# UNUSUAL PATTERNS OF HYBRIDIZATION INVOLVING A NARROW ENDEMIC RHODODENDRON SPECIES (ERICACEAE) IN YUNNAN, CHINA

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BRIEF COMMUNICATION

# Unusual patterns of hybridization involving a narrow endemic *Rhododendron* species (Ericaceae) in Yunnan, China[1]

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- *Premise of the study*: One potential threat to rare species is genetic swamping caused by hybridization, but few studies have quantified this threat. *Rhododendron cyanocarpum* is a narrow endemic species that occurs sympatrically with potentially interfertile congeners throughout its range within Yunnan, China. We searched the entire distribution of *R. cyanocarpum* for hybrids and examined the patterns of hybridization to assess potential threat from hybridization.
- *Methods*: In a comprehensive field survey, we detected only one instance of hybridization involving *R. cyanocarpum*, with *R. delavayi*, at Huadianba near Dali. Material of both species and putative hybrids was examined using morphology, chloroplast DNA, nuclear ribosomal DNA, and Bayesian analysis of AFLP profiles.
- *Key results*: Of 10 putative hybrids, two were $F_1$'s and at least seven were $F_2$'s. Four backcrosses to *R. delavayi* were detected among material with *R. delavayi*-like morphology within the hybrid zone. Backcrosses to *R. cyanocarpum* were not detected. Therefore $F_2$'s outnumbered all other classes within the hybrid zone, a situation not previously confirmed for plants and extremely rare generally. Hybridization was asymmetrical, with *R. delavayi* as the maternal parent in all but one of the hybrids detected.
- *Conclusions*: Although natural hybridization is common in *Rhododendron*, it is rare in *R. cyanocarpum* and is apparently not accompanied by backcrossing toward *R. cyanocarpum*. Hence, there is no immediate risk of genetic swamping, unless habitat disturbance increases and changes the patterns of hybridization. Our study is the first to report a plant hybrid zone dominated by $F_2$ hybrids. This pattern might contribute to species barrier maintenance.

**Key words:** Ericaceae; habitat disturbance; hybrid zone; narrow endemic species; *Rhododendron cyanocarpum*; *Rhododendron delavayi.*

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 (Rieseberg and Gerber, 1995; Arnold, 1997; Rieseberg, 1997; Rieseberg and Carney, 1998; Soltis and Soltis, 2009). However, for rare species, it can also bring about extinction through genetic swamping (Levin et al., 1996; Rhymer and Simberloff, 1996; Vilà et al., 2000; Wolf et al., 2001). Therefore, for rare species that occur sympatrically with interfertile congeners, barriers to hybridization and interspecific gene flow may be vital to their persistence. Furthermore, because anthropogenic disturbance can promote hybridization (Anderson, 1948; Levin et al., 1996; Rieseberg and Carney, 1998), the threat from hybridization may be increasing for some rare species, but very few studies have sought to quantify or evaluate this threat.

The genus *Rhododendron* L. contains about 1025 species, including many narrow endemics that are sympatric with interfertile congeners throughout their ranges. Within *Rhododendron*, subgenus *Hymenanthes* appears to have undergone rapid radiation within the Himalaya region (Milne, 2004), with nearly 200 species endemic to China and adjacent regions, many of them with extremely limited ranges (Chamberlain, 1982; Fang and Min, 1995; Chamberlain et al., 1996; Wu et al., 2005). All *Hymenanthes* species are diploids ($2n = 26$), and hybridization even between distantly related species is common (Chamberlain, 1982; Milne et al., 1999, 2003; Zhang et al., 2007; Zha et al., 2008, 2010). Even remote habitats in China have mostly been subject to some level of habitat disturbance, but although hybrids involving narrow endemic species have occasionally been reported (Chamberlain, 1982), there has not yet been a systematic examination of how commonly any such species forms natural hybrids or what follows when hybridization occurs. Furthermore, hybrid zones within *Rhododendron* sometimes contain only $F_1$'s, removing any possibility of introgression (Milne et al., 2003; Zha et al., 2010), and progression of hybridization beyond the $F_1$ stage can also be promoted by disturbance

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(Kyhos et al., 1981; Milne et al., 2003). So to determine the likely consequences of hybridization, the class structure of any hybrid population detected must be determined.

*Rhododendron cyanocarpum* (Franch.) W.W.Smith (subsection *Thomsonii* Sleumer) is a narrow endemic, confined to mountain slopes above 3000 m a.s.l. in the Cangshan mountains around Dali, Yunnan Province (Chamberlain, 1982; Wu, 1986; Fig. 1). It occurs sympatrically with other *Hymenanthes* species throughout its range, notably the widespread *R. delavayi* Franch. (subsection *Arborea* Sleumer), but has not previously been known to form natural hybrids. The present study was therefore designed to first extensively survey extant *R. cyanocarpum* populations and seek evidence of hybridization. Should hybrids be detected, we then aimed to examine the hybrid zone to determine population structure and direction of crossing and any threat of genetic swamping to *R. cyanocarpum*.

## MATERIALS AND METHODS

***Location and identification of hybrids involving Rhododendron cyanocarpum***—Between 2007 and 2008, we examined all known populations of *R. cyanocarpum,* covering both the east and west slopes of the Cangshan mountains, i.e., Ganchaiqing, (25°52′N, 99°58E), Huadianba (25°52′ , 99°59′E), Guogaishan, (25°51′ , 100°02′E), Xiaohuadian, (25°51 , 100°02′E), Yangbi, (25°42′N, 100°05′E) and Dianshitai, (25°40′ , 100°06′E). At each site, the species forms a single, often broad population.

