

## Asymmetric hybridization in *Rhododendron agastum*: a hybrid taxon comprising mainly $F_1$ s in Yunnan, China

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- **Background and Aims** *Rhododendron* (Ericaceae) is a large woody genus in which hybridization is thought to play an important role in evolution and speciation, particularly in the Sino-Himalaya region where many interfertile species often occur sympatrically. *Rhododendron agastum*, a putative hybrid species, occurs in China, western Yunnan Province, in mixed populations with *R. irroratum* and *R. delavayi*.
- **Methods** Material of these taxa from two sites 400 km apart (ZhuJianYuan, ZJY and HuaDianBa, HDB) was examined using cpDNA and internal transcribed spacer (ITS) sequences, and amplified fragment length polymorphism (AFLP) loci, to test the possibility that *R. agastum* was in fact a hybrid between two of the other species. Chloroplast trnL-F and trnS-trnG sequences together distinguished *R. irroratum*, *R. delavayi* and some material of *R. decorum*, which is also considered a putative parent of *R. agastum*.
- **Key Results** All 14 *R. agastum* plants from the HDB site had the *delavayi* cpDNA haplotype, whereas at the ZJY site 17 *R. agastum* plants had this haplotype and four had the *R. irroratum* haplotype. *R. irroratum* and *R. delavayi* are distinguished by five unequivocal point mutations in their ITS sequences; every *R. agastum* accession had an additive pattern (double peaks) at each of these sites. Data from AFLP loci were acquired for between ten and 21 plants of each taxon from each site, and were analysed using a Bayesian approach implemented by the program NewHybrids. The program confirmed the identity of all accessions of *R. delavayi*, and all *R. irroratum* except one, which was probably a backcross. All *R. agastum* from HDB and 19 of 21 from ZJY were classified as  $F_1$  hybrids; the other two could not be assigned a class.
- **Conclusions** *Rhododendron agastum* represents populations of hybrids between *R. irroratum* and *R. delavayi*, which comprise mostly or only  $F_1$ s, at the two sites examined. The sites differ in that at HDB there was no detected variation in cpDNA type or hybrid class, whereas at ZJY there was variation in both.

**Key words:**  $F_1$ -dominated hybrid zone, species barrier, habitat disturbance, *Rhododendron agastum*, *R. irroratum*, *R. delavayi*.

### INTRODUCTION

The key process in speciation concerns the formation and maintenance of reproductive isolating barriers (Coyne, 1994; Levin, 2000; Wu, 2001). One of the keys to understanding the nature of species is to explain how interfertile species remain distinct in spite of forming populations of fertile hybrids. Species barriers could be formed and maintained via the elimination of intermediate forms or hybrids, which are adapted to the habitat of neither parent (Schluter, 1998; Levin, 2000). However, there is now ample evidence that hybrids may, under certain circumstances, have higher fitness than one or both parents (Arnold and Hodges, 1995; Arnold, 1997; Wang *et al.*, 1997; Rieseberg and Carney, 1998; Campbell, 2004; Rhode and Cruzan, 2005). Hybrid fitness can be habitat-mediated (Johnson *et al.*, 2001; Campbell *et al.*, 2005) and may on occasion permit hybrids to occupy habitats distinct from either parent (Cruzan and Arnold, 1993; Arnold, 2004; Whitney *et al.*, 2006). The presence of hybrids in intermediate habitats provides a potential conduit for germplasm to flow between interfertile species, raising

the possibility that some species exist in spite of continuous gene flow between them at loci not subject to selection (Grant, 1981; Wu, 2001). Gene flow can also be prevented, however, if hybridization proceeds only to the  $F_1$  stage and no further, which can occur due to low  $F_1$  fertility or hybrid breakdown, or occasionally due to apparent habitat-mediated superiority of  $F_1$ s over other hybrid classes (Milne *et al.*, 2003).

Both these hypotheses (hybrid formation with or without ongoing gene flow) could apply to hybrid zones involving members of *Rhododendron* subgenus *Hymenanthes* from south-east Asia. *Rhododendron* × *sochadzeae*, which forms  $F_1$ -dominated hybrid zones, belongs to this subgenus (Milne *et al.*, 2003), so other examples might reasonably be expected within *Hymenanthes* where habitat circumstances are similar. On the other hand, the south-east Asian members of subgenus *Hymenanthes* appear to represent a rapidly radiated group that originated <5 Mya (Milne, 2004), and within which natural hybridization occurs commonly (Chamberlain, 1982). They thus might be a ‘Syngameon’, i.e. a complex of species maintained by selection, that are ecologically highly distinctive but capable of exchanging genetic material (Grant, 1957, 1963, 1981; Seehausen, 2004). To understand the mechanisms by

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which the remarkable diversity of *Hymenanthes* species in south-east Asia arose and are maintained, it is necessary to examine how species barriers are maintained in this group. However, very few of the natural hybrids that occur in this group have been examined in detail. Furthermore, some described species may in fact be hybrids themselves, particularly as large populations of  $F_1$  hybrids can look much like a stabilized species (Milne *et al.*, 2003), and this may obscure the true pattern of species diversity in south-east Asian *Hymenanthes*.

*Rhododendron agastum* Balf. f. et W. W. Smith was originally described as a species (Balfour, 1917; Chamberlain, 1982), but more recently has been treated as a hybrid between *R. delavayi* Franch. (= *R. arboreum* ssp. *delavayi*) and *R. decorum* Franch. based on morphological (Chamberlain *et al.*, 1996) and molecular (Zhang *et al.*, 2007a) evidence. Hybrids between these species closely resemble *R. agastum* in general appearance (Zha *et al.*, 2008), although they tend to follow *R. decorum* in having 6–9 corolla lobes and 11–16 stamens (Zhang *et al.*, 2007a). This is in contrast to the type specimen of *R. agastum* and all other material of this species at the KIB herbarium, which instead resemble *R. delavayi* in having five corolla lobes and ten stamens (Chamberlain, 1982). Furthermore, some populations of *R. agastum* occur where *R. decorum* is rare or absent, but where *R. delavayi* and *R. irroratum* Franch. occur together, sometimes in large numbers (H. G. Zha, R. I. Milne, pers. observ.). *R. irroratum* has the same corolla lobe and stamen numbers as *R. agastum*, and hence at least some populations of *R. agastum* may in fact be *R. delavayi* × *R. irroratum*. *Rhododendron delavayi*, *R. decorum* and *R. irroratum* are members of subsections *Arborea*, *Fortunea* and *Irrorata*, respectively (Chamberlain, 1982; Chamberlain *et al.*, 1996), so no two of these would appear to be sister species; however, phylogenetic analysis

has yet to reveal their precise relationships to one another (R. I. Milne, unpubl. data). All these species have the chromosome number  $2n = 26$  (Zhang, 2007). In a previous paper (Zha *et al.*, 2008), we examined hybrid populations between *R. delavayi* and *R. decorum*; the present study considers *R. agastum* populations where the putative parents are *R. delavayi* and *R. irroratum*, at sites where *R. decorum* is rare or absent.

Because *R. agastum* populations do not display the morphological variability normally associated with segregating post- $F_1$  (after first generation) hybrids (H. G. Zha, pers. observ.), these populations might provide a second example of an  $F_1$ -dominated hybrid zone within *Rhododendron* subgenus *Hymenanthes*. As with the parent species of *Rhododendron* × *sochadzeae* (Milne *et al.*, 2003), *R. delavayi* and *R. irroratum* have distinct but overlapping altitude ranges, i.e. 1500–3000 and 2500–3500 m, respectively, and the known range of *R. agastum* is intermediate (2200–3350 m) (Chamberlain, 1982). *R. delavayi* is a remarkably widespread species with five subspecies extending from north-west India to Thailand and GuiZhou in south-west China. In contrast, *R. irroratum* has a far more limited range mostly within south-west China, occurring from western GuiZhou to northern Yunnan provinces, although it is common within this range (Chamberlain, 1982; Fig. 1). The distribution given for *R. agastum* in the Flora of China is almost identical to that of *R. irroratum* (Fang *et al.*, 2005; Fig. 1), although it is much less abundant and appears to only occur sporadically, in the company of *R. delavayi* and one or both of *R. irroratum* and *R. decorum* (T. L. Ming, Kunming Institute of Botany, China, pers. comm.). The distribution of *R. decorum* is also similar (Chamberlain, 1982; Fig. 1). Ecologically, *R. irroratum* and *R. delavayi* occupy similar habitats to one another and often occur mixed together where their altitudinal ranges overlap, whereas *R. decorum* tends to prefer more shady

