Annals of Botany **120**: 51–61, 2017
doi:10.1093/aob/mcx039, available online at https://academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

Asymmetrical natural hybridization varies among hybrid swarms between two diploid *Rhododendron* species

Li-Jun Yan[1,2,3,4], Kevin S. Burgess[5], Richard Milne[6], Chao-Nan Fu[1,3], De-Zhu Li[1,2,3,*] and Lian-Ming Gao[1,*]

[1]Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China, [2]Germplasm Bank of Wild Species in Southwest China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China, [3]University of Chinese Academy of Sciences, Beijing 100049, China, [4]College of Vocational and Technical Education, Yunnan Normal University, Kunming 650092, China, [5]Department of Biology, College of Letters & Sciences, Columbus State University, University System of Georgia, 163A LeNoir Hall, Columbus, GA 31907-5645, USA and [6]Institute of Molecular Plant Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3JH, UK

**For correspondence. Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei Road, Kunming, Yunnan 650201, China. E-mail: gaolm@mail.kib.ac.cn or dzl@mail.kib.ac.cn*

Received: 9 November 2016 Returned for revision: 29 December 2016 Editorial decision: 3 March 2017 Accepted: 14 March 2017
Published electronically: 21 April 2017

• **Background and Aims** The extent to which hybridization leads to gene flow between plant species depends on the structure of hybrid populations. However, if this varies between locations, species barriers might prove permeable in some locations but not in others. To assess possible variation in hybrid population structure, the magnitude and direction of natural hybridization between two Chinese endemic species, *Rhododendron spiciferum* and *Rhododendron spinuliferum*, were evaluated.

• **Methods** Thirteen nuclear microsatellite markers were employed to characterize 566 individuals collected from 15 non-allopatric populations and nine allopatric parental populations. Chloroplast DNA (cpDNA) sequences were obtained from a subset of samples. Genetic structure and direction of gene flow was determined using a combination of STRUCTURE and NEWHYBRIDS analysis.

• **Key Results** Nuclear analysis revealed that parental taxa formed two genetically distinct clusters and hybrids shared the genetic background of both parents and did not form a separate genetic lineage. Overall, hybrid swarms were dominated by early- and later-generation hybrids, with a significantly higher proportion of hybrids (59·6 %) possessing >50 % R. spiciferum-like nuclear germplasm. The cpDNA analysis further indicated that a significantly greater proportion of hybrids (61·1 %) possessed the R. spiciferum cpDNA haplotype.

• **Conclusions** Gene flow between *R. spiciferum* and *R. spinuliferum* was found to be bidirectional in 14 of the 15 hybrid swarms and asymmetrical in six hybrid swarms. Asymmetrical gene flow was evident for only nuclear DNA (nDNA) in two populations, for only cpDNA in three populations, and for both nDNA and cpDNA in one population. Collectively, the variation in genetic structure found among the 15 hybrid swarms suggests that introgression rather than hybrid speciation is a more likely outcome of hybridization between these hybridizing taxa.

Key words: Hybridization, genetic structure, *Rhododendron*, hybrid swarm, microsatellite, *trnL-F*.

INTRODUCTION

The impact of hybridization on the formation and persistence of plant species is diverse and can vary depending on the genetic, demographic and geographical structure of the hybridizing taxa involved (Anderson, 1949; Grant, 1981; Rieseberg and Wendel, 1993; Arnold, 1997, 2006; Liu *et al*., 2014). On the one hand, hybridization can be a destructive force resulting in the extinction of rare species after hybridizing with native congeners (Levin *et al*., 1996; Rhymer and Simberloff, 1996; Wolf *et al*., 2001; Buerkle *et al*., 2003), invasive species (Huxel, 1999) or wild relatives of crop species (Ellstrand *et al*., 1999; Ellstrand, 2003; Haygood *et al*., 2003). On the other hand, hybridization can be a creative force leading to adaptation and sometimes subsequent hybrid speciation by the accumulation of adaptive genetic variation (Anderson, 1948; Harrison, 1990; Arnold, 1992; Rieseberg, 1997; Rieseberg and Carney, 1998; Abbott *et al*., 2013). Apart from blurring species' boundaries and producing evolutionary noise (Slatkin, 1985, 1987; Soltis and Soltis, 2009), in some cases hybridization can have a neutral effect with species barriers maintained due to the production of sterile or unfit hybrid offspring (Arnold, 2006). Depending on the hybridizing taxa involved, an assessment of the evolutionary outcome of hybridization may provide important insight in predicting species persistence and hybrid speciation, as well as the maintenance of biological diversity.

Studies of the genetic structure and ecological dynamics of hybrid swarms, defined as complex mixtures of parental forms, F_1 hybrids, backcross types and segregation products (Grant, 1981), are central to our understanding of the evolutionary processes associated with hybridization. Various barriers to gene flow between the parent species can exist even where hybrids occur, and the type of barriers that apply will depend on the structure of the hybrid swarm (Tiffin *et al*., 2001).

