

Research Article

Evidence for natural hybridization between *Primula beesiana* and *P. bulleyana*, two heterostylous primroses in NW Yunnan, China

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Abstract Natural hybridization was assumed to play an essential role for the diversification of *Primula*; however, only one study of hybridization in the region of the Himalayas has been undertaken. In the present study, we examined another natural hybrid zone where morphologically putative hybrids as well as *P. beesiana* Forrest, *P. bulleyana* Forrest, and *P. poissonii* Franch. co-occurred. We used molecular data to confirm the parental species of putative hybrids and the unidirectional hybridization pattern between *P. beesiana* and *P. bulleyana*. Moreover, with reference to synthetic F₁s, most hybrids examined are possibly advanced generations, although the possibility of F₁ hybrids currently examined could not be completely excluded. In addition, pollinator observations on experimental arrays of transplanted parental species showed interspecific pollen flows during visitations of shared pollinators, indicating an incomplete pre-zygotic barrier between *P. beesiana* and *P. bulleyana*. Seed productions from both flower morphs of putative hybrids were significantly lower than parental species, suggesting lower reproductive success in these hybrids. Combined with the evidence of recent habitat disturbance in the study area, we might witness the early process of hybridization between *P. beesiana* and *P. bulleyana*.

Key words natural hybridization, *P. beesiana*, *P. bulleyana*, pollinator behavior, reproductive success, synthetic F₁s.

Natural hybridization is a common phenomenon in plants and has significant roles in plant speciation through the transfer of genetic adaptations, origin of new species, and the reinforcement of reproductive barriers (Anderson, 1948; Ellstrand & Elam, 1993; Rieseberg, 1997; Milne et al., 2003; Milne & Abbott, 2008; Soltis & Soltis, 2009; Ma et al., 2010a; Abbott et al., 2013). Hybrid speciation occurs at either the homoploidy level or the polyploidy level. Compared to polyploidy hybridization, homoploid hybridization appears to be less common and occurs mainly between closely related taxa. So far, only about 20 cases of homoploid hybrid species are well documented in plants (Gross & Rieseberg, 2005). It is assumed that homoploid hybrid speciation would have greatly reduced fitness in early generation hybrids, whereas this may not be the case in early generations in polyploidy hybridization (Mallet, 2007; Rieseberg & Willis, 2007).

Heterostyly is perhaps the most distinctive feature in *Primula* L. (Hildebrand, 1863; Richards, 1993). Heterostyly is a genetic polymorphism in which plant populations comprise two (distyly) or three (tristyly)

mating types that differ in the reciprocally arranged female and male reproductive organs (Barrett, 2002). In the case of distyly, the two kinds of flowers are referred to as pins (P) and thrums (T), respectively (Darwin, 1877). In pin and thrum flowers, male and female reproductive organs are positioned reciprocally, so that anthers are placed low in the corolla of pins and high in the corolla of thrums, whereas stigmas have the opposite configuration.

Primula is a large genus comprising more than 500 species distributed throughout regions of the Northern Hemisphere (Hu & Kelso, 1996; Richards, 2002). The region of the Himalayas and western China has the greatest diversity of *Primula*. Even though hybridization was assumed to play a key role in the diversification of *Primula*, only one study focusing on hybridization in the region of the Himalayas has been carried out (Zhu et al., 2009). Therefore, more research into hybridization of the region's genera is needed.

From 2005, field investigations involving *P. beesiana* Forrest and *P. bulleyana* Forrest for potential horticultural application were carried out and a possible hybrid zone was observed in Heishui River in Jade Dragon Snow Mountain in Lijiang, NW Yunnan, China (Wu & Zhang, 2010). To decide the possible occurrence of other hybrid zones, all recorded populations of the parental species (three *P. bulleyana* and

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nine *P. beesiana*) were further checked on the basis of distribution information provided from the Chinese Virtual Herbarium (<http://www.cvh.org.cn>). However, no other location for the presence of intermediate morphologies was detected. In the single hybrid zone examined, *P. bulleyana* uniformly has yellow flowers and *P. beesiana* has purple flowers, whereas putative hybrids show intermediate flower color from orange to pink. It was noted that some flowering individuals of *P. poissonii* Franch. also existed in the hybrid zone (Fig. 1); therefore, some plants of *P. poissonii* were also sampled for this study.

The present study was carried out to address the following issues.