Hybrids were found only at a single locality at Huadianba, 3200 m a.s.l. At this locality were two other members of subgenus *Hymenanthes*, i.e., *R. delavayi* and *R. alutaceum* Balf. f. et W.W.Smith. Based on morphology (Table 1), the hybrids appeared to be intermediate between *R. cyanocarpum* and *R. delavayi*, whose flowering periods (April to May) overlapped. By contrast, *R. alutaceum* differs from *R. cyanocarpum*, *R. delavayi*, and the putative hybrids at this site in its white to pale pink corolla and much later flowering time (June to July); it was therefore eliminated as a putative parent. *Rhododendron delavayi* is a very widespread species, which contrasts sharply with the restricted range of *R. cyanocarpum* (Fig. 1). Based on examination of the putative parent species, *R. cyanocarpum* and *R. delavayi*, in the field, nine morphological characters were identified that consistently distinguished them from the putative hybrids (Table 1). Using these characters, only 10 putative hybrids were identified in this hybrid zone, despite careful searching and inspection of every *Hymenanthes* plant detected.

***Collection of plant material***—A 40 × 40 m area was marked out in May 2009, and all healthy *Rhododendron* accessions from within this area were mapped and collected (Fig. 2). These comprised 10 putative hybrids, 10 *R. cyanocarpum*-like accessions and 21 *R. delavayi*-like accessions. In addition, a further 17 *R. cyanocarpum*, and 10 *R. delavayi* accessions were collected from the same site but outside this marked area, to provide indicators of morphology and molecular profiles of the pure parental species. From all collected accessions, leaves were desiccated using silica gel and self-sealing polythene bags. Voucher specimens for all putative hybrids and some of the parental accessions were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN) (Appendix 1).

***DNA extraction, PCR amplification, and sequencing***—Genomic DNA was extracted from all collected leaves using a modified CTAB protocol (Doyle and Doyle, 1987). The nrDNA ITS region from 68 accessions was amplified using primers ITS4 and ITS5 (White et al., 1990). The chloroplast *trnC-trnF* and *trnH-psbA* spacer of 37 accessions, comprising 10 putative hybrids and 27 pure parental species, were amplified and sequenced (Taberlet et al., 1991; Kress et al., 2005) to determine the direction of hybridization,. The reaction mix contained 0.625 U AmpliTaq DNA polymerase, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.3 umol/L primer, and 20–60 ng genomic DNA. PCR reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norfolk, Connecticut, USA). The PCR conditions were as follows: 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 finally 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. DNA sequences were obtained using an ABI 3700 automated sequencer (Perkin Elmer).

Purified PCR products of ITS were cloned into Promega's pGEM-T System I vector according to the manufacturer's protocol. Thirty-one clones of ITS sequence from 10 putative hybrids were obtained, and plasmids were prepared using Sangon's protocols. Contiguous DNA sequences were edited using the program SeqMan (DNASTAR package, Madison, Wisconsin, USA) and sequences aligned using Clustal_X (Thompson et al., 1997). Primers ITS4 and ITS5 were used for accessions to double-check nucleotide site polymorphisms and the accuracy of the sequence.

***AFLP marker generation***—We used AFLP markers to examine the 10 putative hybrids, plus five accessions each of *R. cyanocarpum* and

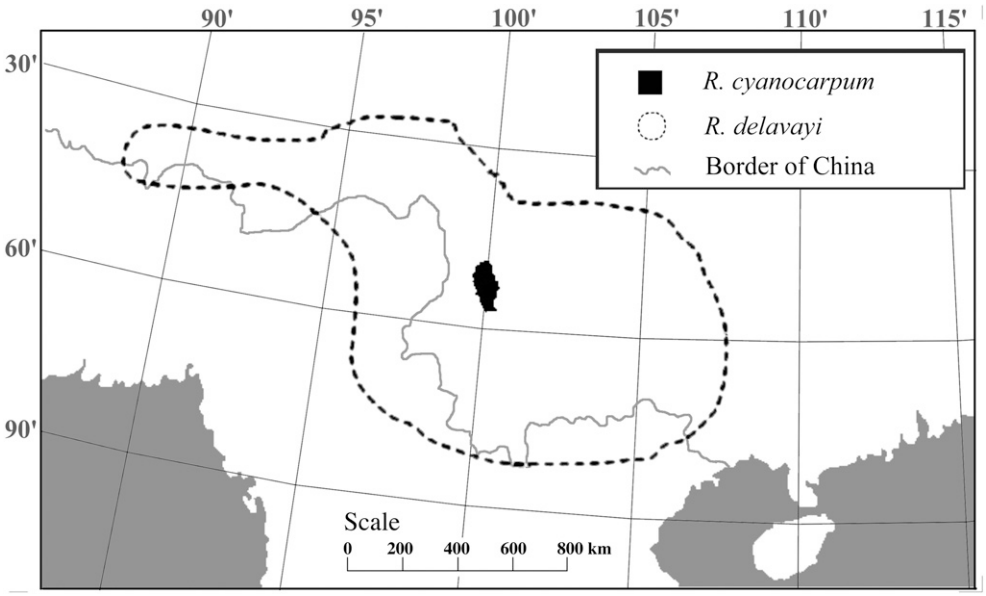


Fig. 1. Distributions of *R. delavayi* and *R. cyanocarpum* in and around southwest China.

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

| Morphological character | *R. delavayi* | *R. cyanocarpum* | Putative hybrid |
|---|---|---|---|
| Leaf shape | long-lanceolate | suborbicular | oblong-elliptical |
| Leaf length/width ratio | 2.61–3.56 | 1.08–1.53 | 1.64–2.23 |
| Ventral leaf surface indumentum | dense | glabrous | thin |
| Calyx length (mm) | 1.1–1.6 | 4.0–7.1 | 4.2–6.9 |
| Calyx persistence in mature capsule | No | Yes | Yes |
| Corolla color | deep red | pink | red |
| Flowers per inflorescence | 17–21 | 5–8 | 7–12 |
| Fruit indumentum | dense | sparse | sparse |
| Carpel number | 10 | 5–6 | 6–9 |

*R. delavayi.* We were unable to examine a larger set of accessions because resources were limited.