Where hybrids are fertile, hybrid swarms commonly have few or no F_1 hybrids, and are composed primarily of later-generation hybrids, with backcrosses to one or both parents often dominating (Arnold and Hodges, 1995; Arnold, 1997; Arnold *et al*., 1999, 2001; Burke and Arnold, 2001; Fogelqvist *et al*., 2015). In some cases, however, fertile F_1s may dominate by apparently outcompeting later-generation hybrids (e.g. backcross and F_2 offspring), which can in turn effectively prevent interspecific gene flow (Kyhos *et al*., 1981; Milne *et al*., 2003; Kameyama *et al*., 2008; Milne and Abbott, 2008; Christe *et al*., 2016). Therefore, determining the genetic structure of hybrid swarms serves as a critical first step in evaluating the outcomes of hybridization with respect to species formation and persistence.

Hybridization is more common in rapidly diversifying taxa (Grant *et al*., 2005; Mallet, 2007; Gourbière and Mallet, 2010). The genus *Rhododendron* is a large, long-lived woody plant genus that has a history of recent rapid adaptive radiation (Milne *et al*., 2010). For example, the *Rhododendron* sect. *Vireya* diverged in the last 15 million years (Goetsch *et al*., 2011) and most of the Sino-Himalayan members of *Rhododendron* subgenus *Hymenanthes* diverged in the past 5 million years (Milne, 2004). Natural hybridization is common in *Rhododendron*, due to relatively weak reproductive barriers among closely related species and the predominance of outcrossing (Milne *et al*., 2010; Yan *et al*., 2015), although selfing is observed to be possible in some species (Escaravage *et al*., 1997). Hybrid swarm structure within the genus appears to be atypical relative to other plant groups, because F_1 hybrids are a dominant component in some hybrid swarms (Milne *et al*., 2003; Zha *et al*., 2010), whereas in other cases hybrid swarms appear to comprise F_1s plus unidirectional backcrosses (Milne and Abbott, 2008), or a combination of F_1 and F_2 hybrids (Ma *et al*., 2010, 2016). Hybrid swarms comprising solely backcrosses and later-generation hybrids have rarely been documented in the genus, despite being common in other genera, such as *Populus* (Lexer *et al*., 2007) and *Silene* (Minder *et al*., 2007). Previous studies in *Rhododendron* have found large differences in composition between hybrid zones, for the same species pair, although these results are limited to two or three hybrid swarms per species (Milne *et al*., 1999; Zhang *et al*., 2007; Milne and Abbott, 2008; Tagane *et al*., 2008; Zha *et al*., 2010; Marczewski *et al*., 2015). Therefore, a systematic study looking at a large number of potential hybrid swarms would substantially improve our understanding of the plausible range of outcomes that may result from gene flow between interfertile *Rhododendron* species in the wild.

The hybrid *Rhododendron* ×*duclouxii* is reported to occur in the mountains of central Yunnan, southwest China, which is a global biodiversity hotspot, and has been suggested to be a morphologically intermediate 'hybrid species' between *R. spiciferum* and *R. spinuliferum* (Handel-Mazzetti, 1936). Its hybrid status was recently confirmed using both chloroplast *trnL-F* and nuclear internal transcribed spacer (ITS) sequences, as well as morphological analysis (Yan *et al*., 2013), although this assessment was based on surveying only a few hybrid swarms. The distribution of *R. spinuliferum* is wider than that of *R. spiciferum*, with the latter primarily distributed within the area of *R. spinuliferum* (Fig. 1). Where the two species co-occur, a large number of putative hybrid swarms exist across a wide geographical area (300 × 200 km across; Fig. 1), providing an opportunity to examine the hybrid status of *R.* ×*duclouxii* and to compare the magnitude and direction of hybridization across many hybrid swarms. By so doing, we may determine whether hybrid swarm formation, structure and persistence, and hence mechanisms of species barrier maintenance, vary according to local conditions.

A hybrid species should be genetically different from its parental species and be recognized as a self-contained and self-perpetuating genetic lineage (Sun *et al*., 2014). Therefore, determining the genetic structure of hybrids is important for the evaluation of the process of hybrid speciation. In the present study, we evaluated the genetic structure of 24 populations including 14 hybrid swarm populations using 13 nuclear microsatellites and sequences of the chloroplast DNA (cpDNA) *trnL-F* region to address the following questions: (1) Does *R.* ×*duclouxii* constitute a hybrid species, or does it comprise populations of different generations of hybrids, i.e. hybrid swarms? (2) If the latter, what is the genetic structure of *R. spiciferum* and *R. spinuliferum* hybrid swarms, and do they show evidence of asymmetrical hybridization? (3) Do the magnitude and direction of hybridization vary among locations?

