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Letter to the Editor

Natural hybridization and reproductive isolation between two *Primula* species

Running Title: Hybridization between two Primula species

Yanping Xie[1, 2], Xingfu Zhu[1], Yongpeng Ma[3], Jianli Zhao[4], Li Li[1, 2] and Qingjun Li[4*]

[1] Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, China

[2] Graduate University of Chinese Academy of Sciences, Beijing 10049, China

[3] Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

[4] Laboratory of Ecology and Evolutionary Biology, State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming 650091, China

[*]Correspondence: Qingjun Li (qingjun.li@ynu.edu.cn)

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Summary

Natural hybridization frequently occurs in plants and can facilitate gene flow between species, possibly resulting in species refusion. However, various reproductive barriers block the formation of hybrids and maintain species integrity. Here, we conducted a field survey to examine natural hybridization and reproductive isolation (RI) between sympatric populations of *Primula secundiflora* and *P. poissonii* using ten nuclear simple sequence repeat (SSR) loci. Although introgressive hybridization occurred, species boundaries between *P. secundiflora* and *P. poissonii* were maintained through nearly complete reproductive isolation. These interfertile species provide an excellent model for studying the RI mechanisms and evolutionary forces that maintain species boundaries.

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Natural hybridization is common in plants, and has many evolutionary consequences. Introgressive hybridization increases species diversity and ecological adaptability (Jensen et al. 2005; Abbott et al. 2013), and excessive introgressive hybridization results in gene flow and, eventually, species refusion which blear species boundary (Rieseberg and Ellstrand 1993; Runyeon Lager and Prentice 2000). By contrast, reproductive isolation (RI) blocks the formation of hybrids and pomotes species isolation (Rogers and Bernatchez 2006; Baack et al. 2015). Most studies on plant RI have focused on only one or a few particular barriers to limit interspecific gene flow, although there are exceptions (e.g., Scopece et al. 2013; Baek et al. 2016; Ma et al. 2016). To determine how species boundaries are maintained between hybridizing species, it is important to understand both the causes and results of hybridization (Furches et al. 2013) and the reproductive barriers that determine the relationship between species boundaries and hybridization of taxa (Widmer et al. 2009; De hert et al. 2012).

Primula L. is a genus of flowering plants with a heterostylous breeding system and extreme species richness, particularly in the eastern Sino-Himalaya region (between 90° and 100°E and 25° to 30° N) (Richards 2003). Only two cases of natural hybridization have been reported in this region (Zhu et al. 2009; Ma et al. 2014). Interspecific hybridization between *P. secundiflora* Franchet and *P. poissonii* Franchet was identified using nuclear internal transcribed spacer (ITS) sequences (Zhu et al. 2009). However, the status of the hybrid individuals and interspecific RI were not mentioned. To explore the consequence of hybridization and the maintenance of species boundaries between these two species, we identified the genetic structure of 110 individuals in the sympatric populations using ten SSR (simple sequence repeats) loci. In addition, we conducted field experiments in Shangri-La to evaluate the contribution of various reproductive barriers (pre-pollination isolation: phonological and pollinator-mediated isolation; post-pollination isolation: seed number, viability and germination) to the total RI between these two species (File S1).

The number of alleles per locus ranged from 5 to 11 (average 7.9); the allele size range and number of alleles per locus are shown in Table S1. Results from the NEWHYBRIDS program suggested that 97 of the 100 morphological parental individuals were pure parental species (with posterior probabilities of $\geq$ 90.7%), while the remaining three individuals were backcrosses to *P. poissonii*. All ten hybrids were backcrosses to *P. poissonii* (with posterior

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probabilities of ≥ 85.8%; Figure 1A). We assigned individuals that had been previously morphologically identified as *P. secundiflora* to one cluster with high probability (q = 0.993 ± 0.001) using the STRUCTURE software and those that had been previously morphologically identified as *P. poissonii* to the other cluster with a similarly high probability (q = 0.985 ± 0.005). The mean estimated proportion of *P. secundiflora* was 0.340 ± 0.017 in the ten hybrids (Figure 1B). *P. poissonii* and *P. secundiflora* individuals were separated into two clusters in PCoA (Figure 2).