The AFLP procedure was carried out according to the Beckman Coulter protocol with only minor modifications as described by Reisch (2007). Double digestion of genomic DNA was performed for 2 h at 37°C in a 20-μL mix using 2 units (U) of MseI and 10U of EcoRI. Following this, adapters were ligated to DNA in a 21-μL volume for 2 h at 37°C using 2 U of T4 DNA Ligase (Shanghai Sangon Biological Engineering Technology, Shanghai, China). Preselective polymerase chain reactions were run in a reaction volume of 25 μL. PCR parameters were chosen as follows: 2 min at 94°C; 25 cycles of denaturing at 94°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min; followed by 2 min at 72°C and ending with 30 min at 60°C. Diluted 20× preselective products underwent selective PCR with the following three primer combinations: E-AAC/M-CTG, E-ACT/M-CAG, E-AAC/M-CAA. Selective amplifications were run in a 25-μL volume, and PCR reactions were performed with the following touchdown profile: 2 min at 94°C; 10 cycles of denaturing at

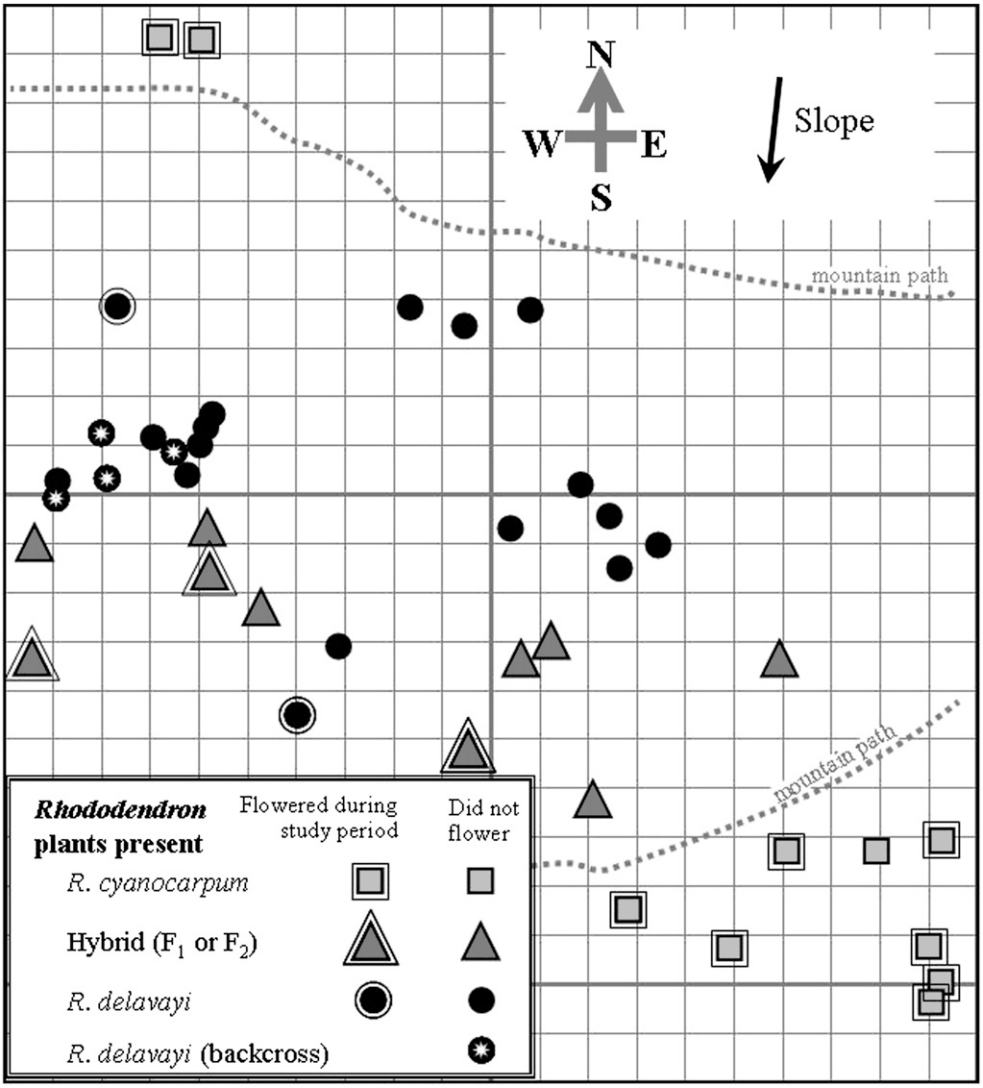


Fig. 2. Map of the hybrid zone at Huadianba between *R. delavayi* and *R. cyanocarpum*, showing positions of hybrid individuals and those of the parent species and the putative hybrids.

94°C for 20 s, annealing for 30 s at 66°C and then reduced by 1°C for the next 10 cycles, elongation at 72°C for 2 min, followed by 25 cycles for denaturing at 94°C for 20 s, annealing at 56°C for 30 s, and 2 min elongation at 72°C; ending with a final extension for 30 min at 60°C. Finally, the PCR products were added to a mixture of Sample Loading Solution (Beckman CoulteR, Fullerton, California, USA) and CEQ Size Standard 400 (Beckman Coulter). The fluorescence-labeled selective amplification products were separated by capillary gel electrophoresis on an automated sequencer (CEQ 8000, Beckman Coulter).

