

Natural Hybridization between *Rhododendron delavayi* and *R. cyanocarpum* (Ericaceae), from Morphological, Molecular and Reproductive Evidence

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Abstract

The natural hybridization that occurs between two sympatric species of *Rhododendron* subgenus *Hymenanthes* in Yunnan, China, was investigated. In field observations, it was noted that the putative hybrids between *R. delavayi* Franch. and *R. cyanocarpum* (Franch.) Franch. ex W.W. Sm. had intermediate morphologies. On the basis of morphology, chloroplast DNA (*trnL-rpl32*) and nuclear DNA (*waxy*), hybrids and parental species were identified. Hybridization occurred in both directions, but was asymmetrical, with *R. delavayi* as the major maternal parent. Reciprocal hand pollination treatments showed that either species, as pollen donor or pollen receiver, could produce fruits. It was noted that fruit set varied among treatments. The same pollinators (bumblebees) were shared in both parental species. From these results, we conclude that individuals with intermediate morphologies are indeed of hybrid origin from natural hybridization between *R. cyanocarpum* and *R. delavayi*. Furthermore, we presume the hybridization at the study site could have been initiated by habitat disturbance in the 1950s, and we may hence witness the early stages of hybrid swarm formation.

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Introduction

Hybridization may have several evolutionary consequences including the origin and transfer of genetic adaptations, the origin of new ecotypes or species and the reinforcement or breakdown of reproductive barriers (Anderson 1948; Ellstrand and Elam 1993; Whitham et al. 1994; Rieseberg 1997; Milne et al. 2003; Milne and Abbott 2008; Soltis and Soltis 2009). Most empirical work on species barriers has focused on isolating mechanisms that either limit hybrid formation or reduce the intrinsic fitness of hybrids (Arnold 2000; Burke and Arnold 2001; Milne et al. 2003). Hence, even if the potential exists

for the production of fertile hybrids, hybrid swarms rarely occur naturally. However, species range changes and environmental disturbance, mostly due to human habitat alteration, often facilitate hybrid formation or establishment (Mallet 2005).

Morphologically, hybrids typically display a mosaic of parental and intermediate characters, although extreme and novel characters appear quite often in the hybrid phenotype (Zhang et al. 2007a). The study of natural hybridization has been highly facilitated and advanced by the development of molecular methods. Combined with both sequences of parentally inherited nuclear DNA and maternally inherited chloroplast DNA, it is feasible to distinguish the paternal and

maternal parents of the hybrids in angiosperms (King et al. 2001; Baumel et al. 2002; Zhang et al. 2007a; Zha et al. 2008, 2010).

Rhododendron L. contains approximately 1 025 species, of which 24 subsections with 225 species belong to subgenus *Hymenanthes* (Chamberlain 1982; Fang and Min 1995; Chamberlain et al. 1996; Wu et al. 2005). Moreover, all of the subgeneric species are diploids ($2n = 26$) (Min and Fang 1990; Zhang et al. 2007a). This subgenus appears to have undergone rapid radiation within the Himalaya region (Milne 2004). As a result of a knowledge of the weak reproductive barriers in *Rhododendron*, many studies on natural hybridization have been conducted in this genus (Milne et al. 1999, 2003; Zhang et al. 2007a; Milne and Abbott 2008; Zha et al. 2008, 2010).

R. delavayi Franch. is included within subsect. *Fortunea* Sleumer. and *R. cyanocarpum* (Franch.) Franch. ex W.W. Sm. within subsect. *Thomsonii* Sleumer. Both species are included within the subgenus *Hymenanthes* (Chamberlain 1982; Hu and Fang 1994). *R. delavayi* is a remarkably widespread species, whereas *R. cyanocarpum* occurs only in the Cangshan mountains around Dali, northwestern Yunnan (Chamberlain 1982; Wu 1986). In field observation, some individuals were examined that had intermediate characters and were distributed sympatrically with *R. delavayi* and *R. cyanocarpum*. Hence, this study focused mainly on the detection of the putative hybrids and their status. Specifically, three main questions are addressed: First, to test whether these morphologically intermediate individuals are of hybrid origin from *R. delavayi*

and *R. cyanocarpum* via morphological and molecular evidence; second, if hybrids are proven to exist then it is necessary to determine whether the hybridization is unidirectional or bidirectional, and to establish which the usual paternal or maternal parent is. Finally, through preliminary pollination experiments to examine reproductive barriers and determine whether certain pollinators were shared, a better understanding of natural hybridization processes in *Rhododendron* could be established.

