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Morphological and molecular evidence of natural hybridization between two distantly related *Rhododendron* species from the Sino-Himalaya

HONG-GUANG ZHA[1], RICHARD IAN MILNE[2] and HANG SUN[1]*

[1]*Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China*
[2]*Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JH, UK*

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Rhododendron (Ericaceae) is a large woody genus in which hybridization may play an important role in evolution and speciation, particularly in the Sino-Himalayan region, where many interfertile species often occur sympatrically. Natural hybridization between *Rhododendron delavayi* Franch. (= *R. arboreum* ssp. *delavayi*) and *Rhododendron decorum* Franch., which belong to different subsections of subgenus *Hymenanthes,* was investigated. Material of *R. delavayi* and *R. decorum* and their putative hybrids was collected from the wild. On the basis of morphology, chloroplast DNA, nuclear ribosomal DNA, and AFLP profiles, hybrids and parental species were identified. Hybridization occurred in both directions, but was asymmetrical, with *R. delavayi* as the major maternal parent in the hybrid zone. Most of the hybrids possessed intermediate phenotypes, and amongst the 15 hybrids detected were six F1s, two F2s, one first-generation backcross to *R. delavayi*, and two first-generation backcrosses to *R. decorum*. This indicates that, if *Rhododendron* underwent rapid radiation in this region, it did so in spite of permeable species barriers. *Botanical Journal of the Linnean Society*, 2008, **156**, 119–129.

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INTRODUCTION

Hybridization is a process that occurs in many groups of organisms, but appears to be particularly prevalent in plants (Anderson, 1949; Stebbins, 1959; Arnold, 1992; Rieseberg & Wendel, 1993; Ellstrand, Whitkus & Rieseberg, 1996). There is no question that natural hybridization occurs widely in vascular plants and plays an important role in their evolution (Arnold, 1997; Rieseberg & Carney, 1998). Recent estimates indicate that at least 25% of plant species, mostly the youngest species, are involved in hybridization and potential introgression with other species (Mallet, 2005). However, the frequency of hybridization varies dramatically between families (Ellstrand *et al*., 1996), and may therefore contribute variably to the evolutionary process amongst taxa.

Rhododendron (Ericaceae) is an example of a large woody genus in which hybridization may play an important role in evolution and speciation. The very large numbers of horticultural hybrids in existence (over 1000; Bean, 1976) testify to the weakness of genetic barriers towards hybridization in this genus, and the few studies of natural hybridization in *Rhododendron* confirm this pattern (Kron, Gawen & Chase, 1993; Milne *et al*., 1999; Kobayashi *et al*., 2002; Milne, Terzioglu & Abbott, 2003). Where several *Rhododendron* species occur in sympatry, these have provided an opportunity to investigate the extent of natural hybridization within the genus. For example, in north-east Turkey and the adjacent Caucasus, four species of subsection *Pontica*, subgenus *Hymenanthes* occur in sympatry, and molecular markers have

*Corresponding author. E-mail: hsun@mail.kib.ac.cn

shown that at least five of the six possible hybrid combinations between them occur (Milne *et al*., 1999). Moreover, one hybrid (*R. ponticum* × *R. caucasicum*), which occurs in quantity wherever the parents are sympatric (Chamberlain, 1982), was found to comprise mostly or all fertile F1s at one site, indicating that species barriers might be maintained by the novel and unexpected mechanism of habitat-mediated selection against the second hybrid generation (Milne *et al*., 2003). Other studies have examined *Rhododendron* hybrid zones in North America (Kron *et al*., 1993) and Japan (Kobayashi *et al*., 2002). However, there has not yet been a study on natural hybridization from the centre of distribution for *Rhododendron*, which is the Sino-Himalaya and south-west China.

Rhododendron subgenus *Hymenanthes* contains about 225 species, all but a handful of which occur in the eastern Himalayan region. These species often occur in close sympatry; for example, 67 species occur in a small area of approximately 100 × 150 km in the eastern Himalaya (Chamberlain, 1982). Only 14 natural hybrids have been identified on the basis of morphology in the field within subgenus *Hymenanthes* (Chamberlain, 1982); however, the true extent of hybridization is almost certainly much greater in parts of the Himalaya where species boundaries appear to be incomplete. Actively speciating species complexes occur within this area (Argent *et al*., 1998) and, in many cases, clear morphological boundaries amongst species have not been determined. Molecular data also indicate that most species of subgenus *Hymenanthes* were derived relatively recently, through rapid radiation (Milne, 2004). A knowledge of how sympatric Himalayan *Rhododendron* species interact in the wild is vital in order to understand how a genus can radiate into large numbers of species in spite of an apparent lack of genetic barriers to interbreeding between them. Therefore, molecular examinations of *Rhododendron* hybrid zones in this region are urgently required.