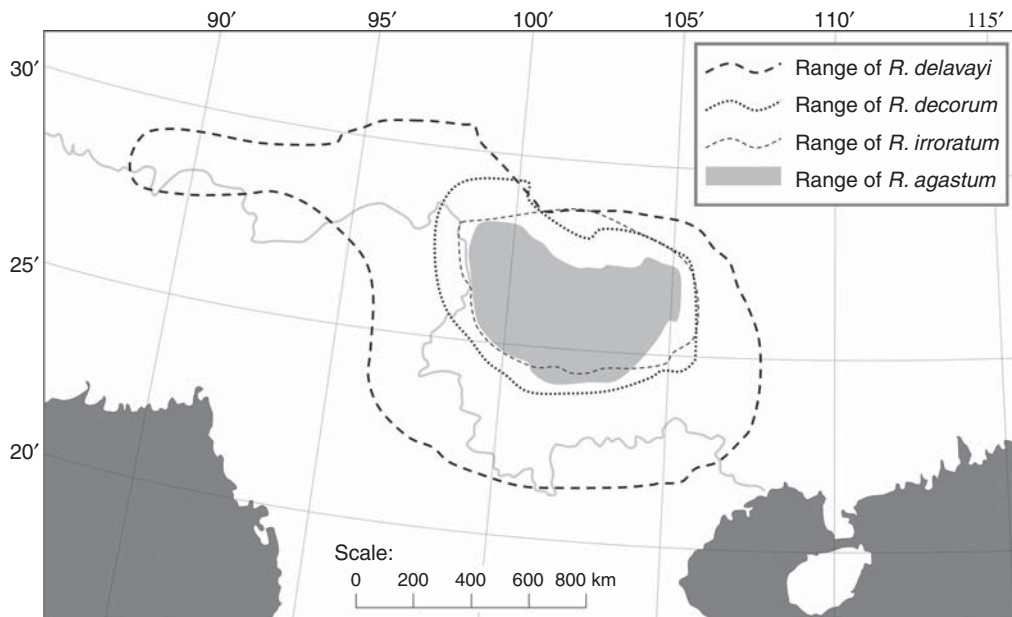


FIG. 1. Known distributions of *Rhododendron delavayi*, *R. decorum*, *R. irroratum* and *R. agastum* in and around south-west China.

and humid conditions, at least in Yunnan Province, and rarely tends to mix with the other two species.

In the current study, therefore, two sites where *R. agastum* occurs together with *R. delavayi* and *R. irroratum* were examined in order to determine: (1) whether *R. agastum* is the hybrid of *R. delavayi* and *R. irroratum* at these sites; (2) whether *R. agastum* populations contain mostly or only *F<sub>1</sub>* hybrids; (3) whether there is variation in which species serves as maternal parent; and (4) whether there is a difference in population structure between the two sites. Of the sites examined, ZhuJiangYuan (north-east Yunnan) was subject to considerable anthropogenic disturbance whereas HuaDianBa (western Yunnan) was almost undisturbed by human activity. Both sites were also surveyed to determine whether *R. decorum* was present, and where it was, material of this species was also collected for comparison.

To answer the above questions, cpDNA (trnL-F and trnS-trnG) and internal transcribed spacer (ITS) sequences were obtained from the three taxa, and the genotype class of a sample of plants of the three taxa at each site was determined by means of a Bayesian analysis (Anderson and Thompson, 2002), conducted on variation revealed by amplified fragment length polymorphism (AFLP) markers. In addition, the fertility of *R. agastum* was determined via *in vitro* germination of naturally set seed gathered from wild material.

## MATERIAL AND METHODS

### *Sampling, site description and DNA extraction*

The two *Rhododendron agastum* localities chosen for examination were HuaDianBa (HDB), near Dali, west Yunnan (25°52'N, 99°58'E, approx. 2900 m) and ZhuJianYuan (ZJY), north-east Yunnan (25°54'N, 103°55'E, approx. 2300 m). All collections were made in April, 2006.

Because habitat disturbance has long been known to promote hybridization (Anderson, 1948, 1949; Anderson and Stebbins, 1954; Rieseberg and Carney, 1998), and in certain cases to have a profound effect on which hybrid classes are present (Kyhos *et al.*, 1981; Milne *et al.*, 2003), the population structure of *R. agastum* might vary according to the level of habitat disturbance, so the two sites chosen for this study had very different levels of habitat disturbance. At HDB, the natural vegetation type is well preserved, with natural forest more or less intact, whereas ZJY has been subject to a long period of habitat disturbance and deforestation, with tree cover now completely removed from most of the area, although the *Rhododendrons* themselves have been left intact to attract tourists. At HDB, *R. agastum* was present in a small area on a forested hillside slope, whereas at ZJY it occurred scattered around the top of a large hill.

At HDB, *R. agastum* could only be found within a small area (about 400 m<sup>2</sup>) mixed with *R. delavayi* and *R. irroratum*. Morphological characters such as corolla colour, ventral leaf surface indumentums and young shoot indumentums were used to distinguish these three taxa in the field. From this site we collected all 14 individuals of *R. agastum* that were found and examined, plus nine of *R. delavayi* and ten of *R. irroratum*, sampled at random. No other *Hymenanthes* species could be found within several

hundred metres of this location, although 20 *Rhododendron* species including ten *Hymenanthes* have been recorded from the area. The only plants found of *R. decorum* were two individuals 1000 m away from the *R. agastum* population; these were both sampled to determine whether they were involved in the parentage of *R. agastum*.

At ZJY, the dominant plant species were *Castanopsis orthacantha* and *Pinus yunnanensis*, and the *Rhododendron* species *R. delavayi* and *R. irroratum*. Also present were *R. aberconwayi* (subgenus *Hymenanthes*), *R. racemosum* and *R. simsii* (other subgenera), and *R. agastum*, which occurred sporadically in the company of *R. delavayi* and *R. irroratum*. Despite repeated searches, *R. decorum* could not be found and appeared therefore to be absent at ZJY. It is possible that this species occurred here in the past, but disappeared relatively recently due the progressive deforestation of this site over recent centuries, which removed the shaded and humid habitats that this species prefers. From this, the possibility had to be considered that a now extinct population or *R. decorum* contributed to the parentage of hybrids at this site. From this site, 21 individuals of *R. agastum*, 14 of *R. irroratum* and ten of *R. delavayi* were sampled at random.

From each accession, desiccated leaf material (approx. 1 g of fresh leaf mass to approx. 25 g of coarse silica gel) for DNA extraction was collected, and voucher specimens were deposited in the Herbarium at Kunming Institute of Botany, Chinese Academy of Sciences. DNA was extracted from silica-gel-dried leaf tissue using a modified CTAB method (Kobayashi *et al.*, 1998). DNA quality and concentration were assessed by agarose gel electrophoresis with known concentrations of uncut lambda DNA (Takara, Dalian, China).

### *Determination of rDNA genotypes and chloroplast haplotypes*

The ITS region including the intervening 5-8S coding region of the nuclear ribosomal DNA (approx. 700 bp) of all the sampled individuals was amplified using primers ITS1 and ITS4 (White *et al.*, 1990). The chloroplast trnL-F and trnS-G regions were amplified using universal primer pairs ('C' and 'F' primers from Taberlet *et al.*, 1991, and trnS and trnG from Hamilton, 1999); the former was used in a previous study on natural hybridization in *R. delavayi* and *R. decorum* (Zha *et al.*, 2008). Each of the three reactions was carried out in a final volume of 50 µL containing 20 ng template DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 400 pmol of each primer and 1 U of Ex-taq (Takara). All amplifications were performed using a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) with the following conditions: 4 min at 94 °C (one cycle); 1 min at 94 °C, 1 min at 55 °C (53 °C for trnS-G amplification) 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. The complete ITS with 5-8S region resolved on agarose as a single sharp band and was sequenced directly from all sampled *R. delavayi*, *R. irroratum* and *R. agastum* individuals. Direct sequencing was performed on both strands on an ABI PRISM 3730 Sequencer using the same primers that were used for the PCR amplifications. The alignment was

performed using the program ClustalX v.1.83 (Thompson *et al.*, 1997). If *R. agastum* were determined to be hybrids of *R. delavayi* and *R. irroratum* by rDNA genotyping and AFLP analysis, a  $\chi^2$  test was conducted to determine if the ratio of *R. delavayi* to *R. irroratum* cpDNA (trnL-F or trnS-G sequences) among the hybrids differed significantly from a 1 : 1 ratio.

#### AFLP analysis

AFLP markers provide reliable diagnostic loci at varying taxonomical levels, and can be relatively easily generated in sufficient numbers to distinguish between genealogical classes in hybrid populations, especially in the case of weakly differentiated source populations (Miller, 2000; Campbell *et al.*, 2003). Although co-dominant markers are twice as informative per locus as dominant markers, they are much more costly to produce, and it is possible to distinguish between  $F_1$ ,  $F_2$  and backcross hybrids with <5% classification error using fewer than 30 diagnostic AFLP loci (Miller, 2000).