Raw data were collected and analyzed with the CEQ Size Standard 400 using the CEQ 8000 software (Beckman Coulter). Individuals were scored for the presence or absence of each fragment in binary mode (1/0) in crv-files. Bins were built using the AutoBin option with a peak height of 800 and a bin width of 2. Fragments were then assigned to bins with a selective height and checked manually. When ambiguous electropherograms were detected, the AFLP procedures were repeated to test for reproducibility. In the AFLP data matrix, the presence of a band was scored as 1, whereas the absence of the band was coded as 0. From this was produced a binomial (0/1) data matrix, representing the scores for AFLP markers across all examined accessions.

***Bayesian analysis of AFLP data***—Many recent papers (e.g., Hänfling et al., 2005; Llopart et al., 2005; Gow et al., 2006; Mercure and Bruneau, 2008; Milne and Abbott, 2008; Smulders et al., 2008; Zha et al., 2008, 2010) have used the program NewHybrids version 1.1, which employs a Bayesian analysis to identify hybrids within natural hybrid zones (Anderson and Thompson, 2002) and to determine their classes. This method can be used with dominant data such as AFLP markers and is capable of identifying hybrids even when the markers are not completely species-specific. Using this program requires certain assumptions about the markers used: that they are unlinked, not subject to selection, and were at linkage equilibrium in the parent species before hybridization.

The default settings of this program assign posterior probabilities for six possible classes (parents, $F_1$, $F_2$, backcross 1 each way), assuming that only two generations of crossing have occurred, which can rarely be verified, but this setting is nonetheless commonly used. This problematic assumption is avoided by using the modified settings of Milne and Abbott (2008), which allow four generations of crossing and group hybrids into six categories (parents, $F_1$-like, $F_2$-like, backcrosses each way). Because it allows for more possibilities, the latter is far more conservative in class assignment. For the present paper, the number of generations is uncertain, so we analyzed the AFLP data using both settings. In each case, posterior probabilities were evaluated after 100 000 iterations of Markov chain Monte Carlo (MCMCs), after a burn-in of 10 000 iterations, without using any prior information of individual or allele frequency.

To provide an indication of the proportion of each parent's germplasm in each hybrid, the same data matrix was also analyzed using the program Structure version 2.3.1 (Hubisz et al., 2009), following the methods of Falush et al. (2007). We adopted the admixture model with correlated allele frequencies (Lepais et al., 2009; Salvini et al., 2009; Zalapa et al., 2009). No prior knowledge of the species was included in the analyzed data set. To determine the optimal number of groups ($K$), we ran Structure with $K$ varying from 1 to 10, with five runs for each $K$ value. Previous studies have found that, in many cases, the posterior probability for a given $K$ increases slightly, even after the real $K$ is reached (Dan et al., 2009). Therefore, we used Evanno et al.'s (2005) ad hoc statistic, $\Delta K$, to determine the true value of $K$. Our parameters were 10 000 burn-in periods and 10 000 MCMC repetitions after burn-in. For the most likely number of clusters ($K = 2$), we used the ANCESTDIST command in Structure to generate 90% credible intervals for the admixture coefficients for each accession. The analysis was independently run 10 times, and the average values across all runs were taken for each of the best estimate, lower limit, and upper limit for admixture coefficients for each accession.

## RESULTS

***Morphological characters***—Of the nine morphological characters by which *R. cyanocarpum* and *R. delavayi* may be distinguished, the 10 putative hybrids were intermediate between these species for six (Table 1), but for the three other characters they matched *R. cyanocarpum*, i.e., mature capsule indumentum, calyx persistence, and calyx length (Table 1). Therefore, morphological evidence strongly supports hybrid status for these 10 accessions.

***Chloroplast DNA* trnC-trnF *and* trnH-psbA *sequences***—Among 10 accessions of *R. delavayi* and of 17 *R. cyanocarpum*, eight and nine variable sites were found in the *trnC-trnF* and *trnH-psbA* chloroplast regions, respectively. These sites all distinguished the haplotype of *R. delavayi* from that of *R. cyanocarpum* (Table 2). No variation was detected between accessions within either species. Among the 10 putative hybrids, the sequences of nine hybrids were identical to that of *R. delavayi* (GenBank accessions HM636523 and HM636525); however, a single accession (P6) had sequences identical to *R. cyanocarpum* (GenBank accessions HM636522 and HM636524; Table 3). Therefore, hybridization between these species is bidirectional but strongly asymmetrical.

***Nuclear ribosomal DNA ITS sequences and clones of the hybrids***—Within the nrDNA ITS region, seven sites were polymorphic. All accessions had either the *R. cyanocarpum* ITS type, or the *R. delavayi* ITS type, or were additive at all variable sites (GenBank accessions HM636518 and HM636521; Table 4). The *R. cyanocarpum* ITS type was present in all accessions of this species examined, both within and outside the hybrid zone. The *R. delavayi* ITS type was present in all accessions of this species outside the hybrid zone and in 15 accessions from within the hybrid zone. Of the remaining six *R. delavayi* accessions within the hybrid zone, two could not be sequenced but the other four were additive at all variable ITS sites. Similarly, all 10 putative hybrids were additive at all variable ITS sites. Based on this, there is evidence of backcrossing and putative introgression toward *R. delavayi* within but not outside the hybrid zone, but no such evidence for *R. cyanocarpum.*

Using multiple clones per individual, we found that six of the 10 putative hybrids contained the ITS types of both species. Of the other four, P6 and P9 had only a single clone, so only one ITS sequence type could be recovered (in both cases, *R. cyanocarpum,* Table 3), whereas for P3, both of the two clones created had the *R. delavayi* ITS type. However, the detection of seven *R. delavayi*-like clones from accession P8 (a putative $F_1$, see below) might indicate that the cloning process was biased against incorporating *R. cyanocarpum* DNA for this one individual. No other ITS types except those of *R. delavayi* or *R. cyanocarpum* were detected from cloned hybrid ITS.