This study examines the natural hybridization between *Rhododendron delavayi* Franch. and *Rhododendron decorum* Franch., which belong to different subsections (*Arborea* and *Fortunea*, respectively) within section *Ponticum*, the only section in subgenus *Hymenanthes* of *Rhododendron* (Sleumer, 1980; Chamberlain, 1982). These species are both morphologically distinctive. *Rhododendron delavayi*, sometimes referred to as *R. arboreum* ssp. *delavayi* (Chamberlain, 1982), is a remarkably widespread species, with five subspecies extending from north-west India to Thailand and GuiZhou in south-west China. By contrast, *R. decorum* occurs only in south-west China and north-east Burma. A putative hybrid zone of *R. delavayi* and *R. decorum* was detected using morphological characteristics in a preliminary survey in north-west Yunnan, Hengduan Mountains (south-west China). At this site, *R. delavayi* and *R. decorum* were the two dominant *Rhododendron* species, and many individuals with an intermediate phenotype between *R. delavayi* and *R. decorum* were observed (H. Sun, pers. observ.).

The study of natural hybridization has been facilitated and advanced by the development of molecular markers. The AFLP method was developed with the aim of combining the advantages of restriction fragment length polymorphism (RFLP) and arbitrary primer methods (Vos *et al*., 1995). On the basis of the selective amplification of genomic restriction fragments, it can be highly informative and reproducible, and is suitable for the assessment of genetic differences from the individual up to species level (Rieseberg, 1998; Mueller & Wolfenbarger, 1999; Sunnucks, 2000), and also for the identification of the origin of hybrids and natural hybridization studies in many species (Han *et al*., 2000; Emelianov, Marec & Mallet, 2004; Wu & Campbell, 2005). The ability to produce a large number of highly reproducible markers from any species using small quantities of DNA, without lethal sampling, and without previous sequence knowledge (Mueller & Wolfenbarger, 1999), is the major advantage of AFLP markers. The internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) has been proven to be an excellent source of informative within-genus sequence variation for a range of plant species (Baldwin *et al*., 1995), and has also been widely employed in studies of hybrid speciation and natural hybridization (Appels & Dvorak, 1982; Rieseberg *et al*., 1996; King *et al*., 2001; Baumel *et al*., 2002; Denda & Yokota, 2003; Garcia-Maroto *et al*., 2003; Hegarty & Hiscock, 2005). In comparison with AFLP data, sequences from the ITS region not only allow the identification of parents, but may also provide the age of the hybrid taxa (Widmer & Baltisberger, 1999). Maternal inheritance of the chloroplast genome predominates in angiosperms (Harris & Ingram, 1991; Olmstead & Palmer, 1994), making chloroplast DNA (cpDNA) sequences an ideal marker for the identification of maternal species in studies of natural hybridization.

In this study, morphological characteristics were used to identify putatively pure material of the two parental species, and putative hybrids, in the field. Molecular evidence of hybridization between *R. delavayi* and *R. decorum* was then sought by examining these plants using a combination of nuclear (ITS and AFLP) and cytoplasmic (cpDNA) genetic markers.

MATERIAL AND METHODS

Sampling and site description

The study site was a hybrid zone in the SiBaoShan Nature Reserve, 77 km north of Dali (Tali) city in

Table 1. Morphological characteristics of *Rhododendron delavayi*, *R. decorum*, and the putative hybrids between these species

	Flower colour	Leaf shape	Ventral leaf surface	Young shoot surface
R. delavayi	Carmine	Long-lanceolate	Woolly	Tomentose
Putative hybrid	Pink	Intermediate	Very thin indumentum	Tomentose
R. decorum	White to pale pink	Oblong-elliptical	Glabrous	Glabrous

Yunnan, China, 26°21′N, 99°50′E, 2430 m above sea level. According to the zones of *Rhododendron* distribution in South-East Asia defined by Chamberlain (1982), the site was in zone C of area 14, and falls within the core area of occurrence of subgenus *Hymenanthes* in the region. The site was the top of a small mountain with oak/pine woodland. Apart from *R. delavayi*, *R. decorum*, and their putative hybrids, the only other *Rhododendron* species present was the distantly related *R. racemosum* (subgenus *Rhododendron*). Hence, from the species present and their morphology, it was considered to be highly unlikely that this or a species other than *R. delavayi* or *R. decorum* could be involved in the parentage of the hybrids present.