1. We used sequence data to test whether these intermediate morphology plants were truly hybrid derivatives and which species were involved in the hybridization.
2. After identifying parental species, F₁s were synthesized by hand cross-pollinations between identified parental species as control to detect the direction of the hybridization and comparison with hybrids from the field.
3. Experimental array was carried out on transplanted parental species to check if some of their pollinators were shared and if their foraging behaviors contribute to hybridization.
4. Seed production between hybrids and parental species were assessed from the hybrid zone.

1 Material and methods

1.1 Study species and study site

Primula beesiana, *P. bulleyana*, and *P. poissonii* are perennial herbs that usually occur in alpine habitat in

SW China. All these species and hybrids are diploid, and have the same chromosome number ($2n = 22$, Zhu et al., 2001; Ma YP et al., 2013, unpublished data). They flower from late May until mid-July and flowering times completely overlap (Wu & Zhang, 2010). Flower buds bloom from the basal umbel to the distal umbel sequentially. The three species have similar ecological conditions of growth, being found in marshy mountain meadows and on the sides of ditches and streams at 2500–3300 m a.s.l. *Primula beesiana* and *P. bulleyana* are structurally very similar, with flower color as the only distinguishing character. In *P. beesiana*, the flowers are rose-carmine and yellow-eyed with an orange tube, whereas in *P. bulleyana* the flowers are red in bud and deep orange when open. Therefore, the two plants can easily be confused without the presence of flower. However, *P. poissonii* has many flower traits (e.g., less flower numbers per inflorescence, larger flowers, efarinose in the whole plant) that can be distinguished from both *P. beesiana* and *P. bulleyana*. It should be noted that *P. beesiana* generally grows well in open habitat whereas *P. bulleyana* is well adopted in shade habitat (Ma YP et al., 2013, unpublished data).

1.2 Synthesis of F₁ hybrids

In the present study, to scientifically detect the direction of the hybridization pattern, we synthesized F₁s to test the hypothesis of maternal inheritance and further compared nuclear ribosomal DNA (nrDNA) of these synthetic F₁s to the pattern of putative hybrids. As molecular data readily excluded the possibility of *P. poissonii* as a parental species (see Results), we carried out four pollination treatments to obtain F₁ hybrids between *P. beesiana* and *P. bulleyana*, that is: (i) *P. beesiana* (P)_♀ × *P. bulleyana* (T)_♂; (ii) *P. beesiana* (T)_♀ × *P. bulleyana* (P)_♂; (iii) *P. bulleyana* (P)_♀ × *P. beesiana* (T)_♂; and (iv) *P. bulleyana* (T)_♀ × *P. beesiana* (P)_♂, in which

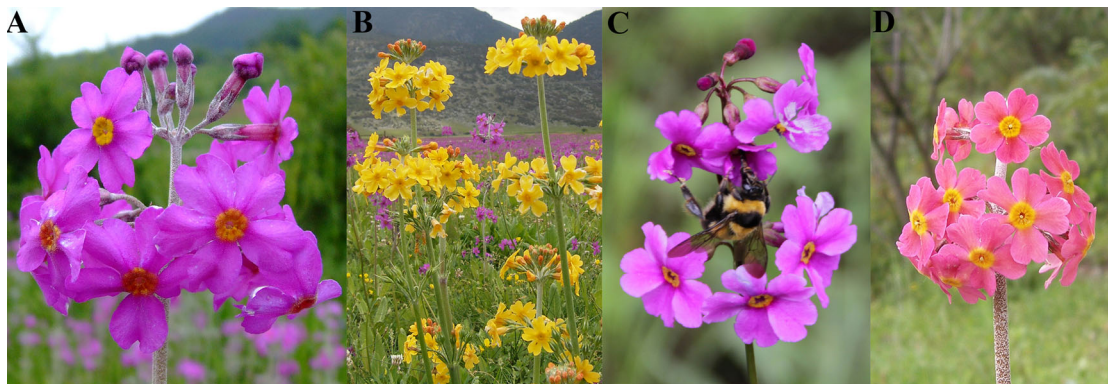


Fig. 1. Photographic images of *Primula beesiana* (A), *P. bulleyana* (B), *P. poissonii* (C), and the putative hybrids (D).