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

| | Sequence region and codon position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *trnC-F* | | | | | *trnH-psbA* | | | | | | | | |
| Haplotype [a] | 289 | 308 | 310–311 | 313–315 | 827 | 78 | 90 | 103 | 109 | 126 | 132 | 162 | 167 | 243 |
| D | T | T | TT | TTT | T | A | C | C | C | A | A | T | T | C |
| C | G | A | AA | AAA | C | T | A | A | A | T | C | A | C | G |

[a] All accessions of *R. delavayi* examined had haplotype D; all accessions of *R. cyanocarpum* had haplotype C.

TABLE 3. Summary of molecular results for all accessions examined.

| Morphological | Accession code | Number of accessions [a] | AFLP results | cpDNA haplotype | ITS sequence type | Number of clones with ITS type of species | |
|---|---|---|---|---|---|---|---|
| | | | | | | *R. cyanocarpum* | *R. delavayi* |
| Outside hybrid zone | | | | | | | |
| *R. cyanocarpum* | RC1,4,8,9,20,23,26,29,31,32,33,34 | 12 | – | C | C | – | – |
| *R. cyanocarpum* | RC3,13,22,27,30 | 5 | *R. c.* | C | C | – | – |
| *R. delavayi* | RD1,2,3,6,7 | 5 | *R. d.* | D | D | – | |
| *R. delavayi* | RD4,5,8,9,10 | 5 | – | D | D | – | – |
| Within hybrid zone | | | | | | | |
| Putative hybrid | P1 | 1 | $F_2$ | D | C+D | 1 | 1 |
| Putative hybrid | P2 | 1 | $F_2$/Bc*D* | D | C+D | 3 | 2 |
| Putative hybrid | P3 | 1 | $F_2$ | D | C+D | 0 | 2 |
| Putative hybrid | P4 | 1 | $F_2$ | D | C+D | 1 | 2 |
| Putative hybrid | P5 | 1 | $F_2$ | D | C+D | 2 | 1 |
| Putative hybrid | P6 | 1 | $F_2$ | C | C+D | 1 | 0 |
| Putative hybrid | P7 | 1 | $F_1$ | D | C+D | 4 | 1 |
| Putative hybrid | P8 | 1 | $F_1$ | D | C+D | 0 | 7 |
| Putative hybrid | P9 | 1 | $F_2$ | D | C+D | 1 | 0 |
| Putative hybrid | P10 | 1 | $F_2$ | D | C+D | 1 | 1 |
| *R. cyanocarpum* | RC35-44 | 10 | – | – | C | – | – |
| *R. delavayi* | RD11,23,26,30 | 4 | – | – | C+D | – | – |
| *R. delavayi* | RD24,25 | 2 | – | – | – | – | – |
| *R. delavayi* | RD12-22,27-29,31 | 15 | – | – | D | – | – |

[a] See Table 5 for details. $F_2$/Bc*D* indicates an accession identified as $F_2$ by NewHybrids but which might have been an $F_2$ or a backcross to *R. delavayi* according to Structure.

***AFLP analysis***—We generated 90 polymorphic AFLP markers, of which 47 were present in all accessions of one parent species and absent from all accessions of the other, and a further 15 were present in one parent species only, though not in all accessions thereof.

Analysis of AFLP data using NewHybrids confirmed the identity of the *R. delavayi* and *R. cyanocarpum* accessions examined, when either setting was used. Using the default NewHybrids settings, eight hybrids were identified as $F_2$'s with posterior probabilities of 98% or more, whereas the other two (P7 and P8) were classified as $F_1$'s with 96–98% probability (Table 5). When the highly conservative 45-class setting was used, the probabilities dropped, to between 63 and 91% for the putative $F_2$'s and 86–87% for the putative $F_1$s. On this setting, for putative $F_2$'s the next most likely class was always backcross to *R. delavayi*, with 5–24% probability, whereas for putative $F_1$'s the only other class with >1% probability was $F_2$ (Table 5). It should be noted that accessions identified as $F_2$'s by this method could potentially be $F_3$'s or another complex class containing roughly equal proportions of parental germplasm; they are henceforth referred to as $F_2$s' for simplicity.

In the Structure analysis of AFLP data, the value of $\Delta K$ was 80.91 for $K = 2$, 13.32 and 18.38 for $K = 3$ and $K = 4$, respectively, and <2.21 for all values of $K$ higher than 4 (Appendix S1; see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.1000018/DC1). Therefore $K = 2$ best represents the data. Following 10 independent Structure runs with $K = 2$, individuals morphologically identified as *R. delavayi* were assigned to one cluster with high probability ($q = 0.987 \pm 0.022$), whereas those morphologically identified as *R. cyanocarpum* were assigned to the other cluster with similarly high probability ($q = 0.997 \pm 0.001$). Therefore these clusters were determined to respresent *R. delavayi* and *R. cyanocarpum*, respectively. Among individuals morphologically identified as hybrids, the lowest estimated proportion of *R. cyanocarpum* germplasm was (0.235–)0.329 (–0.458) in accession P2, whereas the highest was in (0.494–) 0.575(–0.655) accession P4. A backcross to *R. delavayi* would have ~25% *R. cyanocarpum* germplasm, and accession P2 was the only one whose credible interval overlapped this value (Table 5). A backcross to *R. cyanocarpum* would have ~75% *R. cyanocarpum* germplasm, but no accession's credible interval overapped this value, and the upper limit for no accession was higher than 0.678. The expected proportion of *R. cyanocarpum* germplasm in $F_2$'s would be (with some variation) 50%, a value that fell within the credible intervals for all hybrid accessions except P2, P7, and P8. However, accessions P7 and P8 were identified as $F_1$'s by Newhybrids, and if this is the case, then the proportions of *R. cyanocarpum* germplasm estimated by Structure for these, and possibly all, hybrid accessions might be a slight underestimate.