Fifty *Rhododendron* accessions were selected at random for examination, and were divided into three morphological groups: *R. delavayi*-like (18 individuals), *R. decorum*-like (18 individuals), and putative hybrids (14 individuals). Four morphological characters were used to distinguish between these groups: corolla colour, leaf shape, ventral leaf surface indumentum, and young shoot indumentum (Table 1). Accessions in the same group were always at least 10 m apart to minimize the possibility of sampling the same genet twice. From each accession, desiccated leaf material (~1 g of fresh leaf mass to ~25 g of coarse silica gel) for DNA extraction was collected, and voucher specimens were deposited at the Herbarium at Kunming Institute of Botany. All collections were made in May 2005.

DNA extraction and AFLP analysis

DNA was extracted from silica gel-dried leaf using a modified cetyltrimethylammonium bromide (CTAB) method (Kobayashi *et al*., 1998). DNA quality and concentration were assessed by agarose gel electrophoresis with known concentrations of uncut lambda DNA (Takara). A dilution test was carried out to determine the optimal amount of DNA for amplification.

AFLP analysis was performed essentially as described by Vos *et al*. (1995), with modifications by Gilbert *et al*. (2002), except that the *Eco*RI primers were not radioactively labelled and, instead, silver staining was used to visualize the AFLP bands. Primers and adapters were synthesized by Sangon Company, China. Enzymes were obtained from Amersham Pharmacia Biotech, unless otherwise stated. Genomic DNA (50 ng) was digested using both *Eco*RI and *Mse*I enzymes (5 U each in a final volume of 30 μL), and adapters (0.1 μM E-adapter and 1.0 μM M-adapter) were ligated to the resulting fragments. Then, 5 μL of digested DNA from a 1 : 10 dilution with sterile distilled water was used for polymerase chain reaction (PCR) pre-amplification employing primers (0.5 μM *Eco*RI and 0.5 μM *Mse*I primers), complementary to the E- and M-adapters, carrying one selective nucleotide at the 3′-end. In total, 30 cycles were performed at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA), after an initial cycle of 65 °C for 5 min. The pre-amplification products were diluted 1 : 10 with sterile distilled water and used as template for selective amplification, employing 0.6 μM *Eco*RI and 0.1 μM *Mse*I primers, with three selective nucleotides at the 3′-end, with the following thermal cycling conditions: 94 °C for 2 min; one cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; 12 cycles which were identical except that the annealing temperature was reduced each cycle by 0.7 °C; 23 additional cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s; final stage of 72 °C for 5 min. In total, six primer combinations were used: E-ACT/M-AAG, E-AGA/M-CCA, E-AGC/M-ACC, E-AAC/M-CTG, E-AGA/M-CGT, and E-ACT/M-ACA. The amplified products were mixed with an equal volume of AFLP loading buffer (98% formamide, 10 mM ethylenediaminetetraacetic acid, 0.01% xylene cyanol, and 0.01% bromophenol blue), and 5 μL of each sample was electrophoresed on a 6% denaturing polyacrylamide gel in 1 × TBE buffer (Tris-Borate-EDTA) at 65 W for approximately 2 h. AFLP bands were visualized by silver staining of the gel, as described in Bassam, Caetanoanlles & Gresshoff (1991).

Analysis of AFLP data

AFLP bands were scored manually as zero for the absence and one for the presence of a band. Co-migrating bands within a gel between different

individuals were considered to be homologous. Only the polymorphic bands were used in subsequent analyses, as the inclusion of monomorphic bands made no difference to the overall relationship between individuals.

Based on the same set of polymorphic AFLP markers, two different methods were used for analysis. Firstly, principal co-ordinate analysis (PCO) using GenAlEx 6.0 (Peakall & Smouse, 2006). As described in Young *et al*. (2001), using the polymorphic AFLP markers, this analysis determined the genetic relationship of the two species, and allowed us to distinguish hybrid from non-hybrid samples. In this application, individuals intermediate and well separated from the distinct *R. delavayi* and *R. decorum* were assumed to be hybrids. Second, for each individual, we estimated the posterior probability that it belonged to *R. delavayi*, to *R. decorum*, or to early generation hybrid classes (F1, F2, or backcross) using a Bayesian method to analyse the polymorphic AFLP markers. This procedure uses a Markov Chain Monte Carlo method and is implemented in the program 'NewHybrids' (Anderson & Thompson, 2002; http://ib.berkeley.edu/labs/slatkin/eriq/index.htm). Posterior distributions were evaluated after 10^5 iterations of the Monte Carlo Markov Chains, after a burn-in of 10^5 iterations, without using any prior information of individual or allele frequency. Individuals were assigned to one of the six genotypic classes if $P \geq 0.95$. Marginal probabilities of hybrid classes beyond the second generation become increasingly difficult to calculate, and so the 'NewHybrids' program does not normally attempt to identify them (Anderson & Thompson, 2002). Therefore, individuals that could not be assigned to one of the six parent/early generation hybrid genotypic classes with $P \geq 0.95$ were not identified as having a specific genotype, but might be later generation hybrid derivatives.