Results

Morphological identification

On the basis of field investigation, we found that six characters can be identified that easily distinguish the putative hybrids and parental species (Figure 1; Table 1). Specifically, these putative hybrids had four intermediate characters between *R. cyanocarpum* and *R. delavayi*, i.e. leaf shape, ventral leaf surface indumentum, corolla color and flowering period. The two other characters matched *R. cyanocarpum*, i.e. calyx persistence in mature capsule and fruit indumentum. It is therefore concluded that morphological evidence supports hybrid status for the 10 accessions selected.

Chloroplast DNA *trnL-rp132* sequences

Among 10 accessions of *R. delavayi* and 17 accessions of *R. cyanocarpum*, four variable sites were found in the

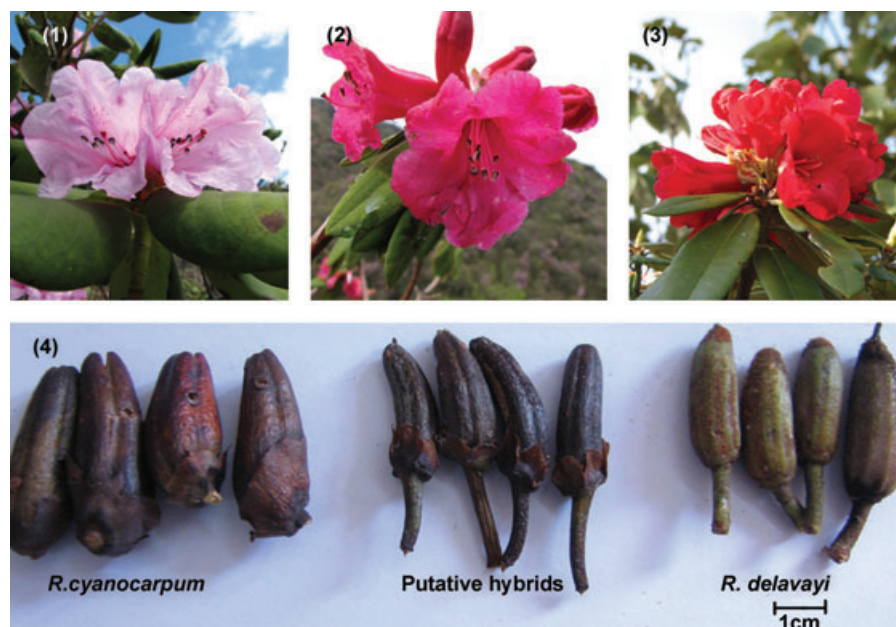


Figure 1. Flowers and fruits of *Rhododendron delavayi*, *R. cyanocarpum* and the putative hybrids.

Table 1. Morphological characters of *Rhododendron delavayi*, *R. cyanocarpum* and the putative hybrids

Morphological character	<i>R. delavayi</i>	<i>R. cyanocarpum</i>	Putative hybrid
Leaf shape	Long-lanceolate	Suborbicular	Oblong-elliptical
Ventral leaf surface indumentum	Dense	Glabrous	Thin
Flowering period	March to May	April to May	Late April
Calyx persistence in mature capsule	No	Yes	Yes
Corolla color	Deep red	Pink	Red
Fruit indumentum	Dense	Sparse	Sparse

trnL-rpl32 sequences. These sites all distinguished the haplotype of *R. delavayi* from that of *R. cyanocarpum* (Table 2). The variation was consistent within detected species. Among the 10 putative hybrids, the sequences of nine hybrids were identical with *R. delavayi* (GenBank accession number GU979812), however, a single accession (P₆) had sequence identical to *R. cyanocarpum* (GenBank accession number GU979810; Table 2). Hence most putative hybrids had *R. delavayi* as the plastid donor parent, but a single accession had *R. cyanocarpum* as plastid donor. Therefore, hybridization between these species is bidirectional but strongly asymmetrical.