MATERIALS AND METHODS

Sampling and DNA isolation

A total of 566 adult individuals were sampled across 24 natural populations in central Yunnan Province, China (Supplementary Data Table S1; Fig. 1). Each individual was identified in the wild as *Rhododendron spiciferum*, *Rhododendron spinuliferum* or hybrid based on the four morphological characters outlined in Supplementary Data Table S2: corolla colour, corolla shape, corolla lobe position and the amount of leaf indumentum. Our sampling included nine allopatric populations, each comprising one parental species, plus 15 non-allopatric populations (14 hybrid swarms and one sympatric population in which the two parental species co-occurred but no hybrids were observed). For each population, individuals were sampled randomly, separated by a minimum of 5 m to minimize the chance of repeated sampling of the same clone. For the hybrid swarm populations, accessions of parental species and hybrids were collected across the centre of the area occupied by the swarm, i.e. where hybrids were most concentrated. In total, from the allopatric populations, 27 *R. spiciferum* individuals were sampled from three populations and 52 *R. spinuliferum* individuals were sampled from six populations. A total of 93 *R. spiciferum*, 141 *R. spinuliferum* and 253 putative hybrids (*R.* ×*duclouxii*) were sampled across the 15 non-allopatric populations. From each individual, fresh, healthy leaves were immediately dried in the field using silica gel. Genomic DNA was isolated with a modified CTAB method (Doyle and Doyle, 1987) and for each accession a corresponding specimen was collected and deposited at the Herbarium of Kunming Institute of Botany (KUN), Chinese Academy of Sciences.

Nuclear microsatellite locus selection and genotyping

Each sample was genotyped for 13 nuclear microsatellite loci tested previously by Yan *et al*. (2014). Details of loci examined

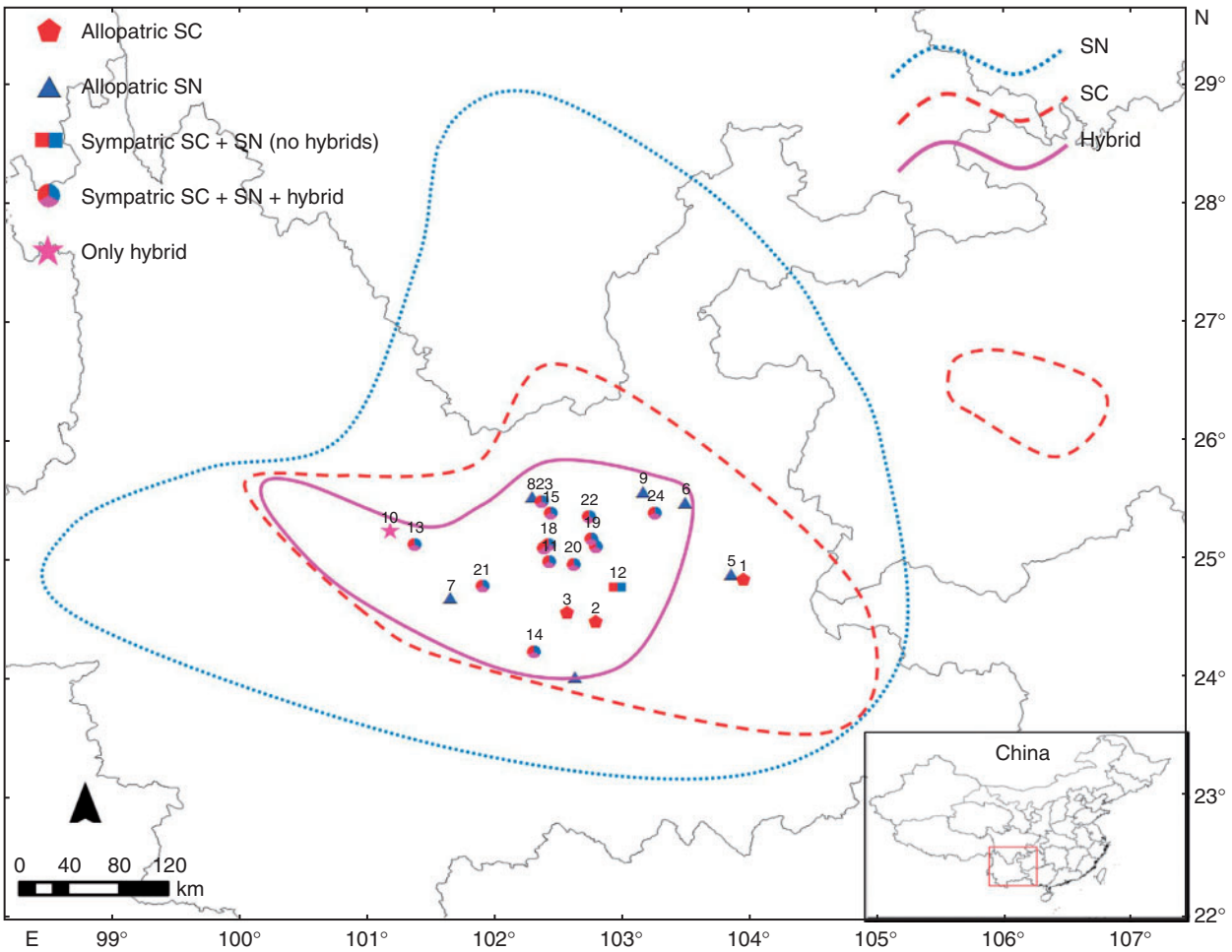

FIG. 1. Geographical distribution of 24 *Rhododendron* populations used in this study outlined in the geographical distribution range of *R. spiciferum*, *R. spinuliferum* and hybrid. SC, *R. spiciferum*; SN, *R. spinuliferum*.