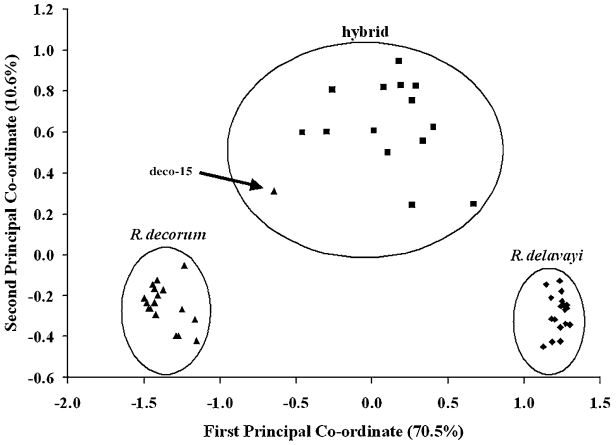

Figure 1. Plot of the first two principal co-ordinate scores calculated using the genotype frequencies of 83 polymorphic AFLP loci in *Rhododendron delavayi* (diamonds), *R. decorum* (triangles), and putative hybrids (squares). Large circles represent the area bounded by all individuals within a group. The groupings match exactly the three morphological categories assigned, except for accession deco-15.

Determination of rDNA genotypes and chloroplast haplotypes

The ITS region and intervening 5.8S coding region (approximately 700 bp) of all the sampled individuals were amplified using primers ITS1 and ITS4 (White *et al*., 1990). To determine the direction of hybrid mating, the chloroplast *trn*L gene and *trn*L-*trn*F intergenic spacer (about 900 bp) were amplified using the 'C' and 'F' primers (Taberlet *et al*., 1991). Both reactions were carried out in a final volume of 50 μL containing 20 ng of template DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP, 400 pmol of each primer, and 1 U of Ex-taq (Takara). Both amplifications were performed using a PTC-100 thermocycler (MJ Research) with the following conditions: 4 min at 94 °C (one cycle); 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C (33 cycles); and 10 min at 72 °C (one cycle).

PCR products were purified using an agarose gel DNA purification kit (Takara), following the manufacturer's instructions. Sequencing was performed with BigDye Terminator 1.1 (Applied Biosystems) on an ABI PRISM 3730 Sequencer using the same primers as used for the PCR amplifications. The alignment was performed using the program ClustalX version 1.83 (Thompson *et al*., 1997). A chi-squared test was conducted to determine whether the ratio of *R. delavayi* to *R. decorum* cpDNA *trn*L-*trn*F sequences amongst the hybrids differed significantly from 1 : 1.

RESULTS

AFLP markers and hybrid identification

AFLP analysis of all the *R. delavayi* and *R. decorum* samples and putative hybrids generated 83 polymorphic markers with the six primer combinations. Additional polymorphic markers were present, but could not be scored, either because of faint, inconsistent amplification or the inability to differentiate two or more fragments of a similar molecular mass.

PCO using 83 polymorphic markers revealed distinct *R. delavayi* and *R. decorum* clusters and the presence of intermediate individuals (Fig. 1). The first two principal co-ordinates, which accounted for 81.15% of the variance (70.53% and 10.61% for the first and second axes, respectively), clearly separated