♂ and ♀ represented pollen donor and pollen receiver. As the hybrid zone seemed to be easily disturbed by grazing and tourists, we introduced the two parental species to Lijiang Alpine Botanical Garden (LABG; 27°00'15.0"N, 100°10'70.7"E; 3250 m a.s.l.) for these pollination treatments. At least 30 flowers were randomly selected from five plants and then were applied to each treatment. In September, seeds were collected, cleaned, and then sown and incubated at 20 °C with a 12:12 h light:dark cycle. Those that germinated were then transferred to a greenhouse for cultivation and subsequent sampling.

1.3 DNA sampling and sequencing

In total, 56 individuals were sampled. These comprised 23 intermediate forms in the hybrid zone, plus 10 accessions of each parent from the same site but at least 1000 m away from the hybrid zone, to provide indicators of morphology and molecular profiles of the pure parental species (Ma et al., 2010b). The remaining eight synthesized F₁s and five accessions of *P. poissonii* were also included for further molecular analysis.

Genomic DNA was extracted from all collected leaves using a modified CTAB protocol (Doyle & Doyle, 1987). The nrDNA internal transcribed spacer (ITS) region was amplified with universal primers ITS4 and ITS5 (White et al., 1990). To determine the direction of hybridization, the *trnL-rpl32* intergenic region of chloroplast DNA (cpDNA) was amplified using primers described by Shaw et al. (2007). DNA sequences were obtained using an ABI 3700 automated sequencer (Perkin Elmer, Norfolk, CT, USA). Contiguous DNA sequences were edited using SeqMan (DNASTAR package) and sequences aligned using CLUSTALX (Thompson et al., 1997).

1.4 Pollinator observation and interspecific pollen transfer

As the hybrid zone was easily disturbed, pollinator observation was carried out on transplanted parental species in LABG in 2010. It is only approximately 5 km between the hybrid zone and LABG, and both parental species were also examined close to LABG; therefore, LABG offers a good indicator of pollinators in the field. Moreover, all pollinators observed in LABG were also found in the hybrid zone (Ma YP et al., 2013, unpublished data).

To test whether insects transfer pollen between *P. beesiana* and *P. bulleyana*, a plot was set up in LABG, within which 10 *P. beesiana* and 10 *P. bulleyana* were replanted in a 2 × 5 grid structure, with plants 1 m apart and alternating between the two species. Pollinator observation was carried out from

09:00 to 17:00 on two consecutive sunny days on June 13–15, 2010. We recorded the numbers of three visitation types: (i) only visitations to *P. beesiana*; (ii) only visitations to *P. bulleyana*; and (iii) interspecific visitations. Insect visits were recorded only if they were observed to visit at least two individuals within the plot in succession.

1.5 Reproductive success

In June of 2010, two to six flowers that were randomly selected from 10 flowering plants from each morph of parental species and hybrids in the hybrid zone were marked. When mature in October, fruits were collected and seeds were counted for evaluating the reproductive success of hybrids and parental species under natural conditions.

1.6 Data analysis

All data were examined for normality and homogeneity of variance with a sample Kolmogorov–Smirnov test (Ma et al., 2012). To compare seed production between flower morphs within species, independent sample *t*-tests were used. For detecting significant differences in seed production between species, data were analyzed with linear mixed-effect models with “species” and “flower morph” as fixed factors. Tukey’s post hoc tests were then carried out to compare these treatments. All statistical analyses were carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

2 Results

2.1 Chloroplast DNA *trnL-rpl32* sequences

The variation was consistent within detected species. Among 10 accessions of *Primula bulleyana* (GenBank accession No. KF934441), 10 accessions of *P. beesiana* (GenBank accession No. KF934440) and 5 accessions of *P. poissonii* (GenBank accession No. KF934442), 24 variable sites were found in the *trnL-rpl32* sequences. These sites clearly distinguished one haplotype of each species from another (Table 1). Specifically, 14 different sites were found between *P. bulleyana* and *P. beesiana* whereas 11 sites from *P. poissonii* were found that differentiate to *P. bulleyana* and 17 to *P. beesiana* (Table 1). Among the 23 putative hybrids (GenBank accession No. KF934443) and eight synthetic F₁ hybrids (GenBank accession No. KF934444), all the sequences of these hybrids were identical with *P. bulleyana*. It was noted that all synthetic F₁ hybrids had *P. bulleyana* as the maternal species.