as well as multiplex PCR reaction annealing temperatures are shown in Supplementary Data Table S3. PCR products were directly analysed on a 3730xl Sequence Analyser (Applied Biosystems, Foster City, CA, USA), using a LIZ GeneScan-500 size standard. Resulting electropherograms were visualized and converted to diploid genotypes using automated allele-calling implemented in GENEMARKER v.4.0 (SoftGenetics LLC, State College, PA, USA). All automated genotyping was re-checked manually. In order to transform the microsatellite dataset for further analysis in additional software packages, all genotypes for each locus and individual were entered in an Excel file following the format of GenALEx 6.501 (Peakall and Smouse, 2012) (Supplementary Data Table S4).

Chloroplast DNA genotyping

To determine the cpDNA haplotype of individuals within hybrid swarms and allopatric populations, we sequenced the *trnL-F* region (including the *trnL* intron and *trnL-trnF* intergenic spacer) of the chloroplast genome. Using sequencing protocols outlined in Yan *et al.* (2013), 362 of the 566 accessions sampled were sequenced; based on morphology, these comprised 73 *R. spinuliferum* (20·2 %), 47 *R. spiciferum* (13·0 %) and 242 hybrids (66·9 %). Sequences were edited in Sequencher 5.0 (Gene Codes Corporation) and aligned in Geneious 6.1.6, and all informative sites were summarized in Mega 6 (Tamura *et al.*, 2013). To determine if the proportions of hybrids containing the *R. spiciferum* or *R. spinuliferum trnL-F* haplotype were significantly different from each other, we conducted a series of χ^2 tests in JMP 10 (SAS Institute, 2002).

Population genetic analysis of microsatellite data

Population genetic analyses on microsatellite data were conducted across all sampled individuals. GENODIVE v.2.0b27 (Meirmans and Van Tienderen, 2004) was used to test for departures from Hardy–Weinberg equilibrium, using the heterozygosity-based method (Nei's G_{IS}) and 1000 permutations by locus, as well as by population. Levels of significance were adjusted by Bonferroni correction (Rice, 1989). An assessment of linkage disequilibrium was conducted on each pair of loci using Fisher's exact test, with probability adjusted by Bonferroni correction in ARLEQUIN 3.5.1.3 (Excoffier and Lischer, 2010). In addition, mean allele number per locus (Na),

number of effective alleles (Ne), private allele number (Np), Shannon's information index (*I*), observed heterozygosity (Ho), expected heterozygosity (He) and unbiased expected heterozygosity (uHe) were calculated (Table 1). With individuals assigned to *R. spiciferum*, *R. spinuliferum* or hybrids according to morphology, the basic *F* statistics value (F_{IS}, F_{ST}) between these three groups was evaluated in Fstat v2.9.3.2 (Goudet, 2001). To further evaluate the extent of population differentiation (pairwise F_{ST}) among the three groupings, we analysed the microsatellite data using an analysis of molecular variation (AMOVA) in GENEPOP 4.2 (Rousset, 2008) with default parameter settings.

Parent and hybrid assignment

To explore genetic relationships among parental and hybrid individuals, principal coordinates analysis (PCoA) was conducted. The pairwise genetic distances were evaluated in MICROSATELLITE ANALYSER (MSA) (Dieringer and Schlötterer, 2003) based on Nei's standard genetic distance (Nei, 1978). Distance matrices were executed in GenALEx 6.501 (Peakall and Smouse, 2012) and analysed with the PCoA option. Scatterplots were visualized in two-dimensional space for the first and second, as well as the first and third, principal coordinates.

To estimate the extent of admixture between parental species, all 566 individuals were analysed in STRUCTURE v.2.3.4 (Pritchard *et al*., 2000) using the Bayesian model-based clustering algorithm. Analyses were conducted with a *K* value that varied from 1 to 10, repeating each analysis ten times to evaluate convergence among different runs. The STRUCTURE analysis was conducted under the admixture model with independent allele frequencies. In addition, a burn-in period of 50 000 steps followed by 200 000 Markov chain Monte Carlo (MCMC) iterations were used. We ran STRUCTURE twice. For the first time, no prior species information was included and no popflags were set. The best-fit number of distinct groups (*K*) was estimated by STRUCTURE HARVESTER (Earl, 2012) based on the maximum Δ*K* value (Evanno *et al*., 2005). Based on the optimal *K* value, we ran the second STRUCTURE using prior population information: individuals from allopatric populations were set as known parental species origin (allopatric *R. spinuliferum* individuals: popflag = 1; allopatric *R. spiciferum* individuals: popflag = 2) to classify individuals of unknown origin. In this case, individuals from sympatric populations could be identified more accurately. *Tq* is a threshold value used to determine which class an individual should belong to (De hert *et al*., 2012). The value of *Tq* is important and variable in different taxa: a higher *Tq* value can result in parental taxa misclassified as hybrids and a lower *Tq* can result in hybrids misclassified as parents. *Tq* values used to assign parental and hybrid individuals have varied from 0·80 to 0·95 (Vähä and Primmer, 2006; Araya-Anchetta *et al*., 2013; Starr *et al*., 2013). In this study, we used a relatively strict threshold *Tq* value, 0·9, above which all allopatric parental individuals were correctly assigned except one *R. spinuliferum* sample ($q = 0{\cdot}855$). In addition, the hybrid index was calculated for all 487 individuals from the 15 non-allopatric populations within R with the est.h function from INTROGRESS (Gompert and Alex Buerkle, 2010).