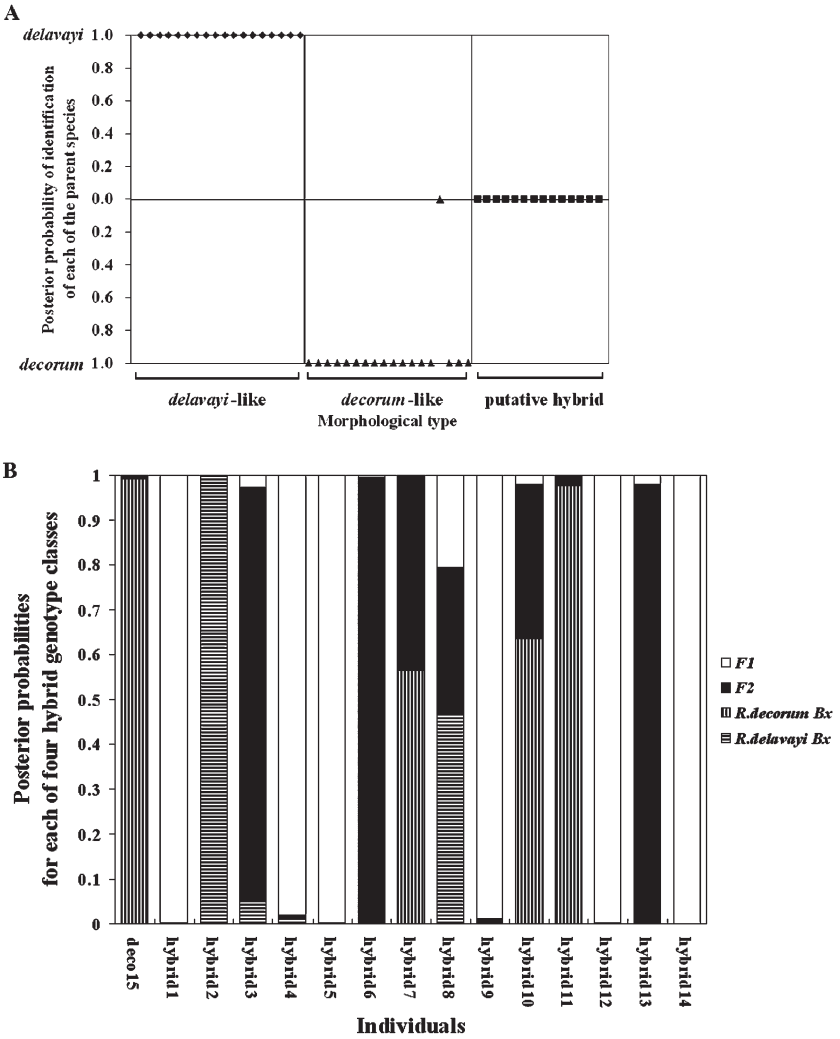

Figure 2. Assignment of genotype class based on Bayesian analysis using the 'NewHybrids' program. A, Posterior probabilities that each accession examined belongs to each of the parent species, i.e. *Rhododendron delavayi* (top) or *R. decorum* (bottom). Accessions are arranged by morphological type, i.e. *R. delavayi*-like (diamonds), *R. decorum*-like (triangles) and putative hybrid (squares). B, Posterior probabilities that those accessions not assigned with >99.9% probability to one of the parent species (i.e. putative hybrids plus accession deco-15) belong to four classes of hybrid derivative, i.e. F1, F2, backcross to *R. delavayi* (*R. delavayi* Bx), and backcross to *R. decorum* (*R. decorum* Bx).

the 50 accessions into three clusters. The clusters matched the morphological groups (*R. delavayi*-like, *R. decorum*-like, and putative hybrids), with one exception: the accession deco-15 which, from morphology, was classed as *R. decorum*-like, clustered with the putative hybrids (Fig. 1). Therefore, deco-15 was transferred to the putative hybrid group, which now contained 15 individuals, whilst the *R. delavayi*-like and *R. decorum*-like (excluding deco-15) groups now contained 18 and 17 plants, respectively.

With the program 'NewHybrids', using the same data set (83 polymorphic AFLP markers), each individual was assigned a posterior probability of belonging to each of the six different genotype classes. Two of these classes were the two pure species *R. delavayi* and *R. decorum*. The 18 probable *R. delavayi* and 17 probable *R. decorum* samples, identified in the field by morphological characteristics, had a posterior probability of more than 0.999 of being either pure *R. delavayi* or pure *R. decorum*. The misidentified individual, deco-15, and all 14 putative hybrids, had a posterior probability of less than 0.001 of being either pure *R. delavayi* or pure *R. decorum* (Fig. 2A). This result is consistent with the PCO analysis. Therefore, deco-15 and all the putative hybrids are confirmed as being hybrids, and the *R. delavayi*-like and *R. decorum*-like groups are classified as pure *R. delavayi* and pure *R. decorum*, respectively.

Table 2. Nucleotide positions in the aligned internal transcribed spacer (ITS) sequences that differ between *Rhododendron delavayi*, *R. decorum*, and their putative hybrids

	Position in the ITS alignment					
Taxon	103	110	123	212	503	514
R. delavayi	Y	C	T	G	C	T
R. decorum	T	G	G	T	T	C
All putative hybrids	Y	S	K	K	Y	Y

Numbers refer to the nucleotide position in the complete alignment. All other positions are identical between the two species and their hybrids. IUPAC ambiguity symbols are used to present polymorphisms (Y = C + T, S = C + G, K = T + G).