TABLE 4. ITS sequence types present in material of *R. delavayi*, *R. cyanocarpum*, and putative hybrids, and the codon positions at which they differ.

| Species | ITS type | Codon position | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 255 | 266 | 557 | 560 | 650 | 663 | 670 |
| *R. delavayi* | DD | A | G | A | C | A | G | G |
| *R. cyanocarpum* | CC | G | A | G | A | C | C | A |
| Putative hybrids [a] | C+D | R(A/G) | R(A/G) | R(A/G) | M(A/C) | M(A/C) | S(G/C) | R(A/G) |

[a] This additive pattern was seen for all 10 putative hybrids and for four accessions with *R. delavayi*-like morphology from the hybrid zone.

TABLE 5. Assignments to genotype classes made by the programs NewHybrids and Structure based on AFLP data.

| Accession | Most likely class (NewHybrids, both methods) | Probability of most likely class (NewHybrids, 6-class method) | Probability of most likely class (NewHybrids, 45-class method) | Second most likely class and its probability (45-class method) [a] | Proportion of *R. cyanocarpum* germplasm (Structure) [b] |
|---|---|---|---|---|---|
| RD1 | *R. delavayi* | 1 | 1 | None | (0–) 0.056 (–0.110) |
| RD2 | *R. delavayi* | 1 | 1 | None | (0–) 0.003 (–0.013) |
| RD3 | *R. delavayi* | 1 | 1 | None | (0–) 0.002 (–0.012) |
| RD6 | *R. delavayi* | 1 | 1 | None | (0–) 0.002 (–0.011) |
| RD7 | *R. delavayi* | 1 | 1 | None | (0–) 0.002 (–0.012) |
| RC3 | *R. cyancarpum* | 1 | 1 | None | (0.988–) 0.998 (–1) |
| RC13 | *R. cyancarpum* | 1 | 1 | None | (0.988–) 0.998 (–1) |
| RC22 | *R. cyancarpum* | 1 | 1 | None | (0.985–) 0.997 (–1) |
| RC27 | *R. cyancarpum* | 1 | 1 | None | (0.979–) 0.996 (–1) |
| RC30 | *R. cyancarpum* | 1 | 1 | None | (0.984–) 0.997 (–1) |
| P1 | $F_2$ | 0.99 | 0.88 | BC-*delayavi* (0.11) | (0.444–) 0.526 (–0.607) |
| P2 | $F_2$ | 1 | 0.90 | BC-*delayavi* (0.05) | (0.235–) 0.329 (–0.458) |
| P3 | $F_2$ | 0.99 | 0.78 | BC-*delayavi* (0.22) | (0.492–) 0.573 (–0.654) |
| P4 | $F_2$ | 1 | 0.77 | BC-*delayavi* (0.23) | (0.494–) 0.575 (–0.655) |
| P5 | $F_2$ | 1 | 0.78 | BC-*delayavi* (0.22) | (0.485–) 0.567 (–0.647) |
| P6 | $F_2$ | 1 | 0.63 | BC-*delayavi* (0.24) | (0.440–) 0.546 (–0.678) |
| P7 | $F_1$ | 0.98 | 0.87 | $F_2$ (0.12) | (0.309–) 0.386 (–0.464) |
| P8 | $F_1$ | 0.96 | 0.86 | $F_2$ (0.13) | (0.331–) 0.410 (–0.492) |
| P9 | $F_2$ | 1 | 0.90 | BC-*delayavi* (0.09) | (0.410–) 0.491 (–0.573) |
| P10 | $F_2$ | 1 | 0.91 | BC-*delayavi* (0.08) | (0.335–) 0.416 (–0.501) |

[a] The second most likely class is given only when the posterior probability for it is at least 5%.

[b] Numbers in parentheses indicate 90% confidence intervals generated using the ANCESTDIST function in Structure. All data in this column are means across 10 runs.

## DISCUSSION

Hybrids between the narrow endemic species *R. cyanocarpum* and the much more widespread species *R. delavayi* were found at Huadianba, near Dali, Yunnan, but at no other site. There, 10 accessions were found to be hybrids by the cpDNA, ITS, and AFLP data. Remarkably, at least seven of the 10 hybrids appeared to be of the $F_2$ class. These are the only natural hybrids involving *R. cyanocarpum* known to science; despite our searches, we did not find hybrids involving *R. cyanocarpum* at any other site within its limited range. Given the well-known weakness of species barriers within *Rhododendron* subgenus *Hymenanthes* (Chamberlain, 1982; Milne et al., 1999, 2003; Zha et al., 2008, 2010), this rarity of natural hybrids is remarkable. It contrasts with the situations involving other hybrids of *R. delavayi* (Zhang et al., 2007; Zha et al., 2008, 2010), and other *Hymenanthes* species in NE Turkey (Milne et al., 1999, 2003), in which hybrids are produced commonly and often in quantity. This rarity of *R. cyanocarpum* hybrids might reflect a relative lack of disturbance to its natural habitats and/or more effective prezygotic isolating mechanisms than exist in many other *Hymenanthes* species.

