, 2001) was used for all statistical analysis. We ran one-way ANOVA tests to compare morphological differences between groups. To compare reciprocity within and between species, relative reciprocity ratios (RRR) were calculated as follows (Richards and Koptur 1993): RRR = (anther height – reciprocal stigma height) / (anther height + reciprocal stigma height). In species that are perfectly reciprocal, the index would approach zero for both organ levels.

MOLECULAR EXAMINATION

Total genomic DNA was extracted by the CTAB method (Doyle, 1991). ITS and *rpl*16 intron were amplified by the polymerase chain reaction (PCR; Mullis and Faloona, 1987) using universal primers ITS1 and ITS4 (White et al., 1990), F71 (Jordan et al., 1996) and R1516 (Baum et al., 1998). PCR reactions were run in a total volume of 25 μL containing 10–40 ng template DNA, 2.5 μL 10 reaction buffers, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP mix, 10 μmol/L of each primer, and 1.25 U Taq polymerase (Takara). PCR reactions were run in a DNA Programmable Thermal Cycler (Biometra, Gottingen, Germany) with initial denaturation at 94°C for 5 min, followed by 34 cycles of 1 min at 94°C, 1 min of annealing at 46°C and 54°C for ITS and *rpl*16, 1 min at 72°C, and a subsequent 10 min final extension at 72°C. PCR products were purified with an agarose gel DNA purification kit (Takara) following the manufacturer's instructions. Sequencing reactions were performed with the dye-terminator cycle-sequencing ready reaction kit and analyzed on an automated sequencer (ABI 3130XL, Applied Biosystems). In addition, ITS of one putative hybrid accession were cloned using vector pGEM-T (Promega, Madison, Wisconsin). Ten positive clones were chosen for sequencing with primers "sp6" and "t7". The resulting sequence trace files were analyzed using Chromas 2.31 (Technelysium Pty Ltd., Tewantin, Queensland, Australia), and the high-quality DNA sequences were aligned using Clustal X 1.81 (Thompson et al., 1997).

RESULTS

MORPHOLOGICAL ANALYSIS

All qualitative morphological features of the putative hybrids appeared to be intermediate between the two parental species (Tab. 1). However, not all quantitative characters measured for the hybrids were shown to be intermediate (Tab. 1; Fig. 2). Heights of stigma and anther in hybrids were intermediate between the two parents or close to one parent, but plant height and corolla tube length were transgressive (higher or longer than both parents). Not only within species, but also between species, the relative reciprocal ratios were tiny (-RRR-<0.2) for both high and low levels (Fig. 3). These ratios fell well within the range observed in other distylous species (Richards and Koptur, 1993), suggesting that the

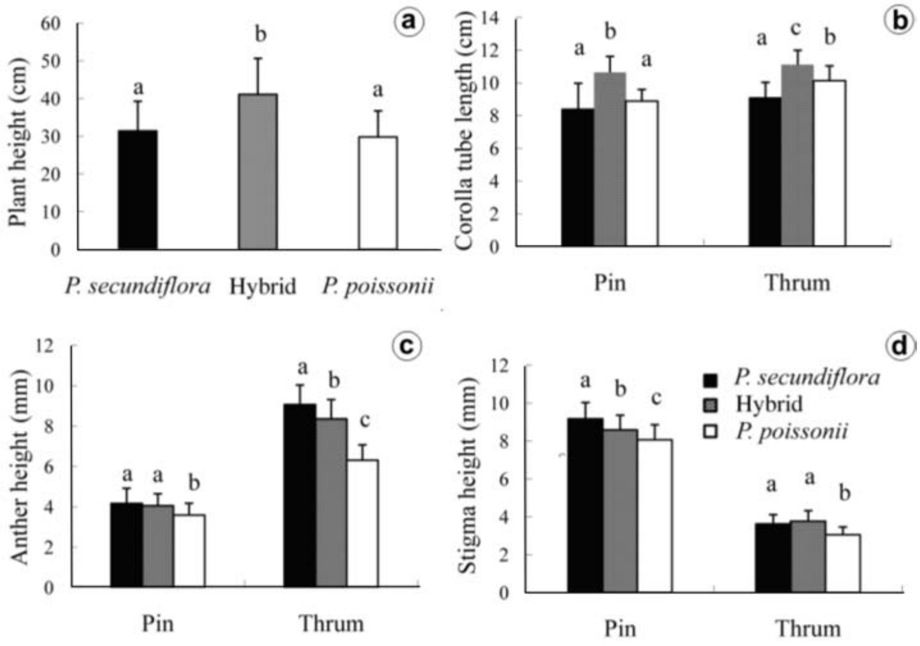

Fig. 2. Statistical comparison of *Primula secundiflora*, *P. poissonii* and their hybrids for plant height (**a**), corolla tube length (**b**), anther height (**c**) and stigma height (**d**). Bars denote means ± SD, and bars with different letters differ significantly ($p < 0.05$).