To check the assignment of all accessions to parents (P_1, P_2) and hybrids, NEWHYBRIDS (Anderson and Thompson, 2002) was used to analyse the same microsatellite dataset including (z0 = *R. spiciferum* and z1 = *R. spinuliferum*) and excluding allopatric population information. Additional parameter settings included a burn-in period of 10 000 generations and 100 000 MCMC iterations. Both 'Jeffrey's like priors' and 'Uniform priors' models were tested, but the Uniform priors model was more stable and was selected for all subsequent NEWHYBRIDS analyses. For a broader classification of individuals into each of the three groups (*R. spiciferum*, *R. spinuliferum* and hybrids), a relatively strict posterior probability of 0·8 was used (Smulders *et al*., 2008). In addition, the 45 classes method for NEWHYBRIDS analysis described by Milne and Abbott (2008) was employed to test for the presence of F_1s.

Table 1. *Total sample number (*N*), mean allele number per locus (Na), number of effective alleles (Ne), private allele number (Np), information index (*I*), observed heterozygosity (Ho), expected (He) and unbiased expected (uHe) heterozygosity and the standard* F *statistics (F_{IS}, F_{ST}) over 13 nuclear microsatellite loci in* R. spiciferum, R. spinuliferum *and hybrids*

Species	*N*	Na	Ne	Np	*I*	Ho	He	uHe	F_{IS}	F_{ST}
R. spiciferum	120	15·154	5·940	14	1·964	0·630	0·765	0·768	0·137	0·054
R. spinuliferum	193	15·769	5·608	19	1·873	0·498	0·728	0·730	0·238	0·111
Hybrids	253	18·154	6·571	27	2·116	0·651	0·812	0·814	0·161	0·051

RESULTS

Population genetic analysis

We were able to genotype 566 accessions for all 13 nuclear microsatellite loci screened across nine allopatric populations and 15 non-allopatric populations: only four accessions (0·05 %) failed for any locus, and in each case it was only for one locus (Rh032, Rh058, Rh060 and Rh086). After Bonferroni correction, all 13 loci were found to be in Hardy–Weinberg equilibrium (Supplementary Data Table S5). All loci were tested for linkage disequilibrium with all other loci, and no pairs of loci were found to exhibit linkage disequilibrium (Supplementary Data Table S6). Based on the analysis of 13 nuclear microsatellite loci in parental species and putative hybrids, the putative hybrids had the highest numbers of alleles (18·154), effective alleles (6·571) and private alleles (27). Additionally, the hybrids showed the highest observed (0·651), expected (0·812) and unbiased expected heterozygosity (0·814) (Table 1). Pairwise F_{ST} values between *R. spiciferum*, *R. spinuliferum* and putative hybrids were lowest between *R. spiciferum* and hybrids (0·035), highest between *R. spiciferum* and *R. spinuliferum* (0·170) and intermediate between *R. spinuliferum* and hybrids (0·062). Hence genetic differentiation between the parental species is significantly higher than that between hybrids and each parental species, and the nuclear genetic background of hybrids is generally closer to *R. spiciferum* than *R. spinuliferum*.

Parent and hybrid assignment

The PCoA analysis showed two clear clusters of parental taxa, with putative hybrids distributed between them (Supplementary Data Fig. S1a, b). All morphologically identified *R. spiciferum* individuals were distinguishable from other morphologically identified *R. spinuliferum* individuals, although many putative hybrid individuals overlapped with *R. spiciferum* or *R. spinuliferum* (Fig. S1a, b).