With this in mind, 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. The most significant period of disturbance to the locality containing the hybrid zone was during 1957–1958, when many trees were felled to aid factory construction (Y. L. Yang, Huadianba Medical Factory, personal communication). From molecular data, we know that hybrids beyond the first generation are present, so if this disturbance did initiate hybridization and the minimum generation time of 12 yr for *R. ponticum* applies here (Cross, 1975), then we may be witnessing the early stages of hybrid zone formation, because no more than four generations could have been completed since 1957 and 1958. If so, more hybrids involving *R. cyanocarpum* would be expected in the future, and hybridization, not presently a threat, might become so. However, it is also possible that this hybrid zone is long-lived and stable.

***Population structure in the* R. delavayi × R. cyanocarpum *hybrid zone***—Among the 10 hybrids examined, two appeared to be $F_1$'s, whereas eight were most likely of the $F_2$ class. If we assume that all hybrids are first or second generation, then these determinations become more certain (>95% posterior probability in each case), and no accession has >1% probability of being a backcross. Without this assumption, the probabilities drop to ~80–90% in most cases, with four accessions having 20–25% chance of being backcrosses to *R. delavayi*, four a 0.05–0.11 probability, and the others <1%.

The Structure analysis, using 90% credible intervals, also rejected the possibility of being backcrosses for all but one of these accessions. The exception, accession P2, had a (0.235–) 0.329(–0.458) proportion of *R. cyanocarpum* germplasm, which appears to favor the hypothesis that it is a backcross to *R. delavayi* over its being an $F_2$. However, if accessions P7 and P8 are indeed $F_1$'s then their credible intervals of 0.309–0.464 and 0.331–0.492, respectively, indicate that the proportion of *R. cyanocarpum* germplasm might be underestimated by Structure in some or all accessions. Based on this, the hypotheses of either an $F_2$ or a backcross to *R. delavayi* for accession P2 cannot be separated based on the Structure results. However, even allowing for this underestimation, none of the hybrid accessions had a high enough proportion of *R. cyanocarpum* germplasm to be backcrosses to this species (Table 5). Based on these analyses together, the 10 hybrids appeared to comprise two $F_1$'s, seven $F_2$'s, and one accession that was either $F_2$ or a backcross to *R. delavayi*.

Backcrosses to *R. delavayi* were certainly present, however, among the individuals of *R. delavayi*-like morphology within the hybrid zone. Of 21 such individuals, four were backcrosses based on ITS data; however, there was no evidence of

backcrossing toward *R. cyanocarpum*. These backcrosses all occurred close together, indicating that they might share a common maternal parent (Fig. 2). Furthermore, among 10 individuals of hybrid morphology, nine had *R. delavayi* as their maternal parent. Hence, the directions of both crossing and backcrossing are strongly biased toward *R. delavayi*. Because *R. delavayi* flowers earlier than hybrids or *R. cyanocarpum*, it would be the more likely maternal parent because *Rhododendrons* are protandrous; however, for the same reason, hybrid stigmas are more likely to receive pollen from the later flowering parent (see Milne and Abbott, 2008). Possibly, therefore, some other mechanism restricts backcross formation toward *R. cyanocarpum*, and perhaps hybridization in this species overall; one such mechanism might be selection toward reinforcement among rare species (Zhou et al., 2008).

The detection of a hybrid zone in which $F_2$'s outnumber both $F_1$'s and backcrosses combined is extremely unusual. The most common pattern for hybrids to be fertile is for most hybrid derivatives to be backcrosses (Barton and Hewitt, 1985; Cruzan and Arnold, 1993; Arnold, 1997; Rieseberg and Carney, 1998; Chung et al., 2005; Lexer et al., 2005; Van Droogenbroeck et al., 2006; Minder et al., 2007). Another pattern, in which $F_1$'s can be the most numerous class, despite their own fertility and the viability of other classes, is fairly common in *Rhododendron* (Milne et al., 2003; Milne and Abbott, 2008; Zha et al., 2010), but very rare in other genera (e.g., Kyhos et al., 1981; Kameyama et al., 2008). In certain cases, hybrid zones may contain many $F_1$'s and backcrosses but very few $F_2$'s (Milne and Abbott, 2008; Zha et al., 2008).

The pattern seen in the current study, in which $F_2$'s are the most abundant class, has to the our knowledge only been detected for stickleback fish (Gow et al., 2006). Among plants, one example exists in which $F_2$'s occur in similar numbers to backcrosses (Smulders et al., 2008), but *R. cyanocarpum* × *R. delavayi* is the only plant example that we know in which $F_2$'s certainly outnumber all other classes in a hybrid zone. Furthermore, both of the aforementioned studies assigned hybrid class using only the less conservative default settings of NewHybrids (Gow et al., 2006; Smulders et al., 2008) and did not expressedly test the hypothesis that the $F_2$'s not only occurred, but they also outnumbered all other classes, meaning the methods in the present study are more stringent.

The simplest explanation for the unusual precedence of $F_2$'s is that much seed produced by $F_1$'s is set by geitonogamy, which is common in *Rhododendron* (Stout, 2007) including *R. cyanocarpum* (Ma et al., personal observation). This would only lead to $F_2$'s being recruited in any quantity if some $F_2$'s had high fitness, because selective pressures among *Rhododendron* seedlings is likely to be extreme (Milne et al., 2003), and numerous seeds of the parents and possibly other hybrid classes would also be falling within the hybrid zone. Therefore, this putatively high fitness of the $F_2$'s might prove to be a special case of the mosaic (Harrison and Rand, 1989) and/or bounded hybrid superiority (Moore, 1977; Arnold, 1997) models of hybrid zone dynamics, in both of which hybrids can have superior fitness in certain conditions. More research is required to determine whether this highly unusual hybrid zone structure is a contributing factor to the absence of backcrossing toward *R. cyanocarpum* and hence to maintaining the genetic purity of this narrow endemic species.