TABLE 1. Morphological traits of *Primula secundiflora*, *P. poissonii* and their putative hybrids; mean ± SD

Trait	*P. secundiflora*	Putative hybrids	*P .poissonii*
Corolla color	Dark crimson	Brilliant dark crimson	Velvety plum-purple
Inflorescence architecture	One or rarely two whorls	Two to five whorls	Two to eight whorls
Farina on stem	Abundant	Absent or a little	Absent
Flower eye	Absent	Inconspicuous dark red eye	Conspicuous golden eye
Plant height	31.43 ± 7.86 cm	41.09 ± 9.56 cm	29.81 ± 6.94 cm
Corolla tube length	8.38 ± 1.59 mm (pin)	10.64 ± 0.97 mm (pin)	8.89 ± 0.70 mm (pin)
	9.07 ± 0.96 mm (thrum)	11.11 ± 0.87 mm (thrum)	10.13 ± 0.90 mm (thrum)
Stigma height	9.17 ± 0.86 mm (pin)	8.59 ± 0.77 mm (pin)	8.06 ± 0.80 mm (pin)
	3.63 ± 0.48 mm (thrum)	3.77 ± 0.55mm (thrum)	3.05 ± 0.41mm (thrum)
Anther height	4.17 ± 0.75 mm (pin)	4.06 ± 0.59 mm (pin)	3.59 ± 0.58 mm (pin)
	9.07 ± 0.96 mm (thrum)	8.35 ± 0.96 mm (thrum)	6.31 ± 0.93 mm (thrum)

reciprocal organs matched well within and between species.

MOLECULAR EXAMINATION

The aligned ITS (including 5.8S) sequence matrix contained 639 characters in total with no indels. We failed to detect any mutation or ambiguity (double peaks) within the two putative parental species, and they were consistent with previous reports, that is, AF396695 (*P. secundiflora*) and AF396692 (*P. poissonii*) (Zhu et al., 2002). There were a total of seven nucleotide mutations (divergence) between the two species (Tab. 2). However, All 26 putative hybrids had double peaks (showing additivity) at the seven nucleotide positions. Two types of ITS sequences were clearly identified by cloning of the hybrid ITS PCR products. The first type is identical to that of *P. secundiflora* and the other one is the same as that of *P. poissonii* (Tab. 2).

The chloroplast sequences obtained were also consistent with previous reports for *P. secundiflora* (AF402520) and *P. poissonii* (AF402517) (Mast et al., 2001), which are consistently differentiated from

TABLE 2. Nucleotide positions in the aligned internal transcribed spacer (ITS) sequences differing between *Primula secundiflora*, *P. poissonii*, and their putative hybrids. Numbers refer to nucleotide position in the complete alignment. IUPAC ambiguity symbols indicate polymorphisms (R = A +G, K = G+T, M = A+C, Y = C+T)

Nucleotide position	52	61	97	215	440	475	567
P. secundiflora	G	A	T	C	C	T	T
P. poissonii	A	G	G	A	A	C	C
All putative hybrids	R	R	K	M	M	Y	Y
Hybrid clone – Type 1	G	A	T	C	C	T	T
Hybrid clone – Type 2	A	G	G	A	A	C	C

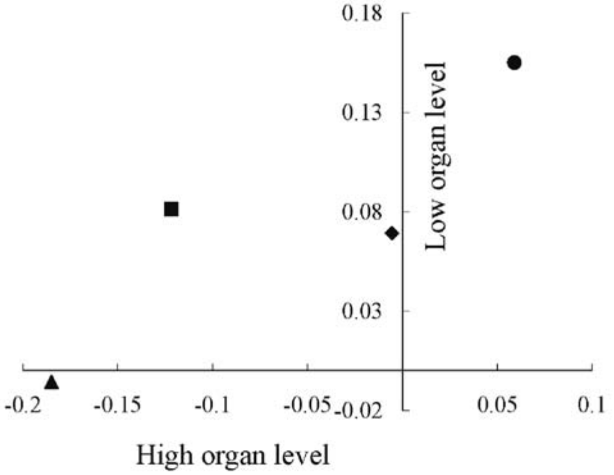

Fig. 3. Relative reciprocity ratios of high and low sex-organ levels in *Primula secundiflora* (♦), *P. poissonii* (■), and between two assumed parental cross species, *P. secundiflora* as mother (▲) or *P. poissonii* as mother (●).

each other at eleven nucleotide positions. Of the 26 hybrids identified by morphology and ITS analyses, 10 (2 thrums, 8 pins) possessed the *P. secundiflora rpl*16 intron haplotype, whereas 16 (10 thrums, 6 pins) possessed the *P. poissonii* haplotype, resulting in a 10:16 ratio, not differing significantly from the 1:1 expectation for no gender bias (null hypothesis).