Based on the ΔK statistics result, the optimal K value was 2, with the two divisions corresponding to the parental species of *R. spiciferum* and *R. spinuliferum* (Supplementary Data Fig. S2). Therefore, we ran the second STRUCTURE analysis with prior allopatric species information. The results showed that the two parental species formed distinct genetic groups and the hybrids showed genetic admixture (Fig. 2). The result of K=3 was also used here to test whether the hybrid individuals formed a separate genetic cluster distinct from either parent, as would be expected if *R.* ×*duclouxii* was indeed a stabilized hybrid species. At $K = 3$, hybrids did not form a distinct cluster but again showed admixture between the parents; instead, *R. spinuliferum* was divided into two groups, one of which mainly comprised individuals from allopatric populations (Fig. 2). There was no obvious morphological distinction between these two intraspecific groups. With the threshold of $Tq = 0{\cdot}9$, all non-allopatric individuals were assigned to a hybrid ($q = 0{\cdot}1 - 0{\cdot}9$), *R. spiciferum* ($q < 0{\cdot}1$) or *R. spinuliferum* ($q > 0{\cdot}9$) for STRUCTURE analysis. Of the 15 investigated non-allopatric populations, 14 contained both parents, plus hybrid individuals, whereas population 10 contained only hybrid individuals; the results for the 15 non-allopatric populations were consistent with morphological identification except in population 12, in which morphologically there were no hybrids, while one *R. spiciferum* was identified as hybrid based on STRUCTURE analysis (Table 2; Supplementary Data Table S7).

Across the 15 non-allopatric populations, based on the Tq value of 0·9, the 487 individuals examined comprised 62 *R. spiciferum* (12·7 %), 329 hybrids (67·6 %) and 96 *R. spinuliferum* (19·7 %) (Fig. 3; Table 2). Among these, 82 individuals were assigned to a different group by STRUCTURE than by morphology; these comprised three morphologically identified hybrids that STRUCTURE classified as *R. spiciferum*, plus 46 morphological *R. spinuliferum* individuals and 33 morphological *R. spiciferum* individuals, all of which STRUCTURE classified as hybrids. Hence no individuals identified by morphology as one parental species were found to be the other. Among hybrids, 196 (59·6 %) were closer to *R. spiciferum* according to their q value, whereas significantly fewer (133 [40·4 %]) were closer to *R. spinuliferum* ($\chi^2 = 12{\cdot}14$, $P < 0{\cdot}001$) (Table 2, Table S7). Across all individuals, hybrid indices calculated in R using INTROGRESS were very similar to STRUCTURE results (Table S7); thus, our discussion is mainly based on the STRUCTURE results. Parental and hybrid assignments across the 15 non-allopatric populations based on NEWHYBRIDS analysis were different from results generated by STRUCTURE, comprising 133 *R. spiciferum* (27·3 %), 243 hybrids (49·9 %) and 111 *R. spinuliferum* (22·8 %). A total of 88 accessions were inconsistently assigned between NEWHYBRIDS and STRUCTURE (Table S7).

According to the 45 classes NEWHYBRIDS analysis, no individual detected had greater than a 16 % chance of being classified as F_1-type, i.e. either F_1 or F_1-like (i.e. a cross between a BC_2 to one species and a pure parental of the other; Milne and Abbott, 2008). This analysis struggled to assign individuals to specific classes with any confidence, which is the expected outcome when many hybrid derivatives of the third generation or beyond are present. Based on summing the probabilities for each class, the analysis estimated that the 566 accessions comprised 143 *R. spiciferum* (25 %), 115 *R. spinuliferum* (20 %), 5 F_1s (1 %), 73 F_2s (or other complex intermediate hybrid

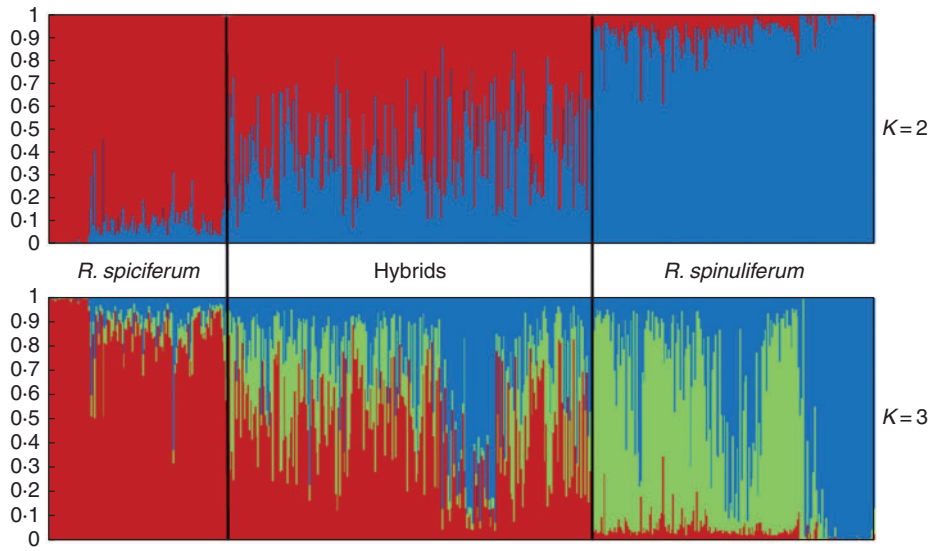

FIG. 2. Results of STRUCTURE analysis for the $K = 2$ and $K = 3$ models of 566 samples, including allopatric population information based on 13 microsatellite loci. All samples were arranged according to morphological identification. The scale on the *y* axis means the rate of the genetic composition for each sample in structure analysis.