Figure 2B shows the posterior probability that deco-15 and all the putative hybrids belong to each of the four different hybrid genotype frequency classes. Six F1s (hybrids 1, 4, 5, 9, 12, and 14), two F2s (hybrids 6 and 13), one *R. delavayi* backcross (hybrid 2) and two *R. decorum* backcrosses (deco-15 and hybrid 11) were determined with a posterior probability of more than 0.95 for each genotype frequency class. No assignment of the remaining individuals (hybrids 3, 7, 8, and 10) could be made with certainty to a single genotype frequency class because of low posterior probability. However, the data confirmed, with more than 98% probability, that accession deco-15 was not pure *R. decorum* (as indicated by morphology), but a backcross to this species.

rDNA GENOTYPES

The complete ITS with the 5.8S region was amplified using the ITS1/ITS4 primer combination, resolved on agarose as a single sharp band, and sequenced directly from all the sampled individuals. The aligned sequence matrix generated a total of 640 characters with no indels or gaps. This comprised the ITS1 (252 bp), 5.8S (164 bp), and ITS2 (224 bp) regions. The 17 individuals in the *R. decorum* group all had identical ITS sequences (GenBank accession DQ295782); this sequence differed by six characters from that of *R. delavayi* (GenBank accession DQ295783), which, likewise, was identical between all 18 accessions examined. Of the six positions that distinguished the two species, four were in ITS1 (103, 110, 123, 212) and two were in ITS2 (503, 514) (Table 2). Position 103 was polymorphic (C/T) (double peaks on the electropherogram) in all the investigated *R. delavayi* individuals, whereas it was always T in *R. decorum* (Table 2). This within-individual polymorphism indicates that *R. delavayi* contains two separate ITS copies that differ by one substitution, which could be a consequence of ancient hybridization between *R. delavayi* and other species.

All of the putative hybrids had double peaks at all six of the polymorphic positions (Table 2), indicating perfect additivity of *R. delavayi* and *R. decorum* ITS types in all of these accessions. ITS data hence confirmed that all of the putative hybrids (including accession deco-15) were indeed hybrid derivatives of *R. delavayi* and *R. decorum*. That no novel ITS genotypes were detected amongst the hybrids indicates that no species other than *R. delavayi* and *R. decorum* were involved in the parentage of these hybrids, even though other *Rhododendron* species were observed within this hybrid zone.

CHLOROPLAST HAPLOTYPES

PCR with the C/F primer combination yielded a single PCR product, and all sampled individuals yielded single products of the same size. Direct sequencing of these PCR products yielded a single haplotype for each amplicon. *Rhododendron delavayi* and *R. decorum* consistently differed from each other at nine nucleotide positions (0.97% bp difference). The sequences from *R. delavayi* and *R. decorum* were deposited in GenBank with accession numbers DQ178247 and DQ178346, respectively.

Of the 15 hybrids identified by AFLP and ITS analyses, 14 possessed the *R. delavayi trn*L-*trn*F haplotype, whereas only one possessed the *R. decorum* haplotype, resulting in a strongly biased ratio of 14 : 1 that was statistically different from the 1 : 1 expectation for no gender bias (null hypothesis) ($\chi^2 = 11.27$, d.f. = 1, $P < 0.05$). This indicates that hybridization is possible in both directions, but, at this site, *R. delavayi* was the usual maternal parent.

DISCUSSION

HYBRIDS BETWEEN *R. DECORUM* AND *R. DELAVAYI*

The molecular data from this study confirm that *R. delavayi* and *R. decorum* form a natural hybrid zone, with the former species the usual maternal parent at the site examined. *Rhododendron decorum* and *R. delavayi* belong to different subsections within *Rhododendron* subgenus *Hymenanthes* (*Fortunea* and *Arborea*, respectively; Chamberlain, 1982), and this study provides the first molecular confirmation that natural intersubsection hybrids occur in this subgenus.

Two nuclear molecular marker systems were employed to examine and characterize putative hybrids in this study, and both confirmed the hybrid nature of all 14 putative hybrids examined, as well as demonstrating that an individual with *R. decorum*-like morphology was also a hybrid. However, the ITS and AFLP markers otherwise provided contrasting results. All hybrid accessions examined showed perfect additivity for all six of the ITS sites by which the two species differed; hence, ITS data provided strong evidence of hybrid origin in all putative hybrids examined, but could not be used to investigate differences between the hybrid individuals or distinguish between hybrid classes. Conversely, AFLP data subjected to Bayesian analysis using the program 'NewHybrids' revealed clear differences between the hybrid individuals, demonstrating that hybrids of at least the first two generations are present, and that backcrossing in both directions occurs. cpDNA markers demonstrated that *R. delavayi* was the maternal parent in all but one of the hybrid derivatives examined, the exception being one of the six F1s.