DISCUSSION

CONFIRMATION OF HYBRIDS

Our field investigation suggested that *P. secundiflora* and *P. poissonii* differ from each other in morphology. Stems of *P. secundiflora* are farinose and possess one or rarely two drooping whorls of flowers. The calyxes of this species are narrowly bell-shaped, with ten stripes alternating blackish-purple and metallic silver, and the corolla is brilliant dark crimson, bell-shaped, and exannulate. By contrast, stems of *P. poissonii* are glabrous and efarinose, and possess two to eight whorls of flowers; the calyxes are cup-shaped, green often with reddish stripes, with short blunt sepal lobes and corollas velvety plum-purple with a conspicuous golden eye, flat-faced (Hu, 1990; Richards, 2002). Our statistical analyses further suggested that these two species differ in a number of quantitative traits, for example stigma and anther heights (Tab. 1; Fig. 2). However, the putative hybrids have stems with a little farina, bearing two to five whorls of flowers, and the calyxes are cup-shaped and dark red. The corollas of these individuals are blunt bell-shaped, dark crimson, with an inconspicuous dark red eye (Fig. 1, Tab. 1). Heights of stigma and anther in these individuals were intermediate between *P. secundiflora* and *P. poissonii* or close to one of them, but plant height and corolla tube length were higher or longer than both species (Fig. 2). The morphological examination suggests that these individuals are offspring of the two species because most morphological traits are intermediate between them. The presence of a few quantitative characters higher or longer than the parents may suggest transgressive expression of the hybrids (Arnold, 1997; Rieseberg and Carney, 1998; Broyles, 2002).

Because nuclear genes are biparentally inherited, hybrids should possess both divergent copies of their putative parents (Sang et al., 1995; Broyles, 2002; Noyes, 2006; Li et al., 2007; Qiu et al., 2008; Zhang et al., 2008). For both *P. secundiflora* and *P. poissonii*, the amplified ITS products could be directly sequenced and clear sequences were obtained. Neither species showed sequence variation between individuals (Tab. 2). However, our direct sequencing of the ITS products of 26 individuals with intermediate morphology suggested that all of them were additive for the seven sites where *P. secundiflora* and *P. poissonii* diverge. Further cloning suggested two types of ITS sequences corresponding to *P. secundiflora* and *P. poissonii* respectively (Tab. 2). These results provide compelling evidence that the individuals with peculiar morphology in northwest Yunnan are hybrids between *P. secundiflora* and *P. poissonii*. Although these two species generally are not found growing in the same area (generally are allopatric), they do occur together at a few sites in northwest Yunnan. During the past two years their flowering phenologies were almost synchronous (early June to early September) and their main pollinators were the same, according to our field observations. Therefore, it is highly likely that these two species could hybridize with each other to produce offspring if their premating reproductive isolation is not complete.

The hybrids between *P. secundiflora* and *P. poissonii* confirmed in this study have two important implications for further work on the genus *Primula*. First, the status of a few specimens in this genus is uncertain because they have intermediate morphology between known species. This study provides substantial evidence for interspecific hybridizations in this genus. Further interspecific delimitation and taxonomic identification of specimens in herbaria could consider the occurrence of hybrids. Second, hybrids may provide a good barrier to interspecific gene flow (i.e., reinforcement) or may promote homogeneity as hybrid bridges (Anderson, 1949; Rieseberg and Carney, 1998; Bleeker and Hurka, 2001). It should be interesting to further investigate the population genetics, ploidy level and reproductive behavior of the hybrids. Such knowledge is needed if we are to assess the process of divergence between *Primula* species and their speciation pattern (Lamont et al., 2003; Tovrsanchez and Oyama, 2004). In the present study, perfect recovery of parental sequences in hybrids was possible because (1) either the hybrids are F1s and the hybridization event occurred recently (this is not the case because both parental species had served as maternal donor) or hybridization was recurrent; or (2) the two parental ITS loci occupied distinct chromosomal positions, thus preventing loss through segregation alone and/or reducing the action of concerted evolution as discussed by Noyes (2006). More informative DNA markers need to be analyzed to diagnose their genetic structure and confirm our results (e.g., Wu and Campbell, 2005; Zhang et. al., 2008). It would be interesting to investigate the fertility of these hybrids. If F1s do not establish, they may actually be sterile. Another possibility would be a change in ploidy level, hindering any backcross with one of the parental species. Also worth evaluating is the mating system of the hybrid plants, since several hypotheses have been proposed with respect to breeding system changes following hybridization events (Stebbins, 1950, 1957; Thompson and Lumaret, 1992).

DIRECTIONS OF HYBRIDIZATION

A substantial number of case studies have suggested that interspecific hybridization is often asymmetric in plants (Xiang et al. 2000; Tiffin et al. 2001; Velde et al. 2004; Zhou et al. 2008). In these studies the maternal composition of the hybrids usually derived from a single parental species. This probably resulted from nuclear-cytoplasmic interactions, X-autosome interactions, maternal effects, asymmetric incompatibility, unidirectional postmating isolation and reinforcement of premating isolation (Turelli and Moyle 2007; Zhou et al. 2008). Here we found that both *P. secundiflora* and *P. poissonii* had served as the maternal donor and that there was no distinct bias of maternal composition for either one of them. These results together suggest that natural hybridization between the two examined species is bidirectional rather than unidirectional. In addition, because intramorphological flowers are incompatible (Barrett and Cruzan, 1994), interspecific hybridization should occur between reciprocal morphs of the two species.

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