Clones of nuclear DNA *waxy* sequences

Using multiple clones per individual, three sites were polymorphic in nuclear *waxy* sequences, and the variation detected was consistent within parental species. These sites all distinguished the haplotype of *R. delavayi* (GenBank accession number GU979813) from that of *R. cyanocarpum* (GenBank accession number GU979811; Table 3). For the putative hybrids, three sequence types were obtained from 31 clones of *waxy* sequences, i.e. *R. cyanocarpum* type (*c*), *R. delavayi* type (*d*) and admixture type (*cd*). The admixture type consisted of two different nucleotide combinations (GenBank accession number GU979814–15; Table 3). We obtained both *c* and *d* types in three individuals (P₂, P₅, P₁₀). For another seven, either one parental sequence type or one admixture type (P₁, P₃, P₆, P₈), or one parental sequence type plus admixture type (P₄, P₉, P₇) were detected (Table 4).

Genetic composition of plant materials

During comparative analysis of both *trnL-rpl32* and *waxy* sequences, three types of genetic composition were obtained, i.e. CC, DD and CD. *R. cyanocarpum* exclusively belonged to CC, whereas *R. delavayi* exclusively to DD. Even if the putative hybrids had three types of *waxy* sequences (Table 3), they all belonged to CD. Among the 10 putative hybrids detected, all contained one chloroplast haplotype and simultaneously had a different *waxy* type. Specifically, P₁–P₅ and P₇–P₁₀ all had chloroplast haplotype D, but they also had *waxy* type *c* or admixture type *cd*, or both. P₆ had both chloroplast haplotype C and *waxy* type *d* (Table 4). Therefore, the molecular evidence supports hybrid status for these 10 accessions.

Pollination treatments and pollinators observation

All six pollination treatments produced fruits, though fruit set among treatments varied. Fruit sets from treatments 2 and 3 were significantly higher than treatments 4 and 6. Fruit sets from treatments 1 and 5 did not exhibit significant differences to the other four treatments (Figure 2). It is therefore concluded that reciprocal pollination treatments favored the possibility of natural hybridization occurrence.

Based on 32 h of observation in 2008, we found 31 visitations from bumblebees (*Bombus* sp.) and only one visitation from butterfly (*Papilio krishna*) approaching to the observed individual of *R. cyanocarpum*. Both bumblebees and butterfly carried pollen as well as making contact with stigmas during visitation. We conclude that the butterfly is a casual pollinator

Table 2. Chloroplast sequences present in material of *Rhododendron delavayi*, *R. cyanocarpum* and putative hybrids, and the codon positions at which they differ

Specimens	Number of accessions	<i>trnL-rpl32</i> - type ^a	Sequence region, and codon position <i>trnL-rpl32</i>			
			238	627	750	751
<i>R. delavayi</i>	10	D	C	T	G	A
<i>R. cyanocarpum</i>	17	C	A	G	T	C
Putative hybrids	1	C	A	G	T	C
	9	D	C	T	G	A

^aAll accessions of *R. delavayi* examined had haplotype D, whereas all accessions of *R. cyanocarpum* had haplotype C.

Table 3. Waxy sequences present in material of *Rhododendron delavayi*, *R. cyanocarpum* and putative hybrids, and the codon positions at which they differ

Specimens	Number of accessions	Number of clones	waxy type	Sequence region, and codon position waxy		
				373	456	731
<i>R. delavayi</i>	5	15	c	T	A	C
<i>R. cyanocarpum</i>	5	14	d	C	T	G
Putative hybrids ^a	10	31	c	T	A	C
			d	C	T	G
			cd ₁	T	A	G
			cd ₂	C	T	C

^aThe putative hybrids had three types of waxy sequences (c, d, cd), with cd₁, cd₂ referring to different nucleotide composition of the admixture sequence.

and bumblebees were the main pollinators. Our observations of *R. delavayi*, found that bumblebees and wasps (*Polistes* sp.) always made contact with stigmas and carried pollen during visitation. In total, there were 47 visitations, all of which resulted in stigma contact. Thirty-six visitations of honeybees were examined in 24 h. However, the pollination efficiency was lower for honeybees because we just observed four visitations simultaneously touching stigmas among the examined 50 visitations, and we thus did not treat honeybees as effective pollinators. Hence bumblebees and wasps were the main pollinators in *R. delavayi*. For the two flowering individuals of putative hybrids, we did not find insects approaching flowers in 12 h of observation. Hence, bumblebees as the same

pollinators of *R. cyanocarpum* and *R. delavayi* also contributed to the possible occurrence of natural hybridization between these species as well.