TABLE 2. *Number of parental and hybrid individuals based on nuclear microsatellite STRUCTURE analysis across 15 non-allopatric populations in Yunnan Province, China.* χ^2 *tests were performed between the number of hybrids having* > *50 %* R. spiciferum*-like germplasm (range 0·10–0·50) or* > *50 %* R. spinuliferum*-like germplasm (range 0·51–0·90), and between the ratio of SC (*R. spiciferum*) to SN (*R. spinuliferum*)* trnL-F *haplotypes for randomly selected hybrid individuals*

Population	Population size	STRUCTURE assignment				Hybrid
		SC 0·00–0·10	SN 0·90–1·00	Hybrid 0·10–0·90	Hybrid (0·10–0·50):(0·51–0·90)	*trnL-F* genotype SC:SN
10	20	0	0	20	7:13	16:3**
11	35	4	2	29	20:9*	12:12
12	22	8	13	1	1:0	–
13	40	2	12	26	16:10	15:7
14	36	8	10	18	10:8	8:7
15	30	2	6	22	14:8	8:9
16	11	2	3	6	3:3	4:2
17	51	2	13	36	23:13	24:11*
18	35	8	5	22	13:9	9:12
19	53	8	5	40	26:14	23:16
20	20	3	4	13	5:8	3:6
21	40	2	6	32	15:17	19:5**
22	40	7	10	23	13:10	10:9
23	34	3	5	26	18:8*	14:12
24	20	3	2	15	12:3*	11:1**
Total	487	62	96	329	196:133***	176:112***

*P<0·05; **P<0·01; ***P<0·001; – no comparison because only one hybrid was found.

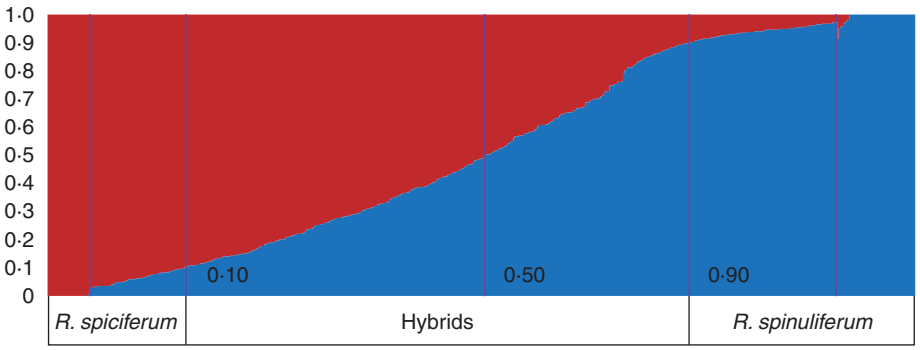

FIG. 3. Results of STRUCTURE analysis for the $K = 2$ model on the 566 samples, including allopatric population information (far left and far right regions of each diagram) based on 13 microsatellite loci. The genetic components from *R. spiciferum* and *R. spinuliferum* are coloured red and blue, respectively. The scale on the *y* axis means the rate of the genetic composition for each sample in structure analysis.

derivatives; 13 %), 164 backcrosses to *R. spiciferum* (29 %) and 66 backcrosses to *R. spinuliferum* (12 %) (Table S7).

Each of the 362 accessions that were sequenced for the *trnL-F* region had either the *R. spiciferum* or the *R. spinuliferum* haplotype previously identified by Yan *et al.* (2013). Across all populations, all individuals identified as *R. spiciferum* based on morphology had the *R. spiciferum* haplotype, as did individuals assigned to *R. spiciferum* in STRUCTURE. Similarly, all individuals identified as *R. spinuliferum* by morphology with the *R. spinuliferum* haplotype were assigned to *R. spinuliferum* in STRUCTURE (Table S6). Among the 329 hybrids assigned by STRUCTURE analysis, 288 had their cpDNA haplotype determined. Of these, a significantly greater proportion (61·1 % [176]) possessed the *R. spiciferum* haplotype relative to the 38·9 % (112) that possessed the *R. spinuliferum* haplotype ($\chi^2 = 14{\cdot}34$, $P < 0{\cdot}001$) (Table 2). In four of the 15 populations with hybrids that were examined, there was a significantly greater proportion of the *R. spiciferum trnL-F* haplotype present among hybrid individuals examined (P10, P17, P21 and P24), while in the other 11 populations there was no significant difference between the proportion of *R. spiciferum* and *R. spinuliferum* haplotypes among hybrid individuals (Table 2).