The total isolation of each species was quite high, i.e., 1.0000 for *P. secundiflora* and 0.9968 for *P. poissonii* when it served as mother donor (Table 1). Post-pollination isolation explained 54.70% and 51.76% of the total isolation for *P. secundiflora* and *P. poissonii*, respectively, which is a little more than that explained by pre-pollination isolation. Pollinator-mediated barriers and low interspecific seed number contributed the most to the total RI. Post-pollination isolation limited interspecific gene flow when pre-pollination isolation was permeable. Detailed information for each barrier was documented in File S2. Although introgressive hybridization had occurred, species boundaries were maintained by multiple reproductive barriers. As the flowering times of the two species were nearly coincident, flowering time represents only a minor reproductive barrier. Pollinator assemblage mediated barriers contributed an asymmetric moderate isolation, with stronger isolation in *P. poissonii*, because all the visits to *P. secundiflora* were from Hymenoptera (bumblebees and *Anthophora* species), whereas about 30% of visits to *P. poissonii* were from Lepidoptera (butterflies). These findings suggest that pre-pollination barriers between *P. secundiflora* and *P. poissonii* were not complete, in such case, post-pollination barriers would work to restrict hybridization. Here we showed that interspecific hybridized F_1 seed numbers were significantly lower than those for the intraspecific crosses, especially when *P. secundiflora* was the maternal donor. Furthermore, embryo development failure was common in seeds produced by inter-specific crossing, and the seed viability resulting from hybridization was significantly lower than that in intraspecific crosses, visible under X-ray as empty seeds and stunted embryos. At the last, low germination rate is a known post-pollination barrier preventing hybridization, and similarly, we found low germination rates for hybrid seeds in both *P. poissonii* and *P. secundiflora*.

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Disturbed habitats might maximize the opportunities for interspecific hybridization (Arnold 1997). A convincing evidence is sunflower hybrid swarms that formed following habitat disturbance due to grazing, and/or trail and road construction (Heiser 1979). In another case, sheep disturbance was belived to be a cause for hybridization of *Psidium socorrense* and *P.* sp. aff. *Sartorianum* (López-Caamal et al. 2014). Grazing activity from livestock is common in the *P. secundiflora* × *P. poissonii* populations, and may have created habitat disturbances and favored the formation of hybrids. Once F_1s arise, they can backcross to parental species, following a classic pattern of natural hybridization (Arnold 1997; Rieseberg and Carney 1998). The differences between the two parental species in heteromorphic incompatibility might explain the occurrence of backcrosses to *P. poissonii*. Viable seed was generally set only when pollination occurred between the pin and the thrum or the thrum and the pin (termed as “legitimate” crosses in *Primula*), but in many species illegitimate pollinations (selfs or crosses between plants of the same morph) result in some seed set (Richards 2003). When crosses happened on *P. poissonii* mothers, more seeds could be produced, while few or no seeds could be formed on *P. secundiflora* mothers. It is possible that the weak heteromorphic incompatibility system in *P. poissonii* provided a greater chance for hetero-specific pollen grains to penetrate their stigmas and styles. Similarly, for another pair of *Primula* species, *P. beesiana* and *P. bulleyana*, where the numbers of F_1 seeds are substantially lower on *P. bulleyana* mothers (Ma et al. 2014).

Overall, despite the sympatry, synchronous flowering times and shared pollinators, we found that *P. poissonii* and *P. secundiflora*,maintained species integrity for long periods of time due to strong RI, reducing the instances of natural hybridization. These naturally hybridizing *Primula* species, with different incompatibilities, offer a unique chance to understand the evolutionary importance of RI in heterostylous species.

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AUTHOR CONTRIBUTIONS

Y. X., X. Z., Y. M., J. Z. and Q. L. designed the research and wrote the manuscript; Y. X., X. Z., and L. L. performed experiments; Y. X. analyzed data and prepared the figures and tables.