Table 1 Chloroplast haplotypes present in materials of *Primula bulleyana*, *P. beesiana*, synthetic F₁s, putative hybrids, and *P. poissonii*, and the codon positions at which they differ

Species	No. of accessions	Sequence region and codon position of <i>trnL-rpl32</i>									
		120	165	241	288	296–297	362	364–365	367–377	391–393	489
<i>P. bulleyana</i> Forrest	10	A	G	T	T	AA	T	--	---G---AA--	GAC	A
<i>P. beesiana</i> Forrest	10	A	G	C	A	--	T	TT	ATAGGTCAATT	GAC	A
Synthetic F ₁ s	8	A	G	T	T	AA	T	--	---G---AA--	GAC	A
Putative hybrids	23	A	G	T	T	AA	T	--	---G---AA--	GAC	A
<i>P. poissonii</i> Franch.	5	G	A	T	A	--	C	--	T-----AA--	AAA	T

-, Indel.

2.2 Nuclear ribosomal DNA ITS sequences

From sequences of nrDNA ITS regions, all accessions of *P. bulleyana* had identical sequences (GenBank accession No. KF934445; Table 2). However, two types were examined in 10 individuals of *P. beesiana*, from which four accessions had identical sequences that differed to *P. bulleyana* in four variable sites (Type b1, GenBank accession No. KF934446) and the other six accessions presented a total of eight polymorphic sites (Type b2, GenBank accession No. KF934447), in which four polymorphic sites were different to both *P. bulleyana* and Type b1 (Table 2).

For the synthetic F₁ hybrids between *P. bulleyana* and *P. beesiana*, two types of ITS sequences were found. Type 1 showed additive at all variable sites between *P. bulleyana* and Type b1 of *P. beesiana* (GenBank accession No. KF934454; Table 2). On the contrary, Type 2 hybrids (GenBank accession No. KF934455) showed high additivity between *P. bulleyana* and Type b2 of *P. beesiana*. Four types of ITS were detected in the examined 23 hybrids, among which 15 (65%) belong to Type h1 (GenBank accession No. KF934451) that has one different site (144) to Type h1 of the synthetic F₁ hybrids. Another five hybrids (Type h2) have identical sequences to

P. bulleyana that might reflect misidentified *P. bulleyana* or repeated backcrosses to *P. bulleyana*. The remaining three hybrids were divided into two types: Type h3 (GenBank accession No. KF934452) might have originated from hybridization between *P. bulleyana* and Type b2 of *P. beesiana*, or possible F₁s of Type 2 (here it was assumed to exist but was not detected in the field) backcrossed to *P. bulleyana*; and Type h4 (GenBank accession No. KF934453) which might have originated from F₁s of Type 1 backcrossed to Type 2 of *P. beesiana* (Table 2).

For the co-occurring species *P. poissonii*, although three types were examined in five samples (GenBank accession Nos. KF934448–KF934450), eight sites that clearly differentiated to *P. bulleyana*, *P. beesiana*, and hybrids therefore excluded the involvement of natural hybridization in the present study (Table 2).

2.3 Combined analysis of cpDNA and nrDNA

As we detected, the cpDNA of *P. bulleyana* and *P. beesiana* was of maternal heritage. If we combined the information with the nrDNA data, all examined putative hybrids except Type h2 should have originated from hybridization between *P. bulleyana* and *P. beesiana* because no combination involving only a

Table 2 Internal transcribed sequence (ITS) types present in material of *Primula bulleyana*, *P. beesiana*, synthetic F₁s, putative hybrids, and *P. poissonii*, and the variable site positions at which they differ

Species	Haplotype	No. of accessions	Sequence region and codon position of ITS													
			144	207	208	221	245	290	313	451	464	515	588	622	636	642
<i>P. bulleyana</i> Forrest		10	C	C	G	C	C	C	C	G	C	C	C	C	A	T
<i>P. beesiana</i> Forrest	Type b1	4	T	C	G	T	C	C	C	A	C	C	C	C	A	C
	Type b2	6	C+T	C+T	G	T+C	C	C	C	A+G	T+C	T+C	T+C	C	A	T+C
Synthetic F ₁ s	Type 1	4	C+T	C	G	C+T	C	C	C	A+G	C	C	C	C	A	C+T
	Type 2	4	C	C+T	G	C	C	C	C	G	C+T	C+T	C+T	C	A	T
Putative hybrids	Type h1	15	C	C	G	C+T	C	C	C	A+G	C	C	C	C	A	C+T
	Type h2	5	C	C	G	C	C	C	C	G	C	C	C	C	A	T
	Type h3	2	C	C+T	G	C	C	C	C	G	C+T	C+T	C+T	C	A	T
	Type h4	1	C	C+T	G	C	C	C	C	G	C	C	C+T	C	A	T
<i>P. poissonii</i> Franch.	Type p1	3	C	C	A	C	C	A	A	G	C	A	C	G+C	C	C
	Type p2	1	C	C	A	C	T	A	A	G	C	A	C	C	C	C
	Type p3	1	C	C	A	C	C+T	A	A	G	C	A	C	G+C	C	C