AFLP analysis was performed essentially as described by Vos *et al.* (1995), with modifications by Gilbert *et al.* (2002) and Zha *et al.* (2008). Primers and adapters were synthesized by Sangon Company (Shanghai, China). Enzymes were obtained from Amersham Pharmacia Biotech, unless otherwise stated. Genomic DNA (50 ng) was digested using both *EcoRI* and *MseI* enzymes (5 U each in a final volume of 30  $\mu$ L) and adapters (0.1  $\mu$ M E-adapter and 1.0  $\mu$ M M-adapter) were ligated to the resulting fragments. Then, 5  $\mu$ L of digested DNA from a 1 : 10 dilution with sterile distilled water (SDW) was used for (PCR) preamplification using primers (0.5  $\mu$ M *EcoRI* and 0.5  $\mu$ M *MseI* 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), after an initial cycle of 65 °C for 5 min. The preamplification products were diluted 1 : 10 with SDW and used as template for selective amplification using 0.6  $\mu$ M *EcoRI* and 0.1  $\mu$ M *MseI* primers, with three selective nucleotides at the 3'-end, with the following thermal cycling conditions: 94 °C for 2 min, followed by one cycle of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, followed by 12 cycles which were identical except that the annealing temperature was reduced each cycle by 0.7 °C, followed by 23 additional cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s; there was then a final stage of 72 °C for 5 min. In total, seven primer combinations were used: E-AAC/M-ACA, E-AGA/M-CCA, E-AGC/M-ACC, E-AGA/M-AAG, 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 EDTA, 0.01% xylene cyanol and 0.01% Bromophenol blue) and 5  $\mu$ L of each sample was electrophoresed on a 6% denaturing polyacrylamide gel in 1  $\times$  TBE buffer at 65 W for approximately 2 h. AFLP bands were visualized by silver staining of the gel, as described in Bassam *et al.* (1991).

AFLP bands were scored manually as 0 for the absence and 1 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. For each individual, the posterior probability that it belonged to parents or to early generation hybrid classes ( $F_1$ ,  $F_2$  or backcross) was estimated using a Bayesian method to analyse the polymorphic AFLP markers. This procedure used a Markov Chain Monte Carlo method and was implemented in the program 'NewHybrids' (v1.1 beta3), which allows distinguishing between  $F_1$  hybrids, different backcrosses or later generation hybrids and does not require that parental populations are sampled separately (Anderson and Thompson, 2002). The program was used with the input files described by Milne and Abbott (2008), which cause the program to consider 45 possible genotype classes, representing every class that could arise after up to four generations of hybridization. This method has the advantage that it avoids the assumption that only early-generation hybrids are present, and hence greatly reduces the possibility that complex hybrids will be wrongly assigned to a class such as  $F_1$ . Posterior distributions were evaluated after  $10^5$  iterations of the Monte Carlo Markov Chains, following a burn-in of  $10^4$  iterations, without using any individual or allele frequency prior information. As in Milne and Abbott (2008), posterior probabilities for the 45 possible classes were pooled into six categories, i.e. parents,  $F_1$ -type (i.e.  $F_1$  or cross between one species and advanced backcross to the other),  $F_2$ -type (i.e.  $F_2$  or complex hybrid not approaching either parent), and backcross-type each way (i.e. hybrid derivative with at least 75% germplasm from one parent, which might be a simple or complex backcross). Individuals were assigned to one of the six genotypic categories if the pooled probability for that category was  $\geq 0.95$ , or where this was not the case, individuals were assigned to a choice of two or more categories if the combined score for these two categories was  $\geq 0.95$ .

*Direct mathematical analysis of AFLP data.* The presence of at least one copy of each parent-specific marker from both parents in each hybrid genet could indicate that all the individuals are heterozygous for all parent-species-specific markers, and therefore must be  $F_1$ s. The only other possibility is that each genet by chance has two copies of every such marker surveyed. By calculating that the probability for this latter possibility was extremely small, Milne *et al.* (2003) demonstrated that all examined individuals of *Rhododendron*  $\times$  *sochadzeae* were  $F_1$ s. Here, the proportion of such markers expressed in hybrids is very high, but not 100% (see Table S1 in Supplementary Data, available online), and so a similar approach was followed here to test alternative hypotheses for each accession against the hypothesis that it was an  $F_1$ , in order to corroborate the results from the NewHybrids analysis.

As with the NewHybrids analysis, the two populations of *R. agastum* were analysed separately from one another. Direct mathematical analysis was used to provide tests of whether each *R. agastum* accession might be (1) an  $F_2$ , or other post- $F_1$  hybrid with 50% germplasm from each species, or (2) a first-generation backcross to one or other parent species. For this analysis only markers that were exclusive to one or other parent species were used (i.e. markers that were present in all accessions of one parent species, but none

of the other). Let  $N_d$  and  $N_i$  be the number of markers exclusive to *R. delavayi* and *R. irroratum*, respectively, and  $d$  and  $i$  the number of markers specific to each species present in each hybrid accession. For this analysis each marker is assumed to be fixed and single copy within each parent species (but see below).

For any  $F_2$  accession, the probability that each marker will be present is 0.75. Hence the probability that it will have  $d$  and  $i$  markers present is given by

$$\frac{0.75^d \times 0.25^{(n_d-d)} \times 0.75^i \times 0.25^{(n_i-i)} \times n_d!/d!}{(n_d-d)! \times n_i!/i!(n_i-i)!} \quad (1)$$

To estimate the probability that an accession is an  $F_2$ , when the alternative hypothesis is that it is an  $F_1$ , the probability was calculated, for each accession, that an  $F_2$  could have at least  $d$  and  $i$  markers present. For *R. irroratum* markers this was done by summing the probabilities for all values of  $i$  between the actual value for each accession (here called  $a_i$ ) and the highest possible value,  $i = n_i$ . Hence the probability that the accession was an  $F_2$ , based on *R. irroratum*-exclusive markers alone, was estimated by

$$\sum_{i=a_i}^{i=n_i} (0.75^i \times 0.25^{(n_i-i)} \times n_i!/i!(n_i-i)!) \quad (2)$$

Likewise for *R. delavayi* markers values of  $d$  between  $a_d$  and  $n_d$  were summed using

$$\sum_{d=a_d}^{d=n_d} (0.75^d \times 0.25^{(n_d-d)} \times n_d!/d!(n_d-d)!) \quad (3)$$

giving an estimate of the probability of being an  $F_2$  based on *R. delavayi*-exclusive markers only. The probability that each accession was an  $F_2$  was hence estimated using the product of these two probabilities:

$$\sum_{i=a_i}^{i=n_i} (0.75^i \times 0.25^{(n_i-i)} \times n_i!/i!(n_i-i)!) \times \sum_{d=a_d}^{d=n_d} (0.75^d \times 0.25^{(n_d-d)} \times n_d!/d!(n_d-d)!) \quad (4)$$

A similar approach was used to test whether an accession might be a backcross. A backcross should have all markers present from one parent species, but from the other, the probability that any marker is present will be  $0.5g$  where  $g$  is the generation of backcrossing. Hence the probability that a backcross to *R. delavayi* contains  $x_i$  markers specific to *R. irroratum* is given by

$$0.5^{gx_i} \times (1 - 0.5^g)^{(1-x_i)} \times n_i!/(x_i)!(n_i-x_i)! \quad (5)$$

and where  $x_i > (1 - x_i)$  (as is the case for all putative hybrid accessions here), the probability drops with increasing generations. For this reason, if an accession can be shown by this method to not be a first-generation backcross, then it follows

that it is even less likely to be a backcross of a later generation. For first-generation backcrosses, the formula for  $g = 1$  simplifies to

$$0.5^{n_i} \times n_i!/(x_i)!(n_i-x_i)! \text{ or } 0.5^{n_d} \times n_d!/(x_d)!(n_d-x_d)! \quad (6)$$

for backcrosses to *R. delavayi* and *R. irroratum*, respectively. As with testing for  $F_2$ s, the possibility of being a backcross in each generation was tested against the alternative possibility of being an  $F_1$ . Hence for each accession, the probability of it being a backcross was estimated by summing the probabilities for all values of  $x_i$  between the actual value for each accession ( $a_i$ ) and the highest possible value,  $x_i = n_i$ . Hence the formula is

$$\sum_{i=a_i}^{i=n_i} (0.5^{n_i} \times n_i!/i!(n_i-i)!) \quad (7)$$

for backcrosses to *R. delavayi*, and

$$\sum_{d=a_d}^{d=n_d} (0.5^{n_d} \times n_d!/d!(n_d-d)!) \quad (8)$$

for backcrosses to *R. irroratum*.

In both these cases, the assumption is made that a marker is fixed in the parent species that it comes from, but it is possible that in some cases the marker-absent allele is present at low frequencies, undetected, in the parent population. If this were so, probabilities for being  $F_2$ s and backcrosses would be over-estimated by the method described. Equally, however, some markers could be multiple-copy, which would cause these probabilities to be under-estimated. Hence the results from these calculations must be interpreted with caution, and treated as informative rather than as absolute probability values.