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SUPPORTING INFORMATION

File S1. Materials and Methods

File S2. Reproductive isolation between *P. poissonii* and *P. secundiflora*

Table S1. Basic allele information for the ten nSSR loci in the two parental species and hybrids

Table S2. Observations of pollinator visits to *P. secundiflora* and *P. poissonii*, and the proportion of visits of each pollinator to each plant species

Table S3. Seed numbers per flower resulting from 16 pollination treatments of the two parental species *P. secundiflora* and *P. poissonii*

Table S4. Effects of cross-pollination treatments (intra- or inter-species, mother species, pin or thrum as mother) on seed production

Figure S1. The sympatric populations of *P. poissonii* and *P. secundiflora* and flowers of *P. poissonii*, hybrid individuals and *P. secundiflora*

(**A**) The sympatric populations and a representative flower of (**B**) *P. poissonii*, (**C**) the natural hybrid, and (**D**) *P. secundiflora*.

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Figure legends

Figure 1. **Bayesian clustering analysis of *P. poissonii*, the hybrids, and *P. secundiflora* using nSSR data**

Clustering results based on the programs (**A**) NEWHYBRIDS and (**B**) STRUCTURE for K = 2.

Figure 2. **Plot of genetic structure (PCoA) based on variation at 10 nSSRs of *P. poissonii*, *P. secundiflora*, and hybrids**

The x-axes and y-axes represent 62.21% and 5.88% of the variance in genetic structure, respectively.

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Table 1. The strength of each reproductive barrier component, and the absolute contribution of this component to total reproductive isolation when *P. secundiflora* and *P. poissonii* served as mothers

	Components of RI		Absolute contribution to total RI	
Reproductive barriers	*P. secundiflora* ♀	*P. poissonii* ♀	*P. secundiflora* ♀	*P. poissonii* ♀
Phenological	0.130	0.111	0.1304	0.1111
Pollinator mediated	0.371	0.416	0.3226	0.3698
Pre-pollination RI			**0.4530**	**0.4809**
Seed number	0.980	0.704	0.5361	0.3654
Seed viability	0.895	0.788	0.0098	0.1211
Seed germination	0.989	0.902	0.0011	0.0294
Post-pollination RI			**0.5470**	**0.5159**
Total RI			**1.0000**	**0.9968**

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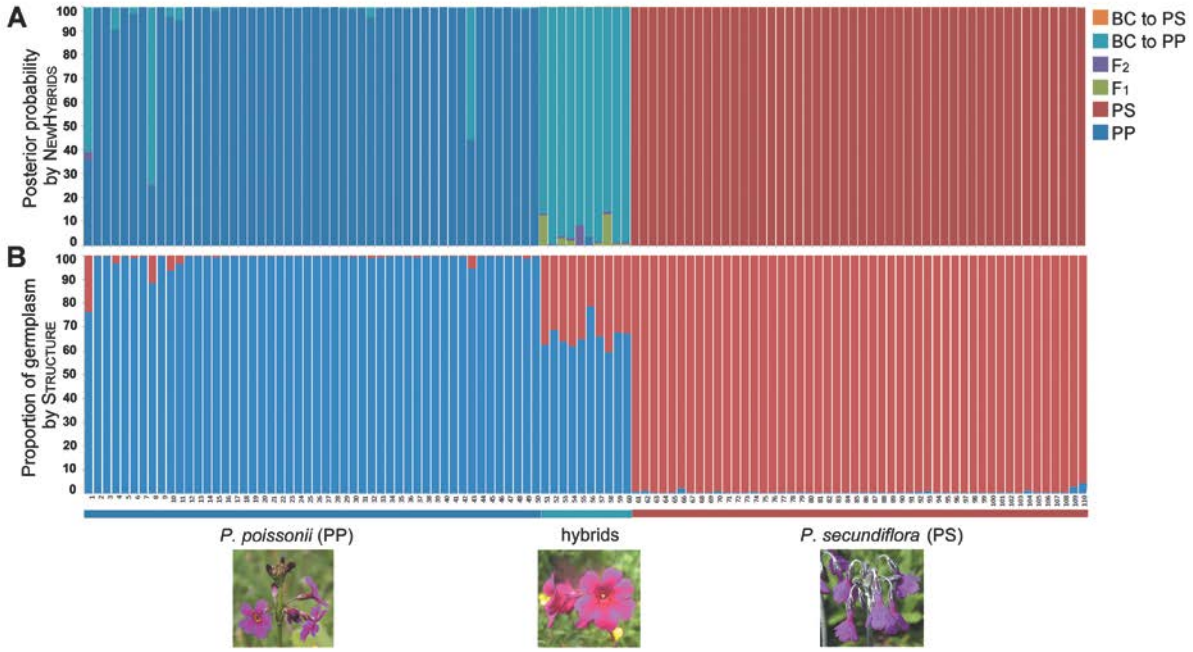