single species can result into the sequence pattern of these detected hybrids. For instance, all types of putative hybrids could be obtained from repeated crosses between Type b2 of *P. beesiana* in examined ITS. However, in such a case, the cpDNA must be the same as *P. beesiana*. Therefore, *P. bulleyana* must be involved in the hybridization.

2.4 Pollinator observation and interspecific pollen transfer

For the plot containing 20 plants each of *P. bulleyana* and *P. beesiana*, 86 instances of bees (*Anthophora* spp.) flying from one *Primula* plant to another were observed. It seems that bees preferred *P. bulleyana* to *P. beesiana* (58 vs. 2). Moreover, 26 instances (30%) were between *P. bulleyana* and *P. beesiana* (Fig. 2; Table 3). Hence, bee behavior was not a strong isolation barrier between parental species. Only 13 instances of other pollinator species visiting more than one flower in this plot were observed but these visitations were always observed within species (Table 3).

2.5 Reproductive success under natural conditions

On average, 96 and 95 seeds were produced from pin and thrum flowers of *P. beesiana*, respectively, and no significant difference was detected between morphs ($t = 0.159$, $P = 0.874$, Fig. 3). However, pin flowers from *P. bulleyana* produced on average 94.82 seeds whereas thrum flowers produced on average 81.84 seeds, and the difference was significant ($t = 2.615$, $P = 0.013$, Fig. 3). For these hybrids, both pin and thrum flowers produce much fewer seeds (pin morph, 53.54 ± 2.07 ; thrum morph, 56.46 ± 2.20 ; $t = 0.951$, $P = 0.344$, Fig. 3) than both morphs of *P. beesiana*

Table 3 Observations of pollinator flights from one *Primula* individual to another, in a plot containing 10 *P. bulleyana* and 10 *P. beesiana* in Lijiang Alpine Botanical Garden (LABG)

Visitation pattern	Bees	Bumblebees	Butterflies	Hawkmoths
<i>P. bulleyana</i> Forrest	58	8	0	0
<i>P. beesiana</i> Forrest	2	0	3	2
Between species	26	0	0	0

($F = 164.869$, $P < 0.001$, Fig. 3) and *P. bulleyana* ($F = 126.566$, $P < 0.001$, Fig. 3).

3 Discussion

The genus *Primula* includes over 200 species in the region of the Himalayas, which is regarded as the geographical origin and current diversity distribution center of *Primula* (Wedderburn & Richards, 1992; Arnold & Richards, 1998; Richards, 2002). Despite lacking evidence, natural hybridization was presumed to play an important role for diversification of *Primula* in this region (Hu, 1990). In addition to the only case of natural hybridization between *P. secundiflora* Franch. and *P. poissonii* that has been confirmed from the region (Zhu et al., 2009), we present the second case of natural hybridization in *Primula* in the region of the Himalayas, on the basis of comprehensive field investigations, completely overlapping flowering times, the shared pollinator and its association with interspecific pollen transfer, and molecular evidence.