Discussion

Hybrids between *R. delavayi* and *R. cyanocarpum*

Sympatric geographic distribution, morphological intermediacy characters and partial fertility are useful evidence for testing hybridization (Gottlieb 1972). These can also be applied to our study. Field observations showed that the putative hybrids located sympatrically with parental species and had

Table 4. Comparative analysis of chloroplast haplotype and waxy type of *Rhododendron delavayi*, *R. cyanocarpum* and putative hybrids

Specimens	cpDNA haplotype ^a	Number of clones with waxy type of species			Presumed genetic composition ^c
		RC (c)	RD (d)	Admixture ^b (cd)	
<i>R. cyanocarpum</i>	C	14	0	0	CC
<i>R. delavayi</i>	D	0	15	0	DD
Putative Hybrid (P ₁)	D	1	0	0	CD
Putative Hybrid (P ₂)	D	1	2	0	CD
Putative Hybrid (P ₃)	D	2	0	0	CD
Putative Hybrid (P ₄)	D	0	3	2	CD
Putative Hybrid (P ₅)	D	2	1	1	CD
Putative Hybrid (P ₆)	C	0	3	0	CD
Putative Hybrid (P ₇)	D	0	2	1	CD
Putative Hybrid (P ₈)	D	0	0	2	CD
Putative Hybrid (P ₉)	D	2	0	3	CD
Putative Hybrid (P ₁₀)	D	2	1	0	CD

^aSee Table 2 for details. ^bSee Table 3 for details. ^cAccession that had one chloroplast haplotype and simultaneously had another waxy type was considered as CD.

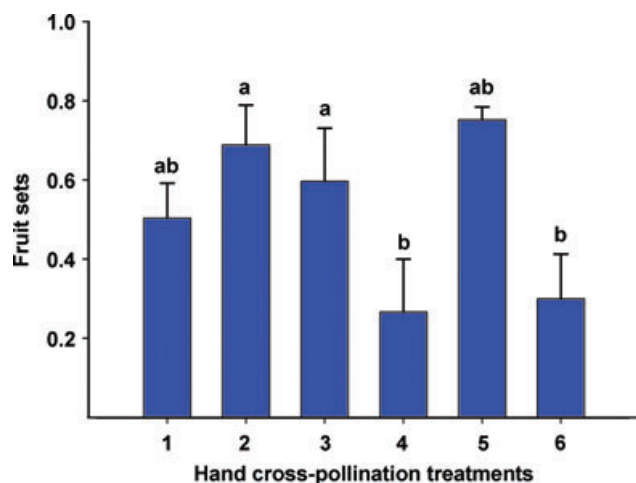


Figure 2. Fruit sets (mean \pm SE) among hand cross-pollination treatments, with the same letter indicating no difference.

intermediate characters. Moreover, the flowering periods of parental species overlapped, providing the possible occurrence of natural hybridization in flower phenology. In addition, reciprocal hand pollinations exclusively produced fruits suggesting that the hypothesis of stringent reproductive isolation between *R. delavayi* and *R. cyanocarpum* is rejected. This has already been proved in cultivated species and other wild species in *Rhododendron* (Chamberlain 1982; Milne et al. 1999, 2003; Zhang et al. 2007b; Milne and Abbott 2008; Zha et al. 2008, 2010). Therefore, due to the preliminary evidence obtained in the field, it is reasonable to deduce the hybrid status for these 10 accessions.

The molecular evidence confirmed the occurrence of natural hybridization from morphological presumption, because genetic admixture between *R. delavayi* and *R. cyanocarpum* was detected from all of the putative hybrids (Table 4). We first detected hybridization in *Rhododendron* using *waxy* gene, which had been proved to be a recombinant gene in *Cymbopogon* and presumed to be feasible for examining hybridization (Mason-Gamer et al. 1998). Furthermore, the *trnL-rpl32* sequences of hybrids matched either the *R. cyanocarpum* type (90%) or *R. delavayi* type (10%) indicating that occurrence of natural hybridization is bidirectional but very symmetrical, and *R. delavayi* as the usual maternal parent. This is the same situation as found with *R. agustum* (Zha et al. 2008). A probable cause for asymmetrical hybridization in this case might be flower phenology. *R. delavayi* flowers earlier than hybrids or *R. cyanocarpum* (Table 1), and it would be the more likely maternal parent because *Rhododendrons* are protandrous; and for the same reason hybrid stigmas are more likely to receive pollen from the later flowering parent (Milne and Abbott 2008; Zha et al. 2010).