Figure. 1

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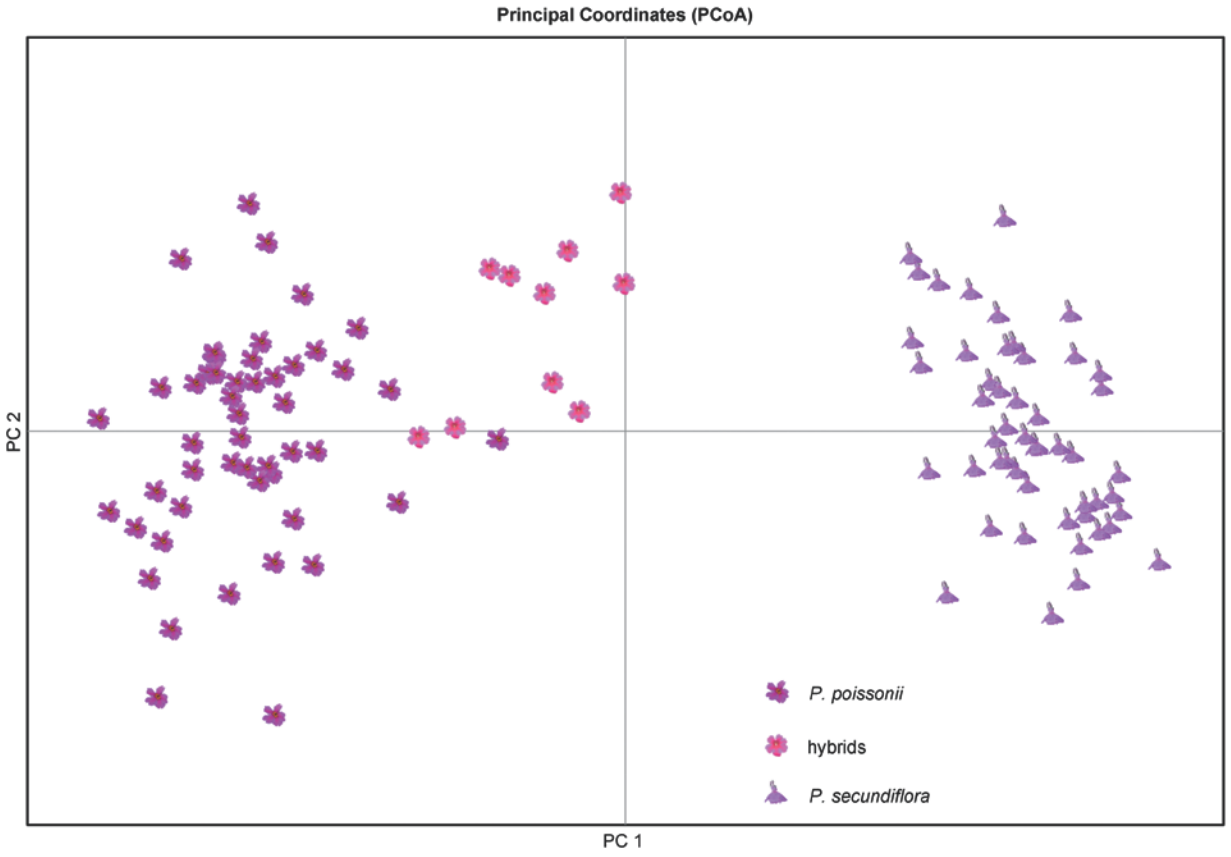

Figure. 2