Complex hybrid derivatives with 50% germplasm from each parent would have the same probability scores as  $F_2$ s using this method, whereas in cases where the proportion of germplasm was unequal, the score would grade towards that for a backcross. The assumption is made that where the combined probabilities of being  $F_2$  or a backcross either way does not exceed 5% then the accession in question is also unlikely to be a complex hybrid derivative.

## RESULTS

### Morphology

At both of the sampling sites, *Rhododendron* individuals in the hybrid swarms could easily be classified into three types, i.e. *R. delavayi*-like, *R. irroratum*-like and *R. agastum*-like, using three morphological characters: corolla colour, ventral leaf surface indumentum and young shoot indumentum. The thin ventral leaf indumentum of *R. agastum*, which was clearly intermediate between those of *R. delavayi* and *R. irroratum*, has been taken as a diagnostic character for *R. agastum* (Chamberlain, 1982). At both populations examined, each morphological character for each *R. agastum*-like individual either matched one of *R. delavayi* or *R. irroratum*, or was intermediate between these two species (Table 1). This was thus

TABLE 1. Morphological traits by which *Rhododendron delavayi*, *R. irroratum*, *R. agastum* and *R. decorum* were distinguished

Character*	<i>R. delavayi</i>	<i>R. agastum</i>	<i>R. irroratum</i>	<i>R. decorum</i> *
Flower colour	Carmine	Pink	White to creamy yellow	White to pale pink
Corolla lobes	5	5 <sup>†</sup>	5	6–8
Stamens	10	10 <sup>†</sup>	10	12–16
Stamen filament	Glabrous	Pubescent at base	Pubescent at base	Pubescent at base
Ovary	Densely fawn-tomentose	Glandular-hairy with strigose hairs	Densely glandular-hairy	Densely glandular-hairy
Style	Glabrous	Glandular to tip or glabrous	Glandular to tip	Glandular to tip
Flowering period	March to May	April to May	March to May <sup>‡</sup>	April to July
Ventral leaf surface	Woolly	Thin indumentum	Glabrous	Glabrous
Leaf shape	Long-lanceolate	Long-lanceolate	Long-lanceolate	Oblong-elliptical

\*These characters are based mainly on our own gatherings from the study sites, except for *R. decorum*, which was not found at ZJY, so the characters listed are based on specimens we collected in HDB, collections in the Herbarium of Kunming Institute of Botany (CAS) and the description in the 'Flora of China'.

<sup>†</sup>All specimens of *R. agastum* collected had five corolla lobes and ten stamens.

<sup>‡</sup>Within the region we collected all our specimens, especially in HDB, *R. delavayi* always flowers 1 month earlier than *R. irroratum*.

TABLE 2. Differences between ITS sequences found in *Rhododendron delavayi*, *R. irroratum*, *R. agastum* and *R. decorum*

ITS-type	Morphology of plants with this type	Locality	Position in the ITS alignment*							
			91	98	111	200	203	211	491	502
DD	<i>R. delavayi</i> -like	HDB/ZJY	Y	C	T	G	Y	G	T	T
DH	<i>R. agastum</i> -like	HDB	Y	S	K	K	Y	G	Y	Y
DZ	<i>R. agastum</i> -like	ZJY	Y	S	K	K	Y	K	Y	Y
HH	<i>R. irroratum</i> -like	HDB	T	G	G	T	C	G	C	C
ZZ	<i>R. irroratum</i> -like	ZJY	T	G	G	T	C	K	C	C
HH	<i>R. decorum</i> -like	HDB	T	G	G	T	C	G	C	C

\*Position numbers are based on the sequence of *R. delavayi*. All other positions are identical between the species.

consistent with *R. agastum* being the hybrid of these two species. By contrast, none of the *R. agastum* plants examined had the higher numbers of corolla lobes and stamens, nor the leaf shape, characteristic of *R. decorum*. This, plus the rarity and apparent absence of *R. decorum* at HDB and ZJY, respectively, made it unlikely that this species was involved in the parentage of *R. agastum* at these sites.

Within each of the *R. delavayi*-like, *R. irroratum*-like and *R. agastum*-like groups, very little morphological variation was observed. This is consistent either with all three being species, or with *R. agastum* comprising only hybrids of  $F_1$  class. It indicates that if backcrosses or segregating hybrid derivatives are present, they are probably so at low frequency.

#### ITS genotypes

The aligned sequence matrix generated a total of 642 characters with no indels or gaps. This comprised the ITS1 (253 bp), 5-8S (164 bp) and ITS2 (225 bp) regions. All individuals in *R. delavayi*-like groups from ZJY and HDB had identical ITS sequences (GenBank accession numbers EF035043 and DQ295783; ITS-type DD; Table 2) and were polymorphic (C/T) at positions 91 and 203. All *R. irroratum* individuals from HDB had identical ITS profiles (GenBank accession number EF035044; ITS-type HH; Table 2); at ZJY all *R. irroratum* accessions except IRR-Z-7 had identical profiles to one another (GenBank accession number EF035045;

ITS-type ZZ; Table 2) but differed from those from HDB in that position 211 was polymorphic (T/G) at ZJY but monomorphic (G) at HDB. However, the two accessions of *R. decorum* examined from HDB had identical ITS profiles to those of *R. irroratum* at this site (Table 2).

The ITS sequences of *R. delavayi* and *R. irroratum* differ at eight positions, six in ITS1 (91, 98, 111, 200, 203, 211) and two in ITS2 (491, 502) (Table 2). Position 91 was polymorphic (C/T) in *R. delavayi*, whereas it was monomorphic (T) in *R. irroratum* and *R. decorum* (Table 2). Hence *R. delavayi* and the ZJY population of *R. irroratum* both contain polymorphic loci indicating that at least two slightly differing copies of ITS are present.

Additive ITS profiles were present in all *R. agastum* accessions examined, and also in one accession (IRR-Z-7) with *R. irroratum* morphology. ITS profiles of *R. agastum* at HDB displayed perfect additivity between types DD (*R. delavayi*) and HH (*R. irroratum* and *R. decorum* at HDB) (ITS type DH; Table 2). Likewise, *R. agastum* at ZJY and accession IRR-Z-7 had additive profiles of types DD (*R. delavayi*) and ZZ (*R. irroratum* at ZJY) (ITS type DH; Table 2). As with the profiles of *R. irroratum*, the profiles of *R. agastum* at HDB and ZJY therefore differed only at position 211 (Table 2). This additivity strongly supports *R. agastum* being the hybrid of *R. delavayi* and *R. irroratum*, although the ITS data alone cannot reject the possibility that it is *R. delavayi* × *R. decorum*.

## Chloroplast haplotypes

PCR reactions with the C/F and trnS/trnG primer combination each yielded a single PCR product of the same size for all sampled individuals. Direct sequencing of these PCR products yielded a single haplotype for each amplicon.

There was no variation within *R. delavayi*-like accessions from HDB or ZJY; all had the same trnS-G haplotypes (GenBank accession number DQ988999), and the same trnL-F sequence as reported for this species in a previous study (Zha et al., 2008, GenBank accession number DQ178247) (haplotype D, Table 3). Regarding *R. irroratum*, there was no variation within either site, but material from HDB (GenBank accession numbers DQ999963 and DQ989004; haplotype Ih; Table 3) differed from that at ZJY (Genbank accession numbers DQ999964? and DQ989005 haplotype Ih; Table 3) by one trnL-F insertion and one point mutation, and by three trnS-G point mutations. This indicates that material of *R. irroratum* is quite distinct between these two sites, and/or that one population has captured the cpDNA of another species. cpDNA of *R. decorum* differed from HDB material of *R. irroratum* by five mutations, and from ZJY material of *R. irroratum* by two mutations (Table 3). Both species differed from *R. delavayi* at 12 or more positions.

At HDB, all *R. agastum*-like accessions had haplotype D, matching *R. delavayi*. However, at ZJY, four *R. agastum*-like accessions had the haplotype Iz, matching local *R. irroratum* populations, while the other 17 accessions had haplotype D. This strongly indicates that *R. agastum* at ZJY is a hybrid of *R. delavayi* and *R. irroratum* and that either species can be the plastid donor, whereas at HDB *R. delavayi* is always the plastid donor. Chloroplast DNA is usually inherited maternally in *Rhododendron* species (Harris and Ingram, 1991), indicating that *R. delavayi* may always be the maternal parent at HDB, while at ZJY the maternal parent may be either species. The strongly biased ratio of 17:4 at ZJY was statistically different from the 1:1 expectation for no gender bias (null hypothesis) ( $\chi^2 = 8.04$ , d.f. = 1,  $P < 0.01$ ), indicating that even here, *R. delavayi* is more commonly the maternal parent. This significant deviation remains if only individuals shown to be  $F_1$ s (see below) are taken into account ( $\chi^2 = 6.36$ , d.f. = 1,  $P < 0.05$ ). The results at ZJY eliminate *R. decorum* as a possible parent for those accessions with haplotype Iz and, based on the close similarity between these four accessions and the others examined for morphology, ITS and AFLP characters, make it unlikely that *R. decorum* is a parent to *R. agastum* at either site.