When a naturally rare species is sympatric and interfertile with a more common one, barriers to crossing in the rare species tend to be stronger, possibly from stronger selection for this in the rarer member of a hybridizing pair of species (Zhou et al., 2008). Our study fits this pattern. Conversely, most known instances where a species faces a threat from genetic swamping involve human interference of some kind (Levin et al., 1996; Rhymer and Simberloff, 1996; Vilà et al., 2000). Even if the detected *R. cyanocarpum* hybrid zone results from human ldisturbance, it currently poses no threat of genetic assimilation. Such a threat is only likely to arise if habitat disturbance increases substantially, for example, as a result of climate change.

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APPENDIX 1. Voucher information for accessions used in this study. The voucher specimens were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

| Accession | Taxon | Voucher number | Latitude, longitude | Collection locality |
|---|---|---|---|---|
| RD1 | *R. delavayi* | MYP-2009-5-12-1 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD2 | *R. delavayi* | MYP-2009-5-12-2 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RD3 | *R. delavayi* | MYP-2009-5-12-3 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RD4 | *R. delavayi* | MYP-2009-5-12-4 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RD5 | *R. delavayi* | MYP-2009-5-12-5 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RD6 | *R. delavayi* | MYP-2009-5-12-6 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD7 | *R. delavayi* | MYP-2009-5-12-7 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD8 | *R. delavayi* | MYP-2009-5-12-8 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD9 | *R. delavayi* | MYP-2009-5-12-9 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD10 | *R. delavayi* | MYP-2009-5-12-10 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD11 | *R. delavayi* | MYP-2009-5-12-11 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD12 | *R. delavayi* | MYP-2009-5-12-12 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD13 | *R. delavayi* | MYP-2009-5-12-13 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD14 | *R. delavayi* | MYP-2009-5-12-14 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD15 | *R. delavayi* | MYP-2009-5-12-15 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD16 | *R. delavayi* | MYP-2009-5-12-16 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD17 | *R. delavayi* | MYP-2009-5-12-17 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD18 | *R. delavayi* | MYP-2009-5-12-18 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD19 | *R. delavayi* | MYP-2009-5-12-19 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD20 | *R. delavayi* | MYP-2009-5-12-20 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD21 | *R. delavayi* | MYP-2009-5-12-21 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD22 | *R. delavayi* | MYP-2009-5-12-22 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD23 | *R. delavayi* | MYP-2009-5-12-23 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD24 | *R. delavayi* | MYP-2009-5-12-24 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD25 | *R. delavayi* | MYP-2009-5-12-25 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD26 | *R. delavayi* | MYP-2009-5-12-26 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD27 | *R. delavayi* | MYP-2009-5-12-27 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD28 | *R. delavayi* | MYP-2009-5-12-28 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD29 | *R. delavayi* | MYP-2009-5-12-29 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RD30 | *R. delavayi* | MYP-2009-5-12-30 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC1 | *R. cyancarpum* | MYP, 2009-5-13-1 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC3 | *R. cyancarpum* | MYP, 2009-5-13-3 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC4 | *R. cyancarpum* | MYP, 2009-5-13-4 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC8 | *R. cyancarpum* | MYP, 2009-5-13-8 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC9 | *R. cyancarpum* | MYP, 2009-5-13-9 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC13 | *R. cyancarpum* | MYP, 2009-5-13-13 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC20 | *R. cyancarpum* | MYP, 2009-5-13-20 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RC22 | *R. cyancarpum* | MYP, 2009-5-13-23 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC23 | *R. cyancarpum* | MYP, 2009-5-13-22 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC26 | *R. cyancarpum* | MYP, 2009-5-13-26 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC27 | *R. cyancarpum* | MYP, 2009-5-13-27 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC29 | *R. cyancarpum* | MYP, 2009-5-13-29 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC30 | *R. cyancarpum* | MYP, 2009-5-13-30 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC31 | *R. cyancarpum* | MYP, 2009-5-13-31 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC32 | *R. cyancarpum* | MYP, 2009-5-13-32 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC33 | *R. cyancarpum* | MYP, 2009-5-13-33 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC34 | *R. cyancarpum* | MYP, 2009-5-13-34 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC35 | *R. cyancarpum* | MYP, 2009-5-13-35 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC36 | *R. cyancarpum* | MYP, 2009-5-13-36 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC37 | *R. cyancarpum* | MYP, 2009-5-13-37 | 25°52'N, 99°59'E' | Huadianba, Dali, Yunnan, China |
| RC38 | *R. cyancarpum* | MYP, 2009-5-13-38 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC39 | *R. cyancarpum* | MYP, 2009-5-13-39 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC40 | *R. cyancarpum* | MYP, 2009-5-13-40 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC41 | *R. cyancarpum* | MYP, 2009-5-13-41 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC42 | *R. cyancarpum* | MYP, 2009-5-13-42 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC43 | *R. cyancarpum* | MYP, 2009-5-13-43 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| RC44 | *R. cyancarpum* | MYP, 2009-5-13-44 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P1 | Putative hybrid | MYP, 2009-5-14-1 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P2 | Putative hybrid | MYP, 2009-5-14-2 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P3 | Putative hybrid | MYP, 2009-5-14-3 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P4 | Putative hybrid | MYP, 2009-5-14-4 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P5 | Putative hybrid | MYP, 2009-5-14-5 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P6 | Putative hybrid | MYP, 2009-5-14-6 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P7 | Putative hybrid | MYP, 2009-5-14-7 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P8 | Putative hybrid | MYP, 2009-5-14-8 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P9 | Putative hybrid | MYP, 2009-5-14-9 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |
| P10 | Putative hybrid | MYP, 2009-5-14-10 | 25°52'N, 99°59'E | Huadianba, Dali, Yunnan, China |