3.1 Evidence for the occurrence of hybridization in sympatric *Primula*

In general, sympatric geographic distribution, overlapping phenologies, and morphologically

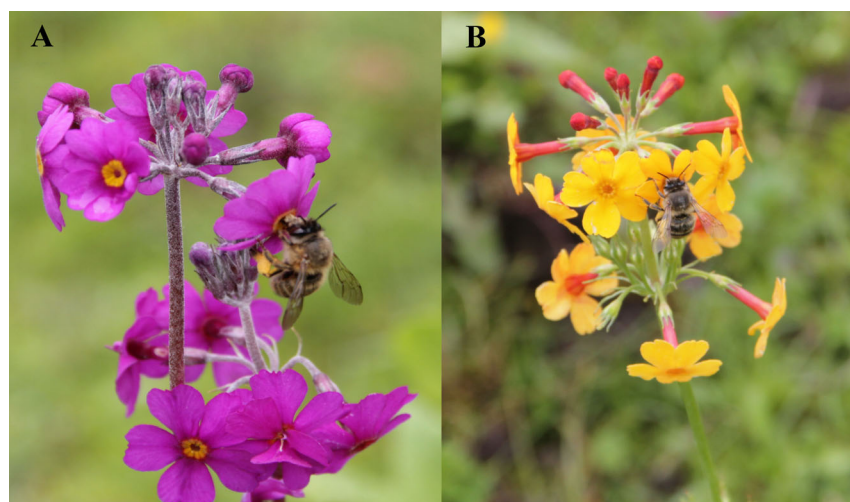


Fig. 2. Main shared pollinators (*Anthophora* spp.) between *Primula beesiana* (A) and *P. bulleyana* (B).

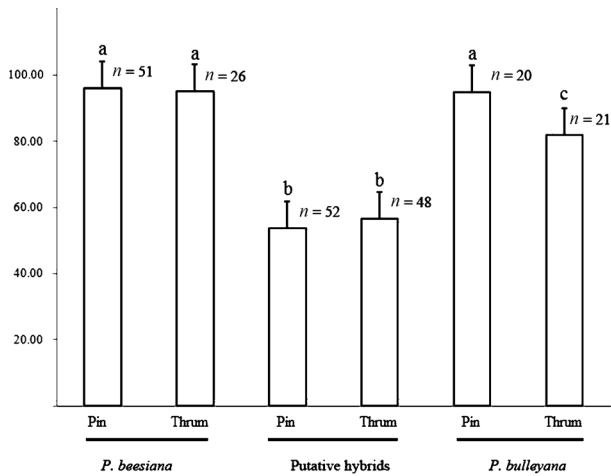


Fig. 3. Seed production of *Primula beesiana*, *P. bulleyana*, and their putative hybrids with different flower morphs in the hybrid zone. The same letter indicates no significant difference ($P = 0.05$). n , number of sample.

intermediate characters are useful indicators for preliminary testing of hybridization (Gottlieb, 1972). However, it seems complex when applying these criteria to our study. As flowering times completely overlapped and flower morphologies among these species were so similar (except flower color, Fig. 1), no strong diagnostic characters could distinguish parental species of these putative hybrids. Furthermore, if repeated backcrosses toward one parental species could occur, flower characters would be expected to be similar to the parental species involved in backcrosses (Arnold, 1997; Abbott et al., 2013).

The occurrence of hybridization between species depends on the strength of both pre- and post-zygotic reproductive barriers (Ellstrand et al., 1996). In flowering plants, the initial stages of pre-zygotic reproductive isolation are often related to the behavior of pollinators (Campbell et al., 2002). Hence, any tendency of pollinators to move within species influences hybridization. Our transplant experiment revealed that not only pollinators were shared (e.g., *Anthophora* spp., Fig. 2) but also interspecific visitation was observed during one trip (Table 3), providing strong evidence for pollen transfer between parental species.

Even if the transference of pollen grains occurs, formation of hybrids can still be prevented by post-zygotic reproductive barriers (Campbell et al., 2002). In the present study, although we did not have enough data for quantifying the reproductive success after interspecific hand pollination, the successful production of F_1 s seedlings in the greenhouse indicates that post-zygotic reproductive barriers did not completely prevent the formation of F_1 s.

The molecular evidence confirmed the occurrence of natural hybridization in 18 of 23 putative hybrids (78%) as a genetic admixture between *P. bulleyana* and *P. beesiana* was detected (Table 2). Furthermore, in the comparison with synthetic F_1 s, molecular data provided interesting findings, as none of the types from the detected putative hybrids was the same as the synthetic F_1 s in terms of ITS sequences. Therefore, two possibilities could exist: (i) most putative hybrids examined here were advanced generation hybrids in the hybrid zone; or (ii) the hypothesis of F_1 s among these examined putative hybrids cannot be rejected, as hybridization between *P. bulleyana* and Type b2 of *P. beesiana* could also produce most of the ITS types in these examined hybrids.