## AFLP data and putative parent species

A total of 119 scorable polymorphic AFLP markers were generated during the analysis. 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.

AFLP analyses were conducted separately and independently for two locations, HDB and ZJY. At HDB, AFLP profiles of *R. delavayi*, *R. decorum* and *R. irroratum* were first compared with those of *R. agastum* to determine whether the profiles of *R. agastum* accessions were additive for any two of the other species. At HDB, all markers present in *R. agastum* were also present in either *R. delavayi* or *R. irroratum*, including 20 markers that were present only in *R. delavayi* and *R. agastum*, and ten that were present only in *R. irroratum* and *R. agastum*. Conversely, there were no markers present only in *R. decorum* and *R. agastum*. Similarly, at site ZJY, *R. agastum* contained 20 and 12 markers that were otherwise only present in *R. delavayi* and *R. irroratum*, respectively, and none that was otherwise only present in *R. decorum* (based on material of *R. decorum* from HDB). At both HDB and ZJY, *R. agastum* does not contain any markers that are not found in either *R. delavayi* or *R. irroratum*. Based on this evidence, *R. agastum* is a hybrid derivative of *R. delavayi* and *R. irroratum*, whereas *R. decorum* is not or only marginally involved. Therefore, *R. decorum*, along with markers exclusive to it, was excluded from the following analysis.

## NewHybrids analysis

The NewHybrids analysis (Fig. 2) indicated that all accessions of *R. agastum* at HDB, and most accessions of it at ZJY, were  $F_1$  hybrids between *R. delavayi* and *R. irroratum*. At HDB, all putative parents examined were confirmed to be the pure parent species with >97% probability, and all hybrids were determined to be  $F_1$ s with >99% probability. At ZJY, however, one individual identified as *R. irroratum* was determined to be a hybrid derivative, although the program could not determine whether it was a backcross to *R. irroratum* or a complex hybrid. Among 21 putative hybrids examined at ZJY, 19 were determined to be  $F_1$ s but the remaining two could not be assigned a class with 95% certainty. Of these two, one had a 91% probability of being an  $F_1$ , whereas the other had a 26% possibility of being an  $F_1$  but a 47% probability of being a backcross to *R. delavayi*.

TABLE 3. Differences between trnL-F and trnS-G sequences of cpDNA haplotypes found in *Rhododendron delavayi*, *R. irroratum*, *R. agastum* and *R. decorum*

Haplotype	Species it is found in	Locality	Position in the trnL-F alignment*						Position in the trnS-G alignment*						
			103	118	289	306	308–313	788	64	103	106	267	406	540	674
D	<i>R. delavayi</i>	ZJY/HDB	G	–	T	T	TTTTTT	A	C	G	C	T	T	G	A
Ih	<i>R. irroratum</i>	HDB	A	A	G	A	AAAAAA	C	C	T	C	–	T	G	C
Iz	<i>R. irroratum</i>	ZJY	A	–	G	A	AATAAA	C	A	T	C	–	G	T	C
C	<i>R. decorum</i>	HDB	A	–	G	A	AATAAA	C	A	T	T	–	G	G	C

\*Position numbers are based on the sequence of *R. delavayi*. All other positions are identical between the species.

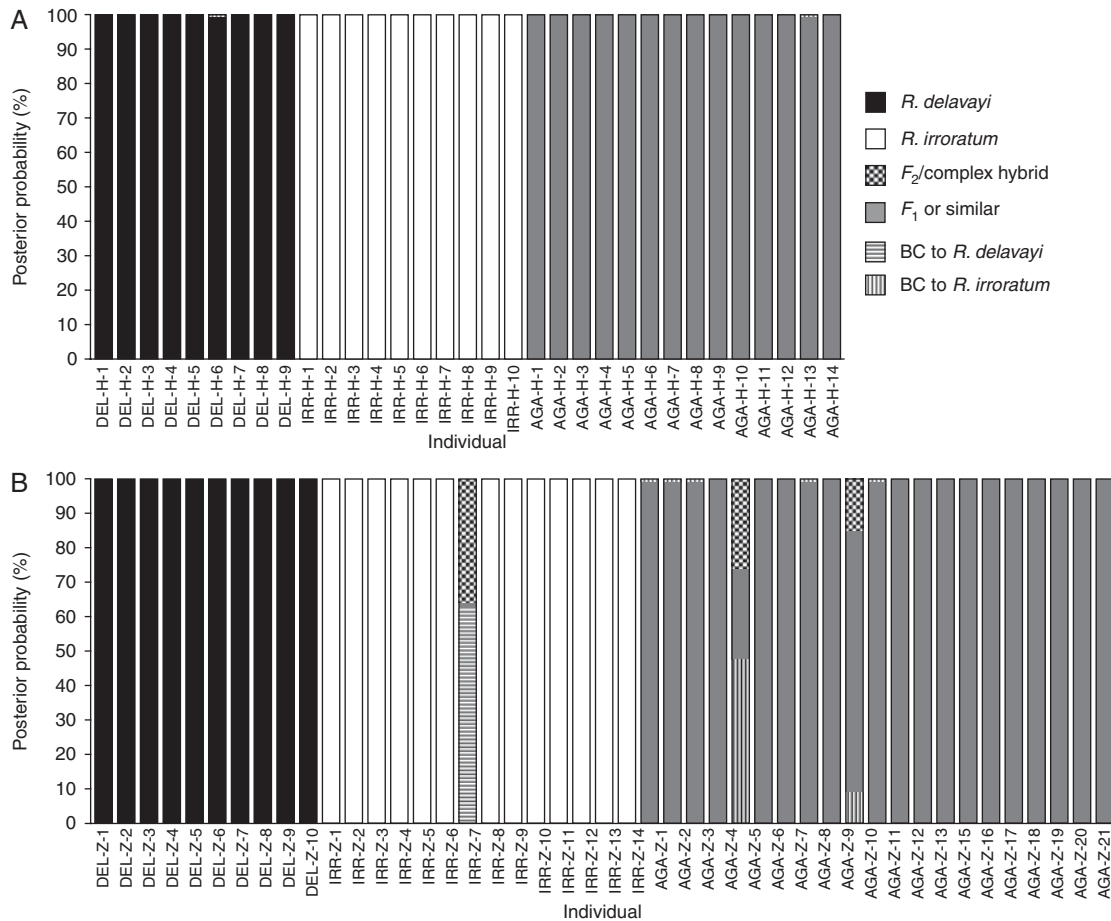


FIG. 2. Output from NewHybrids indicating individual posterior probability of belonging to each parental or hybrid class for all accessions collected in HDB (A) and ZJY (B). For the label of each accession, ‘DEL’ refers to *R. delavayi*, ‘IRR’ to *R. irroratum*, ‘AGA’ to *R. agastum*, ‘H’ to HDB and ‘Z’ to ZJY. For AFLP data for NewHybrids and direct mathematical analysis for both sites see Table S3 in Supplementary Data (available online).

Therefore, there was no evidence for hybridization proceeding beyond the  $F_1$  level at HDB, but at least one, probably two and possibly three accessions examined at ZJY were not  $F_1$ s, so hybridization does proceed beyond the  $F_1$  at this location.

#### Direct mathematical analysis of AFLP data

At HDB, there were 20 markers exclusive to *R. delavayi* and ten markers exclusive to *R. irroratum*. Accessions of *R. agastum* expressed between 17 and 20 of the former, and either nine or ten of the latter. Calculated probabilities for any of these accessions being  $F_2$ s were low, ranging from  $<0.001$  in five accessions to 0.013 in *aga-H-12* (Table S1 in Supplementary Data, available online). Probabilities for being backcrosses to *R. delavayi* were between 0.001 and 0.011, whereas probabilities for being backcrosses to *R. irroratum* were  $<0.001$  in all accessions except *aga-H-10*, in which it was 0.001 (Table S1). Based on this,  $F_2$ s and backcrosses are not present among the sampled material, and hence all *R. agastum* accessions from this site are  $F_1$ s (Table S1 in Supplementary Data).

At ZJY, there were 19 markers exclusive to *R. delavayi* and 11 markers exclusive to *R. irroratum*. Accessions of

*R. agastum* expressed between 16 and 19 of the former, and between seven and 11 of the latter; however, accession *irro-Z-7* expressed only 14 markers specific to *R. delavayi*. For accession *irro-Z-7* the probability for being a backcross to *R. irroratum* was 0.032, whereas in all *R. agastum* accessions the probability of being such a backcross was 0.002 or  $<0.001$  (Table S1 in Supplementary Data). The probabilities recorded for being backcrosses to *R. delavayi*, however, range from  $<0.001$  to 0.274, with four accessions given a probability of  $>0.05$  for being this class (Table S1 in Supplementary Data).