Pollination and hybridization

The possibility of hybridization occurrence between species depends on the strength of prezygotic and postzygotic reproductive barriers, thereby leading to the frequency of hybridization dramatically varied among families (Ellstrand et al. 1996). In flowering plants, the initial stages of prezygotic isolation are often related to the behavior of pollinators (Campbell et al. 2002). Hence, any tendency of pollinators to move within species prevents hybridization. Despite the fact that pollinators observed in the present study varied between *R. cyanocarpum* and *R. delavayi*, bumblebees were found as a common pollinator. Even if pollen grains are transferred between species, formation of hybrids can still be prevented by postzygotic reproductive barriers (Campbell et al. 2002). Reciprocal hand pollinations between *R. cyanocarpum* and *R. delavayi* resulted in fruit set, thereby facilitating production of F_1 hybrids, which act as a bridgehead for further production of later generation hybrids (Arnold 1997; Rieseberg and Carney 1998; Zha et al. 2008). As for the detected hybrids, we do not know what generations each belongs to. However, it is usual for hybrids of *Rhododendrons* consisting of mainly F_1 s (Milne et al. 2003; Milne and Abbott 2008; Zha et al. 2008, 2010), but evidence for introgression is also detected (Kron et al. 1993; Tagane et al. 2008).

Possible reasons and implications for hybrid formation

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 and Carney 1998). In *R. delavayi* \times *cyanocarpum*, 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. To date, we have not observed any evidence indicating that it is a stable, long-term phenomenon. Based on three consecutive years of observation, we found just three hybrids flowering. Observations from these few flowering individuals showed basal diameters within the range of 4.4 cm to 10.3 cm and heights from 56 cm to 175 cm, respectively (YP Ma, unpubl. data, 2009). Such features suggest the recent formation of these hybrids. Many studies that were involved in the occurrence of hybridization may be in a long history because the detected hybrids are of large numbers and sizes as well as occurrence of many sites (populations), even some of them lost one parental species for past local extinction (Dodd and Afzal-Rafii 2004; Lepais et al. 2009; Ortego and Bonal 2009). Within *R. delavayi* \times *cyanocarpum*, this situation can not usually be detected and this may be of significance for the early stage study of speciation. Furthermore, features of these hybrids have also indicated the single hybrid zone formation

was associated with a relatively weak disturbance to its natural habitats. With regard to habitat disturbance, the most significant period of disturbance within the locality containing the hybrid zone occurred during 1957–1958, when many trees were felled to allow the construction of a factory (YL Yang, pers. comm., 2009). This suggests that we are witnessing the early stages of the formation of a hybrid swarm. An additional point of interest and possible concern is that *R. cyanocarpum* is a listed endangered species and although hybridization may be having little impact on this species at present, the situation is dynamic and will change with the occurrence of more hybrids.

Materials and Methods

Location, identification and collection of plant materials

Between 2007 and 2008 we examined all known populations of *Rhododendron cyanocarpum* (Franch.) Franch. ex W.W. Sm., covering both east and west slopes of the Cangshan mountains, i.e. Ganchaiqing (25°52'N, 99°58'E), Huadianba (25°52'N, 99°59'E), Guogaishan (25°51'N, 100°02'E), Xiaohuadian (25°51'N, 100°02'E), Yangbi (25°42'N, 100°05'E) and Dianshitai (25°40'N, 100°06'E). Hybrids were found only at a single locality at Huadianba (HDB), 3 200 m above sea level. At this locality we found two other members of subgenus *Hymenanthes*, i.e. *R. delavayi* Franch. and *R. alutaceum* Balf. f. & W.W. Sm. Based on morphology, the hybrids appeared to be intermediate between *R. cyanocarpum* and *R. delavayi*. In contrast, *R. alutaceum* differs from *R. cyanocarpum*, *R. delavayi* and the putative hybrids in its white to pale pink corolla and much later flowering time (June to July); it was therefore eliminated as a putative parent. Based on examination of the putative parent species in the field, six morphologically diagnostic characters were selected that are easily observed and consistently distinguish them and the putative hybrids (Table 1). Using these characters, only 10 putative hybrids were identified at this site despite careful inspection of every *Hymenanthes* plant detected. Therefore, all of the 10 putative hybrid accessions were collected. In addition, a further 17 *R. cyanocarpum* and 10 *R. delavayi* accessions were collected to provide indicators of morphology and molecular profiles of parental species. From all collected accessions, leaves were desiccated using silica gel and self-sealing polythene bags, and voucher specimens for all putative hybrids and some of the parental accessions were deposited in the herbarium of the Kunming Institute of Botany, the Chinese Academy of Sciences (KUN).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from leaves following a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and