3.2 Importance of synthetic F_1 s for comparison

Two questions can be clarified by comparing the putative hybrids with the synthetic F_1 s. First, the evidence for maternal inheritance was further confirmed. Many studies routinely assume maternal cytoplasmic inheritance due to a lack of available information, despite evidence of bi-parental inheritance in many families (Mogensen, 1996; Zhang & Liu, 2003). Hence, in this study, we also examined eight of the synthetic F_1 s described above to test the hypothesis that cpDNA was normally maternally inherited in this cross. The synthetic F_1 s all have *P. bulleyana* as the maternal species. Second, we also proved that these putative hybrids would be advanced generations after comparison with synthetic F_1 s, although the possibility of F_1 hybrids could not be completely excluded.

3.3 Formation and structure of the hybrid zone

Anderson (1948) emphasized that hybrids were most often associated with disturbed habitats. Hence, increasing levels of habitat disturbance are likely to promote hybridization (Levin et al., 1996; Rieseberg & Carney, 1998). For *P. bulleyana* and *P. beesiana*, the single hybrid zone detected might either be a stable, long-term phenomenon or a relatively recent occurrence, initiated or at least facilitated by habitat disturbance (Ma et al., 2010a). To date, we have not observed any evidence indicating that it is a stable, long-term phenomenon. Conversely, when examined 8 years ago, only few hybrids were observed (Wu & Zhang, 2010), but have now spread to hundreds of individuals (Ma YP et al., 2013, unpublished data). Therefore, we conclude that the hybridization could be recently initiated by habitat disturbance due to road building after 1990, as the Jade Dragon area was developed for tourism.

In general, F_1 formation is a rare event that, once achieved, can permit far larger numbers of later generation hybrid derivatives to form (Arnold, 1997; Rieseberg & Carney, 1998; Ma et al., 2010b), creating the potential for gene transfer between parental species through introgression (e.g., Arnold et al., 2012). Our data support the hypothesis of hybrids that consisted of later generations, as none was the same as the synthetic F_1 s in terms of ITS data. Furthermore, it should be noted that seeds that germinate can flower only 1 and ½ years later. Therefore, combined with recent formation of the hybrids, we could be witnessing the process of hybridization in that advanced generations existed in the hybrid zone, although the hypothesis of no existing F_1 s could not be rejected.

Followed by repeated backcrosses to *P. bulleyana*, more hybrids morphologically similar to *P. bulleyana* were expected to be produced, therefore facilitating gene transfer from *P. beesiana* to *P. bulleyana*. It should be mentioned that we have already monitored some flowering plants of *P. bulleyana* (also might be repeated backcrosses to *P. bulleyana*) growing well in open habitat that is well adopted by *P. beesiana*, in contrast with that typical shade habitat that is generally occupied by *P. bulleyana* (Ma YP et al., 2013, unpublished data). Further study involving genomic analysis for such adaptation transfer by way of repeated backcrosses would provide insight for introgressive speciation.