Accession *irro-Z-7* was either an  $F_2$  or backcross to *R. delavayi* according to NewHybrids, but the mathematical probabilities calculated for each of these classes were low (0.028 and 0.032, respectively), although these probability values were still higher than those for most other hybrid accessions. Hence this accession could be an  $F_1$ ,  $F_2$  or backcross to *R. irroratum* based on molecular data. This discrepancy between the two analyses suggests that the direct mathematical analysis might under-estimate the probabilities for these classes, and/or that NewHybrids might over-estimate them, relative to the probability for being  $F_1$ s. As NewHybrids involves fewer untested assumptions, we favour the former

possibility; however, the approach here will be to assign  $F_1$  class where both NewHybrids and direct mathematical analysis reject all other possibilities, but otherwise to consider any class that is not rejected by both methods as possible for each accession.

Among the 21 *R. agastum* individuals from ZJY, 15 are indicated to be  $F_1$ s by both analyses (Table S2 in Supplementary Data). Of the remaining six, four are  $F_1$ s according to NewHybrids, but of these, two might be  $F_1$ s and two might be backcrosses to *R. delavayi* based on direct mathematical analysis, although the probabilities for these in each case are low (0.052, 0.12, 0.113 and 0.113, respectively; Table S1 in Supplementary Data). Of the remaining three accessions, *aga-Z-10* is given a relatively high probability (0.274) of being a backcross to *R. delavayi*, although NewHybrids gave this accession a 0.75 probability of being an  $F_1$ , so it could belong to either class. Accession *aga-Z-5* could be an  $F_1$ ,  $F_2$  or backcross to *R. delavayi* according to NewHybrids, whereas according to the mathematical analysis it could be an  $F_1$  or  $F_2$  but is unlikely to be a backcross (Table S1 in Supplementary Data).

## DISCUSSION

### *The hybrid nature of R. agastum*

*Rhododendron agastum* was described as a species in 1917 (Balfour, 1917) and was still considered as such in 1982 (Chamberlain, 1982) but by 1996 it was listed as a probable hybrid of *R. delavayi* (Chamberlain *et al.*, 1996), with the second parent suggested to be *R. decorum*. Indeed, hybrids of *R. delavayi* and *R. decorum* do closely resemble *R. agastum* in some morphological characters (e.g. Zha *et al.*, 2008). However, the populations of *R. agastum* examined for the current study lack some of the distinctive morphological characters of *R. decorum*, and instead appeared based on morphology to be hybrids between *R. delavayi* and *R. irroratum*. Furthermore, both *R. agastum* populations examined occurred mixed together with *R. delavayi* and *R. irroratum*, and at one of the sites examined *R. decorum* was rare and found no closer than 1 km to the study population, whereas at the other site *R. decorum* was absent altogether.

Data from the current study indicated clearly that the *R. agastum* populations examined are hybrids, with *R. delavayi* as one parent. Morphological data (especially corolla lobe and stamen number) indicated that the second parent was *R. irroratum*. Caution is normally required in interpreting morphological data in hybrids (Rieseberg and Ellstrand, 1993). However, in this case artificial hybrids between *R. decorum* and *R. delavayi* had the same number of stamens (11–16) and corolla lobes (6–9) as *R. decorum* (Zhang *et al.* 2007a), whereas the numbers of these were ten and five, respectively, in the current study. Based on this, parentage from *R. decorum* seems unlikely, although variation in the dominance of these characters within species cannot be ruled out.

The ITS sequences of *R. irroratum* and *R. decorum* are very similar, so these data confirmed only that the *R. agastum* populations examined are hybrids of one of these two and *R. delavayi*. However, AFLP data from each site demonstrated that the profiles of *R. agastum* were additive for *R. delavayi*

plus *R. irroratum*, but not for *R. delavayi* plus *R. decorum*, strongly indicating that *R. irroratum*, not *R. decorum*, was the second parent. The only caveat to this was the very small sample size for *R. decorum*. A third line of evidence was cpDNA: although most *R. agastum* plants had cpDNA of *R. delavayi*, four at ZJY had cpDNA of the second parent. As *R. irroratum* and *R. decorum* cpDNA data differ at two sites in the trnG-S region it was possible to determine that for the four accessions mentioned, *R. irroratum* was the maternal parent. Hence parentage from *R. irroratum* was proved, but only for a minority of plants; however, these four did not differ from other *R. agastum* in morphology so there was no reason to assume they were unusual in their parentage. Taking morphological, AFLP and cpDNA data together, and considering also the rarity/absence of *R. decorum* at HDB and ZJY, respectively, it can be concluded with some confidence that *R. agastum* is *R. delavayi* × *R. irroratum* at these two sites.

Plants of *R. agastum* determined to be *R. irroratum* × *R. delavayi* are similar in morphology to those of *R. delavayi* × *R. decorum* examined elsewhere, particularly in the ventral leaf surface indumentum, ovary and calyx surface, and corolla colour, which are diagnostic for '*R. agastum*' (Chamberlain *et al.*, 1996; Zhang *et al.*, 2007a; Zha *et al.*, 2008). Hence although our study indicates that some plants known as *R. agastum* are *R. irroratum* × *R. delavayi*, it is very likely that some of the other populations referred to this species may be *R. delavayi* × *R. decorum*, and that *R. × agastum*, as it should more properly be known, in fact currently represents two distinct types of hybrid that are very similar in appearance. Based on the morphology of the hybrids in this study (Table 1) and those generated by Zhang *et al.* (2007a), hybrids of the two combinations can be distinguished by characters such as corolla lobe and stamen number, although further work might be necessary to confirm that these characters are always diagnostic for the hybrids.

The type specimen of *R. agastum* has five corolla lobes and ten stamens, and based on this is very probably *R. delavayi* × *R. irroratum*. Hence the other hybrid combination, *R. delavayi* × *R. decorum*, requires a new name.

### *Population structure in R. × agastum: F<sub>1</sub>-dominated hybrid zones?*

Based on AFLP markers, a Bayesian analysis conducted using the program NewHybrids indicated that all 14 *R. × agastum* hybrids at HDB were  $F_1$ s, and as all putative parents were also confirmed to be such, no other hybrid classes were present among the material examined. Direct mathematical analysis of AFLP data gave similar results, indicating that all hybrids at HDB were  $F_1$ s.

Of 21 putative hybrids at ZJY, however, only 19 were indicated to be  $F_1$ s by the NewHybrids analysis. The other two were shown to be hybrids, and of these one (*aga-Z-10*) was more likely an  $F_1$  than another class (76%) while the other (*aga-Z-5*) was likely to be a complex hybrid, with backcross to *R. delavayi* the most likely possibility. Direct mathematical analysis confirmed that 15 of the 21 hybrids at this site were  $F_1$ s, but returned probabilities of 0.052 and 0.12 that two accessions were  $F_2$ s, probabilities of 0.113 that each of three

accessions including *aga-Z-5* might be backcrosses to *R. delavayi*, and a probability of 0.274 that the remaining accession, *aga-Z-10*, was a backcross to *R. delavayi*. In addition to these, one putative *R. irroratum* accession, *irro-Z-7*, was determined by NewHybrids to be a hybrid of second or later generation, most likely a backcross to this species. That this accession was not pure *R. irroratum* but a hybrid derivative was also indicated by the ITS data. Direct mathematical analysis did not provide strong support for this accession being a class other than  $F_1$ ; however, this accession was very different in morphology from all others identified as  $F_1$ s in this study, and so based on this plus the NewHybrids result, the hypothesis that it is an  $F_1$  can be rejected. In another *Rhododendron* hybrid zone where  $F_1$ s were common but not the only class present,  $F_2$ s were rare to absent whereas backcrosses comprised up to one-fifth of hybrids present (Milne and Abbott, 2008). Based on this, plus its morphological similarity to *R. irroratum*, accession *irro-Z-7* is most likely to be a backcross, but the hypothesis that it is an  $F_2$  cannot be rejected.

Taking the two analyses together, ZJY contained 15 hybrid derivatives that were almost certainly  $F_1$ s, four more that were probable  $F_1$ s, and three of uncertain class, of which at least one (*irro-z-7*) was certainly not an  $F_1$  (Table S2 in Supplementary Data). Hence at ZJY backcrossing to both parent species (and hence introgression) might occur but could not be confirmed.

There were no two individuals of *R. × agastum* detected with identical AFLP profiles, which indicates minimal clonal reproduction in these hybrid populations, and that every hybrid examined was a distinct genet. This is in contrast to *Rhododendron × sochadzeae* and *R. × intermedium*, in which a small number of cloned genets were detected (Milne et al., 2003; Milne and Abbott, 2008), and *Rhododendron ferrugineum* in which clonal reproduction plays a significant role (Escaravage et al., 1998). Hence among hybrid derivatives at HDB, 14 of 14 genets were  $F_1$ s, whereas between 19 and 21 of 22 detected hybrid genets at ZJY were  $F_1$ s (Table S2 in Supplementary Data).