Four morphological characteristics were used as field markers to identify the parent species and putative hybrid individuals. All accessions identified as putative hybrids using these markers were confirmed to be so by molecular data. However, our results also showed that backcrosses can occasionally be misidentified as a parent species individual when morphological markers are used. Fertile seeds found on some of the hybrids indicated that hybridization might be progressing beyond the F1 generation, and morphological variation amongst the hybrids examined indicated that backcrossing and segregation might be occurring. However, the effects of segregation on morphology can be difficult to predict (Rieseberg & Ellstrand, 1993), and, in this case, morphological markers could not be used to determine the class of hybrid of any given individual. Therefore, the morphological markers developed here provide a useful and reliable tool by which hybrids of this combination may be identified by field workers at other sites, but to determine the class of hybrids present requires molecular markers.

Some of the hybrids examined here match closely the description of *R. agastum*, which has been described as a species in subsection *Irrorata* (i.e. a different subsection from either *R. delavayi* or *R. decorum*). This demonstrates both the difficulty of assigning meaningful subgeneric groups to genera prone to frequent hybridization, and also that some *Rhododendron* 'species' currently in cultivation might in fact be early generation hybrid derivatives. However, hybrids whose morphology matches *R. agastum* are also formed by the combination *R. delavayi* × *R. irroratum* (H.-G. Zha, unpubl. data). *Rhododendron irroratum* was not present at the study site. Further work will be required to determine whether all records of *R. agastum* are hybrids, and, if so, which hybrid combination should properly be named '*R.* × *agastum*'.

HYBRID ZONE POPULATION STRUCTURE

The 14 putative hybrids examined provided a sample of the population structure in a hybrid zone between *R. delavayi* and *R. decorum*. Amongst this sample, F1s were the most frequent genotype class (six accessions), whereas only two F2s and one backcross in each direction were detected in this sample. The remaining four accessions were indicated to be hybrids by the PCO plots of AFLP data, but could not be assigned with confidence to any of the early generation hybrid genotype classes mentioned above (F1, F2, backcross 1) by 'NewHybrids'. One might have been a third F2, but the other three, by elimination, were probably hybrid derivatives belonging to later generations. In addition to these 14 accessions, a second backcross to *R. decorum* was detected amongst individuals of *R. decorum*-like morphology examined. From this, the number of backcrosses present is probably higher than this sample suggests, as other backcrosses may exist amongst individuals with parent-like morphology.

Where interfertile plant species form hybrid zones, the normal pattern is for F1s to form a bridgehead from which later generation hybrids can be formed in much larger numbers, producing a swarm of hybrid derivatives of complex parentage (Arnold, 1997; Rieseberg & Carney, 1998; Broyles, 2002). This pattern arises because F1 formation is an event made rare by various barriers to hybrid formation in the parent species (Arnold, 1997; Rieseberg & Carney, 1998; Ramsey, Bradshaw & Schemske, 2003), but, once an F1 is formed, all of its descendants will be post-F1s. In extreme cases, hybrid zones may contain no surviving F1s (Arnold, 1993; Cruzan & Arnold, 1993), although, more commonly, very few F1s are present (Barton & Hewitt, 1985; Nason, Ellstrand & Arnold, 1992;

Arnold, 1997, 2000; Rieseberg & Carney, 1998; Johnson *et al*., 2001; Broyles, 2002). Occasionally, F1s may be present in slightly larger numbers; for example, in *Borrichia frutescens* × *arborescens* hybrid zones, F1s comprise 18% of hybrid derivatives, or 7% of all *Borrichia* plants present (Cattell & Karl, 2004). Furthermore, very few examples are known of hybrid zones where virtually all hybrids are fertile F1s. The hybrid oaks *Quercus kelloggii* × *wislizenii* var *frutescens* occur as F1s at very low frequency (Nason *et al*., 1992). Situations in which F1 hybrids are abundant and dominate the hybrid zone to the exclusion of both parents and other hybrid derivatives are known in two cases, i.e. *Encelia* × *laciniata* (Kyhos, Clark & Thompson, 1981) and *Rhododendron* × *sochadzeae* (Milne *et al*., 2003). This phenomenon might reflect habitat-mediated superiority of F1 hybrids (Milne *et al*., 2003). The situation observed in the current study clearly matches neither extreme, because F1s make up 40% of the hybrids surveyed, and, although this is based on a relatively small sample, it is clear that F1 production is more common here than predicted by some models (Arnold, 1997; Rieseberg & Carney, 1998; Broyles, 2002). Our study falls between the *Borrichia* situation, where 7% of hybrids are F1s, and the extremes, where nearly all hybrids are F1s, and is consistent with a hypothesis that the proportion of F1s present in a hybrid zone can vary from 0% to 100% depending on factors such as the species present, the age of the hybrid zone, and the habitat conditions.