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References

- Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, Butlin RK, Dieckmann U, Eroukhanoff F, Grill A, Cahan SH, Hermansen JS, Hewitt G, Hudson AG, Jiggins C, Jones J, Keller B, Marczewski T, Mallet J, Martinez-Rodriguez P, Möst M, Mullen S, Nichols R, Nolte AW, Parisod C, Pfennig K, Rice AM, Ritchie MG, Seifert B, Smadja CM, Stelkens R, Szymura JM, Väinölä R, Wolf JBW, Zinner D. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* 26: 229–246.
- Anderson E. 1948. Hybridization of the habitat. *Evolution* 2: 1–9.
- Arnold ES, Richards AJ. 1998. On the occurrence of unilateral incompatibility in *Primula* sect. *Aleuritia* Duby and the origin of *Primula scotica* Hook. *Botanical Journal of the Linnean Society* 128: 359–368.
- Arnold ML. 1997. *Natural hybridization and evolution*. New York: Oxford University Press.
- Arnold ML, Ballerini ES, Brothers AN. 2012. Hybrid fitness, adaptation and evolutionary diversification: Lessons learned from Louisiana Irises. *Heredity* 108: 159–166.
- Barrett SCH. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–284.
- Campbell DR, Waser NM, Pederson GT. 2002. Predicting patterns of mating and potential hybridization from pollinator behavior. *The American Naturalist* 159: 438–450.
- Darwin C. 1877. *The different forms of flowers on plants of the same species*. London: John Murray.
- Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* 19: 11–15.
- Ellstrand NC, Elam DR. 1993. Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics* 24: 217–242.
- Ellstrand NC, Whitkus R, Rieseberg LH. 1996. Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences USA* 93: 5090–5093.
- Gottlieb LD. 1972. Leaves of confidence in the analysis of hybridization in plants. *Annals of the Missouri Botanical Garden* 59: 435–446.
- Gross BL, Rieseberg LH. 2005. The ecological genetics of homoploid hybrid speciation. *Journal of Heredity* 96: 241–252.
- Hildebrand F. 1863. *De la Variation Des Animaux et Des Plantes a L'etat Domestique*. Paris: C. Reinwald.
- Hu CM. 1990. *Primula*. In: *Flora Reipublicae Popularis Sinicae*. Beijing: Science Press. 59: 1–277.
- Hu CM, Kelso S. 1996. *Primulaceae*. In: Wu ZY, Raven PH eds. *Flora of China*. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press. 15: 39–189.
- Levin DA, Francisco-Ortega J, Jansen RK. 1996. Hybridization and the extinction of rare species. *Conservation Biology* 10: 10–16.
- Ma YP, Milne RI, Zhang CQ, Yang JB. 2010a. Unusual patterns of hybridization involving a narrow endemic *Rhododendron* species in Yunnan (Ericaceae), China. *American Journal of Botany* 97: 1749–1757.
- Ma YP, Wu ZK, Tian XL, Zhang CQ, Sun WB. 2012. Growth discrepancy between filament and style facilitates autonomous self-fertilization in *Hedychium yunnanense*. *Plant Ecology and Evolution* 145: 185–189.
- Ma YP, Zhang CQ, Zhang JL, Yang JB. 2010b. Natural hybridization between *Rhododendron delavayi* and *R. cyanocarpum* (Ericaceae), from morphological, molecular and reproductive evidence. *Journal of Integrative Plant Biology* 52: 844–851.
- Mallet J. 2007. Hybrid speciation. *Nature* 446: 279–283.
- Milne RI, Abbott RJ. 2008. Reproductive isolation among two interfertile *Rhododendron* species: Low frequency of post- F_1 hybrid genotypes in alpine hybrid zones. *Molecular Ecology* 17: 1108–1121.

- Milne RI, Terzioglu S, Abbott RJ. 2003. A hybrid zone dominated by fertile F_1 s: Maintenance of species barriers in *Rhododendron*. *Molecular Ecology* 12: 2719–2729.
- Mogensen HL. 1996. The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany* 83: 383–404.
- Richards AJ. 1993. *Primula*. 1st ed. London: Batsford.
- Richards AJ. 2002. *Primula*. 2nd ed. London: Batsford.
- Rieseberg LH. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* 28: 359–389.
- Rieseberg LH, Carney SE. 1998. Plant hybridization. *New Phytologist* 140: 599–624.
- Rieseberg LH, Willis JH. 2007. Plant speciation. *Science* 317: 910–914.
- Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany* 94: 275–288.
- Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* 60: 561–588.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins GD. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882.
- Wedderburn FM, Richards AJ. 1992. Secondary homostyly in *Primula* L.; evidence for the model of the ‘S’ supergene. *New Phytologist* 121: 649–655.
- White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelf D, Sninsky J, White T eds. *PCR protocols: A guide to methods and applications*. San Diego: Academic Press. 315–322.
- Wu ZK, Zhang CQ. 2010. Comparative study of pollination biology of two closely related alpine *Primula* species, namely *Primula beesiana* and *P. bulleyana* (Primulaceae). *Journal of Systematics and Evolution* 48: 109–117.
- Zhang Q, Liu Y. 2003. Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species. *Plant Cell Physiology* 44: 941–951.
- Zhu HF, Zhang CQ, Gu ZJ, Gong X. 2001. A karyomorphological study on nine species of *Primula* (Primulaceae). *Acta Botanica Yunnanica* 23: 466–472.
- Zhu XF, Li Y, Wu GL, Fang ZD, Li QJ, Liu JQ. 2009. Molecular and morphological evidence for natural hybridization between *Primula secundiflora* Franchet and *P. poissonii* Franchet (Primulaceae). *Acta Biologica Cracoviensia Series Botanica* 51: 29–36.