$F_1$  hybrids between *R. delavayi* and *R. irroratum* are known to be fertile based on field observation and seed germination tests (H. G. Zha, unpubl. res.), and the presence of non- $F_1$  hybrid derivatives at ZJY. Hence populations of *R. × agastum* at HDB are  $F_1$ -dominated hybrid zones, where selection appears to remove hybrid derivatives of subsequent generations before they reach adulthood (Kyhos et al., 1981; Milne et al., 2003). At ZJY, however, the situation is more complex, with a minority of second- or later-generation hybrid derivatives reaching adulthood, a situation also observed in *Rhododendron × intermedium* (Milne and Abbott, 2008) and *Phyllodoce* (Kameyama et al., 2008). The difference in population structure between ZJY and HDB indicates that there might be some factor affecting the relative fitness of  $F_1$  and other hybrid derivatives between the two sites, of which the most likely is habitat-mediated selection.

#### Asymmetric hybridization and *R. agastum*

Asymmetrical hybridization in plants is relatively common (Barton and Hewitt, 1985; Brubaker et al., 1993; Cruzan and

Arnold, 1994; Arnold, 1997; Caraway et al., 2001; Wu and Campbell, 2005), and various causes for this have been suggested. Unilateral incompatibility (Harrison and Darby, 1955; Lewis and Crowe, 1958; Gore et al., 1990; Harder et al., 1993) can be ruled out as a cause because occasional hybrids with *R. irroratum* as maternal parent do occur at ZJY. Of the parents of *R. × agastum*, *R. delavayi* is self-incompatible and *R. irroratum* is self-compatible (Zhang et al., 2007b), so the greater tendency of the former species to outcross could lead to it producing more hybrid seed; however, it might be more common for the self-compatible species in such situations to be the maternal parent (M. Arnold, University of Georgia, USA, pers. comm.). Pollen of short-styled species tends to be less successful in forming complete pollen tubes in long-styled species, relative to the other way around (Briggs, 1964; Williams and Rouse, 1988); however, based on our specimens no significant difference was found between flower size and style length between the mature flowers of the parent species ( $|t| < t_{0.2}$ ; H. G. Zha, unpubl. res.).

A probable cause for asymmetry in this case is phenology. *Rhododendrons* are protandrous, and differences in flowering time could lead to an earlier flowering species tending to receive mainly heterospecific pollen onto stigmas towards the end of its flowering period, so favouring it as maternal parent for hybrids. At HDB, *R. delavayi* flowers about 1 month earlier than *R. irroratum* (H. G. Zha, pers. observ.), so phenology would make this species the normal maternal parent, as was observed in all *R. agastum* from this site. However, at ZJY the phenology of the species is different, and they have a longer overlap of flowering time, which could account for the less pronounced asymmetry in parentage at this site.

A third possibility is pollinator behaviour. Some pollinators such as *Apis cerana cerana* are common to both species (Zhang et al., 2007b), but *R. delavayi* has bright red flowers, whereas *R. irroratum* has pale flowers. Hence the possibility exists that pollinators commonly switch from *R. irroratum* to the more strikingly coloured *R. delavayi*, but seldom do the reverse; this would account for the asymmetric hybridization seen. The more open habitat at ZJY could affect pollinator behaviour (e.g. perhaps they can see further) and/or which pollinator species are present, either of which could possibly lead to bidirectional crossing at one site but not the other.

#### Between-site comparisons

The *R. agastum* populations at HDB and ZJY differed in two respects: first, the HDB population comprised only  $F_1$ s, whereas other classes were present in small numbers at ZJY; and second, crossing was bidirectional at ZJY, whereas only *R. delavayi* was maternal parent at HDB. The major difference between the two sites was anthropogenic disturbance at ZJY, at which the forest canopy had been completely removed, but not at HDB, where it was intact. Such disturbance has been shown to extend and alter flowering periods in other species (Lamont et al., 2003) and so could account for the increased overlap at ZJY and hence for the occasional hybrids for which *R. irroratum* is maternal parent. Habitat disturbance has long been known to promote hybridization (Anderson, 1948,

1949; Anderson and Stebbins, 1954; Semple and Semple, 1977; Rieseberg and Carney, 1998; Bleeker and Hurka, 2001; Lamont *et al.*, 2003) and in some cases can profoundly affect which hybrid classes are present (Kyhos *et al.*, 1981; Milne *et al.*, 2003), possibly because disturbance can favour segregating hybrid derivatives (Abbott, 1992; Arnold, 1997; Rieseberg and Carney, 1998). This provides a possible explanation for why hybrid generations after the  $F_1$  are present at ZJY but not at HDB. Therefore, in natural conditions where the habitat is undisturbed, as at HDB, hybridization produces only  $F_1$ s with *R. delavayi* mothers, which cannot bring about gene flow between the parent species, and might even reinforce species barriers if  $F_1$ s out-compete other hybrid generations through superior fitness (Milne *et al.*, 2003). Conversely, at ZJY, hybridization may proceed beyond the  $F_1$  and the direction of crossing is variable, making inter-specific gene flow or the evolution of new hybrid taxa a possibility. Further work is now required to confirm that these differences in population structure are indeed due to habitat disturbance at ZJY.

## CONCLUSIONS

This study has shown that at least some populations of *R. agastum* are the hybrid *R. delavayi* × *R. irroratum*, with the former the usual maternal parent and most hybrids being  $F_1$ s. Hybrids of the combination *R. delavayi* × *R. decorum* may also have been referred to as *R. agastum*, but the type for the name *R. agastum* matches *R. delavayi* × *R. irroratum*. Like *R. × sochadzeae* from Turkey, *R. delavayi* × *R. irroratum* forms populations comprising only  $F_1$ s in undisturbed habitats, which means that such hybrid populations could have existed indefinitely without any gene flow between the parents having occurred. That a small number of other hybrid classes occur at the heavily disturbed ZJY site indicates the possibility that human disturbance might bring about a small increase in the incidence of backcrossing, and hence introgression; however, the effect of disturbance on this taxon appears far smaller than is seen in *R. × sochadzeae* (Milne *et al.*, 2003) or *Encelia × laciniata* (Kyhos *et al.*, 1981).

Future work on *Rhododendron* hybrid zones, in both these species and others, should concentrate on comparing population structure in disturbed and pristine habitats, and establishing a causal relationship between habitat disturbance and changes in the pattern of hybridization.

## SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following tables. Table S1: Probabilities for three hybrid genotype categories calculated using binomial distribution. Table S2: Morphological identification, ITS types, chloroplast haplotypes and identity according to NewHybrids analysis of AFLP data, for all accessions examined. Table S3: AFLP data for NewHybrids and direct mathematical analysis for both sites.

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## LITERATURE CITED

- Abbott RJ. 1992.** Plant invasions, interspecific hybridization and the evolution of plant taxa. *Trends in Ecology and Evolution* **7**: 401–405.
- Anderson E. 1948.** Hybridization of the habitat. *Evolution* **2**: 1–9.
- Anderson E. 1949.** *Introgressive hybridization*. New York: John Wiley & Sons.
- Anderson E, Stebbins JR Jr. 1954.** Hybridization as an evolutionary stimulus. *Evolution* **8**: 378–388.
- Anderson EC, Thompson EA. 2002.** A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**: 1217–1229.
- Arnold ML. 1997.** *Natural hybridization and evolution*. New York: Oxford University Press.
- Arnold ML. 2004.** Natural hybridization and the evolution of domesticated, pest and disease organisms. *Molecular Ecology* **13**: 997–1007.
- Arnold ML, Hodges SA. 1995.** Are natural hybrids fit or unfit relative to their parents? *Trends in Ecology and Evolution* **10**: 67–70.
- Balfour B. 1917.** *Rhododendrons of the Irroratum Series*. *Transactions of the Botanical Society of Edinburgh* **xxvii**: 157–220.
- Barton NH, Hewitt GM. 1985.** Analysis of hybrid zones. *Annual Review of Ecology and Systematics* **16**: 113–148.
- Bassam BJ, Caetanoanlles G, Gresshoff PM. 1991.** Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry* **196**: 80–83.
- Bleeker W, Hurka H. 2001.** Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology* **10**: 2013–2022.
- Briggs BG. 1964.** The control of interspecific hybridisation in *Darwinia*. *Evolution* **18**: 292–303.
- Brubaker CL, Koontz JA, Wendel JF. 1993.** Bidirectional cytoplasmic and nuclear introgression in the New World cottons, *Gossypium barbadense* and *G. hirsutum* (Malvaceae). *American Journal of Botany* **80**: 1203–1208.
- Campbell DR. 2004.** Natural selection in *Ipomopsis* hybrid zones: implications for ecological speciation. *New Phytologist* **161**: 83–90.
- Campbell D, Duchesne P, Bernatchez L. 2003.** AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology* **12**: 1979–1991.
- Campbell DR, Galen C, Wu CA. 2005.** Ecophysiology of first and second generation hybrids in a natural plant hybrid zone. *Oecologia (Berlin)* **144**: 214–225.
- Caraway V, Carr GD, Morden CW. 2001.** Assessment of hybridization and introgression in lava-colonizing Hawaiian *Dubautia* (Asteraceae: Madiinae) using RAPD markers. *American Journal of Botany* **88**: 1688–1694.
- Chamberlain DF. 1982.** A revision of *Rhododendron* II. subgenus *Hyemanthus*. *Notes from the Royal Botanic Garden, Edinburgh* **39**: 209–486.
- Chamberlain DF, Hyam R, Argent G, Fairweather G, Walter KS. 1996.** *The genus Rhododendron, its classification and synonymy*. Oxford: Alden Press.
- Coyne JA. 1994.** Ernst Mayr and the origin of species. *Evolution* **48**: 19–30.