Natural hybridization between *R. delavayi* and *R. decorum* was bidirectional and asymmetrical at this site, with *R. delavayi* as the main maternal parent. Asymmetrical hybridization is relatively common in plants (Barton & Hewitt, 1985; Brubaker, Koontz & Wendel, 1993; Cruzan & Arnold, 1994; Arnold, 1997; Caraway, Carr & Morden, 2001; Wu & Campbell, 2005), and has been recorded in a hybrid zone between two deciduous American *Rhododendron* species from subsection *Pentanthera* (Kron *et al*., 1993). In the present study, the probable cause of asymmetry is the difference in flowering times between the two species. The order of flowering is *R. delavayi*, then hybrids, then *R. decorum*. *Rhododendron delavayi* usually starts to flower about 1 month ahead of *R. decorum* (H.G. Zha, pers. observ.), and F1 formation presumably occurs during the period of overlap. Because these taxa are protandrous, this means F1 formation would normally involve *R. decorum* pollen reaching *R. delavayi* stigmas; indeed, the very last *R. delavayi* flowers to open might be exposed only to *R. decorum* and hybrid pollen. Hence, it is unsurprising that *R. delavayi* is the usual maternal parent at this site; indeed, the presence of a single F1 with *R. decorum* as the maternal parent is the more surprising result.

The presence of first-generation backcrosses raises the possibility that introgression of germplasm between these two species might occur, in either direction. However, no germplasm of *R. decorum* was detected in any of the 15 accessions of *R. delavayi* examined. Likewise, amongst 15 accessions of putative *R. decorum* examined, 14 had no *R. decorum* germplasm, excluding that which was determined to be a first-generation backcross. Hence, we found no evidence of introgression, although we cannot rule out the possibility that it occurs.

Species barriers and hybridization in Sino-Himalayan *Rhododendron* species

This study is the first to demonstrate that hybridization between Himalayan *Rhododendron* not only occurs, but also proceeds beyond the F1 stage, raising the possibility that introgression occurs and can move germplasm between species. This has consequences for our understanding of how the diversity of Himalayan *Rhododendron* species arose and is maintained. In some areas, many species may occur together in a single valley, and yet this study shows that even species from different subsections may form hybrid zones. Furthermore, the radiation of subgenus *Hymenanthes* into more than 200 species in the Himalaya and China may be relatively recent (within the last ten million years; Milne, 2004), and appears to still be ongoing in some regions (Argent *et al*., 1998; D. Chamberlain, Royal Botanic Garden Edinburgh, pers. comm.). The results from this study make it clear that this rapid radiation has occurred, and is occurring, in spite of a lack of genetic barriers to gene flow between species.

There are two possible explanations of how radiation and speciation could be occurring in Sino-Himalayan *Rhododendron* species in spite of natural hybridization. The first is that, although introgression may occur, only neutral germplasm passes between the species involved (Wu, 2001; Rieseberg, Church & Morjan, 2004). This form of introgression might not interfere with the speciation process or cause distinct species to alter or become more similar to one another. The second possibility is that the species maintain their distinctness in the wild principally through habitat-mediated barriers to hybrid formation. It has long been known that habitat disturbance favours hybrid establishment (Anderson, 1949; Rieseberg & Carney, 1998; Bleeker & Hurka, 2001; Lamont *et al*., 2003; Tovrsanchez & Oyama, 2004), and, in particular, there is now clear evidence that it creates novel conditions that tend to favour segregating hybrid derivatives (Arnold, 1997; Rieseberg & Carney, 1998), which can be pre-adapted to novel habitat conditions (Rieseberg, Archer & Wayne,

1999; Rieseberg, Baird & Gardner, 2000; Rieseberg *et al*., 2003). Hence, anthropogenic habitat disturbance might have increased the rate of hybridization amongst Sino-Himalayan *Rhododendron* species, so that both the frequency and size of hybrid populations now observed are higher than would have occurred in an environment free from such disturbance.

The study site, in this case, has been subject to deforestation, a form of disturbance that has occurred frequently in the Sino-Himalayan region and may have favoured hybrid establishment. Hence, the level of hybridization detected here might be greater than that which occurred in the past when the ancestors of these species first diverged. If so, the possibility exists that the increased rate of hybridization might cause species barriers amongst some *Rhododendron* species to begin to break down, arresting or even beginning to reverse the process of speciation. More detailed studies are required to determine what effect deforestation and other disturbance might have on species barriers in large genera such as *Rhododendron*, which account for a significant proportion of the species diversity in the Sino-Himalayan region.

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