Doyle 1987). The nrDNA gene *waxy* was amplified using the primers described by Yang et al. (2006). To determine the direction of hybridization, the *trnL-rpl32* intergenic region of chloroplast DNA was amplified using primers described by Shaw et al. (2007).

The reaction mix contained 0.625 U AmpliTaq DNA polymerase, FS (PE Applied Biosystems, Foster City, CA, USA), 1×PCR buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTP, 0.3 μmol/L primer and 20–60 ng genomic DNA. PCR reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norfolk, CT, USA). The PCR conditions included an initial denaturation at 94 °C for 4 min, followed by 30 cycles of 1 min at 94 °C for template denaturation, 1 min at 50 °C for primer annealing, 1.5 min at 72 °C for extension, and finished with an extension step of 10 min at 72 °C. The PCR products were purified using a Sangon Purification kit according to the manufacturer's protocol for sequencing PCR reactions. Purified PCR product of *waxy* genes were cloned into Promega's (Madison, WI, USA) pGEM-T System I vector according to the manufacturer. In total, 15 clones from five *R. delavayi*, 14 clones from five *R. cyanocarpum* and 31 clones from the 10 putative hybrids were obtained respectively, and plasmid preparations were carried out following Sangon's protocols. Contiguous DNA sequences were edited using SeqMan (DNASTAR package) and sequences aligned using Clustal X (Thompson et al. 1997).

Pollination treatments and pollinators observation

In 2009, to investigate if reproductive barriers occurred among these species, randomly selected flower buds were covered with nylon nets after six hand cross-pollination treatments. 1, *R. cyanocarpum* ♀ × *R. delavayi* ♂ (36 flowers); 2, *R. delavayi* ♀ × *R. cyanocarpum* ♂ (31 flowers); 3, *R. cyanocarpum* ♀ × Putative hybrids ♂ (37 flowers); 4, *R. delavayi* ♀ × Putative hybrids ♂ (38 flowers); 5, Putative hybrids ♀ × *R. delavayi* ♂ (21 flowers); 6, Putative hybrids ♀ × *R. cyanocarpum* ♂ (23 flowers), in which ♂ and ♀ represented pollen donor and pollen receiver. Due to the restricted flowers of putative hybrids, six inflorescences were used for treatments 1–4, and three inflorescences for treatments 5–6. Fruits produced by these flowers were counted in October when they were full-sized and after fruit abortion had occurred (Rathcke and Real 1993). For fruit set (total number of fruits produced/total number of flowers per inflorescence), means and standard error were calculated after examination of normal distribution with a one-sample K-S test. One-way ANOVA analysis was used to compare fruit sets among treatments.

We observed flower visitors of *R. cyanocarpum* at least 20 h each year during the flowering period in both 2007 and 2008 at HDB. To examine the main pollinators, we recorded visitations of potential pollinators in a selected individual for a total of 32 h from 10.00 to 14.00 hours for successive days in 2008.

For *R. delavayi*, honeybees have been solely determined to be effective pollinators in eastern Yunnan, China (Zhang et al. 2007b). To track potential pollinators and record insects at HDB, northwestern Yunnan, we carried out observations for a total of 24 h from 10.00 to 16.00 hours on four successive days in 2008. According to our basic observations at HDB, we noticed that honeybees rarely touch stigmas, but carry pollen during visitation. Hence, to clarify the pollination efficiency of honeybees, we also randomly observed 50 visitations of honeybees to flowers and recorded visitations that resulted in contact with stigmas. For the putative hybrids, we found just two individuals flowering in 2008, and we observed the potential pollinators from 10.00 to 16.00 hour over two successive days. All flower visitors were captured and brought to the laboratory for identification. Voucher specimens were deposited in the Kunming Institute of Botany. Insects that collected pollen and contacted stigmas were recorded as pollinators (Li and Huang 2009).

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