- Cruzan MB, Arnold ML. 1993.** Ecological and genetic associations in an *Iris* hybrid zone. *Evolution* **47**: 1432–1445.
- Cruzan MB, Arnold ML. 1994.** Assortative mating and natural selection in an *Iris* hybrid zone. *Evolution* **48**: 1946–1958.
- Escaravage N, Questiau S, Pornon A, Doche B, Taberlet P. 1998.** Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology* **7**: 975–982.
- Fang MY, Fang RZ, He MY, Hu LZ, Yang HB, Chamberlain DF. 2005.** *Rhododendron*. In: Wu ZY, Raven PH. eds. *Flora of China*, vol. 14. Beijing and St Louis, Science Press and Missouri Botanical Garden, 260–455.
- Gilbert (nee Stoker) K, Garton S, Karam M, et al. 2002.** A high degree of genetic diversity is revealed in *Isatis* spp. (dyer's woad) by amplified fragment length polymorphism (AFLP). *Theoretical and Applied Genetics* **104**: 1150–1156.
- Gore PL, Potts BM, Volker PW, Megalos J. 1990.** Unilateral cross-incompatibility in *Eucalyptus*: the case of hybridization between *E. globulus* and *E. nitens*. *Australian Journal of Botany* **38**: 383–394.
- Grant V. 1957.** The plant species in theory and practice. In: Mayr E. ed. *The species problem*. Washington, DC: American Association for the Advancement of Science, 39–80.
- Grant V. 1963.** *The origin of adaptations*. New York: Columbia University Press.
- Grant V. 1981.** *Plant speciation*. New York: Columbia University Press.
- Hamilton MB. 1999.** Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**: 521–523.
- Harder LD, Cruzan MB, Thompson JD. 1993.** Unilateral incompatibility and the effects of interspecific pollination for *Erythronium americanum* and *Erythronium albidum* (Liliaceae). *Canadian Journal of Botany* **71**: 353–358.
- Harris SA, Ingram R. 1991.** Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. *Taxon* **40**: 393–412.
- Harrison BJ, Darby LA. 1955.** Unilateral hybridization. *Nature* **176**: 982.
- Johnson JA, Wesselingh RA, Bouck AC, Donovan LA, Arnold ML. 2001.** Intimately linked or hardly speaking? The relationship between genotype and environmental gradients in a Louisiana *Iris* hybrid population. *Molecular Ecology* **10**: 673–682.
- Kameyama Y, Kasagi T, Kudo G. 2008.** A hybrid zone dominated by fertile  $F_1$ s of two alpine shrub species, *Phyllodoce caerulea* and *Phyllodoce aleutica*, along a snowmelt gradient. *Journal of Evolutionary Biology* **21**: 588–597.
- Kobayashi N, Horikoshi T, Katsuyama H, Handa T, Takayanagi K. 1998.** A simple and efficient DNA extraction method for plants, especially woody plants. *Plant Tissue Culture Biotech* **4**: 72–80.
- Kyhos DW, Clark C, Thompson WC. 1981.** The hybrid nature of *Encelia laciniata* (Compositae: Heliantheae) and control of population composition by post-dispersal selection. *Systematic Botany* **6**: 399–411.
- Lamont BB, He T, Enright NJ, Krauss SL, Miller BP. 2003.** Anthropogenic disturbance promotes hybridization between *Banksia* species by altering their biology. *Journal of Evolutionary Biology* **16**: 551–557.
- Levin DA. 2000.** *The origin, expansion, and demise of plant species*. Oxford: Oxford University Press.
- Lewis D, Crowe LK. 1958.** Unilateral interspecific incompatibility in flowering plants. *Heredity* **12**: 233–256.
- Miller LM. 2000.** Classifying genealogical origins in hybrid populations using dominant markers. *Journal of Heredity* **91**: 46–49.
- Milne RI. 2004.** Phylogeny and biogeography of *Rhododendron* subsection *Pontica*, a group with a tertiary relict distribution. *Molecular Phylogenetics and Evolution* **33**: 389–401.
- Milne RI, Abbott RJ. 2008.** Reproductive isolation between two interfertile *Rhododendron* species: low frequency of post- $F_1$  hybrid genotypes in alpine hybrid zones. *Molecular Ecology* **17**: 1108–1121.
- Milne RI, Terzioglu S, Abbott RJ. 2003.** A hybrid zone dominated by fertile  $F_1$ s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology* **12**: 2719–2729.
- Rhode JM, Cruzan MB. 2005.** Contributions of heterosis and epistasis to hybrid fitness. *The American Naturalist* **166**: E124–E139.
- Rieseberg LH, Carney SE. 1998.** Plant hybridization. *New Phytologist* **140**: 599–624.
- Rieseberg LH, Ellstrand NC. 1993.** What can morphological and molecular markers tell us about plant hybridization? *Critical Reviews in Plant Sciences* **12**: 213–241.
- Schluter D. 1998.** Ecological causes of speciation. In: Howard DJ, Berlocher SH. eds. *Endless forms. species and speciation*. Oxford: Oxford University Press, 114–129.
- Seehausen O. 2004.** Hybridization and adaptive radiation. *Trends in Ecology & Evolution* **19**: 198–207.
- Semple JC, Semple KS. 1977.** *Borrchia* × *cubana* (*B. frutescens* × *B. arborescens*): interspecific hybridization in the Florida Keys. *Systematic Botany* **2**: 292–302.
- Taberlet P, Gielly L, Puatou G, Bouvet J. 1991.** Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997.** The Clustal\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.
- Vos P, Hogers R, Bleeker M, et al. 1995.** AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.
- Wang H, McArthur ED, Sanderson SC, Graham JH, Freeman DC. 1997.** Narrow hybrid zone between two subspecies of big sagebrush (*Artemisia tridentata*: Asteraceae). V. Reciprocal transplant experiments. *Evolution* **51**: 95–102.
- White TJ, Bruns T, Lee S, Taylor J. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ. eds. *PCR protocols. A guide to methods and applications*. San Diego: Academic Press, 315–322.
- Whitney KD, Randell RA, Rieseberg LH. 2006.** Adaptive introgression of herbivore resistance traits in the weedy sunflower *Helianthus annuus*. *American Naturalist* **167**: 794–807.
- Williams EG, Rouse JL. 1988.** Disparate style lengths contribute to isolation of species in *Rhododendron*. *Australian Journal of Botany* **36**: 183–191.
- Wu CA, Campbell DR. 2005.** Cytoplasmic and nuclear markers reveal contrasting patterns of spatial genetic structure in a natural *Ipomopsis* hybrid zone. *Molecular Ecology* **14**: 781–792.
- Wu CI. 2001.** The genic view of the process of speciation. *Journal of Evolutionary Biology* **14**: 851–865.
- Zha HG, Milne RI, Sun H. 2008.** Morphological and molecular evidence of natural hybridization between two distantly related *Rhododendron* species from the Sino-Himalaya. *Botanical Journal of the Linnean Society* **156**: 119–129.
- Zhang JL. 2007.** *Natural hybridization origin of Rhododendron agastum (Ericaceae) in Yunnan, China*. Ph. dissertation, The Graduate School of Chinese Academy of Sciences, Beijing, China.
- Zhang JL, Zhang CQ, Gao LM, Yang JB, Li HT. 2007a.** Natural hybridization origin of *Rhododendron agastum* (Ericaceae) in Yunnan, China: inferred from morphological and molecular evidence. *Journal of Plant Research* **120**: 457–463.
- Zhang JL, Zhang CQ, Wu ZK, Qiao Q. 2007b.** The potential roles of inter-specific pollination in natural hybridization of *Rhododendron* species in Yunnan, China. *Biodiversity Science* **15**: 658–665.