DISCUSSION

Our molecular evidence indicated that '*R.* ×*duclouxii*' comprises natural hybrid individuals between *R. spiciferum* and *R. spinuliferum*. The two parental species are clearly separable based on nuclear microsatellite analysis (Figs 2 and 3), and the hybrids form a complete spectrum of genotypes from one parent to the other (Figs 3–5; Table S7), indicating that backcrossing in both directions is common. In addition, hybrid individuals could have the chloroplast *trnL-F* haplotype of either parent. Hence the hybrids did not form a consistent and self-perpetuating lineage and therefore do not constitute a stabilized hybrid species; instead, the hybrid populations represent a

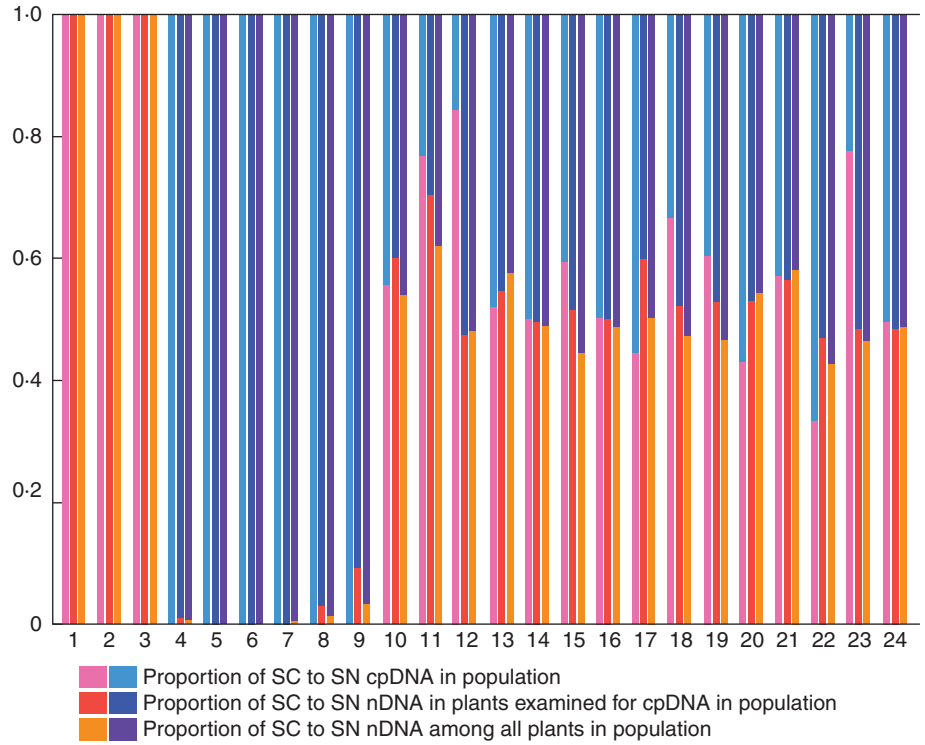

FIG. 4. Relative proportions of *R. spiciferum* (SC) and *R. spinuliferum* (SN) cpDNA haplotype and nuclear DNA composition across all 24 populations examined.

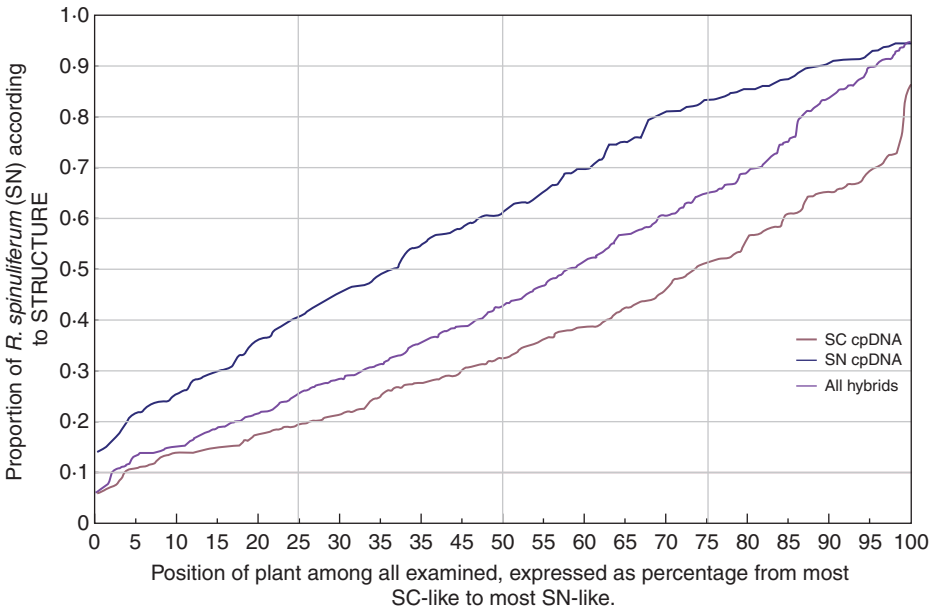

FIG. 5. Proportions of *R. spinuliferum* (SN) to *R. spiciferum* (SC) germplasm calculated by STUCTURE, across all individuals examined in 15 non-allopatric populations (10–24), with individuals arranged from most SN-like on left to most SC-like on right, and expressed as percentiles along the *x*-axis. The purple line shows all individuals examined, and the blue and red lines only those determined to have SN and SC *trnL-F* cpDNA haplotypes, respectively.

series of hybrid swarms. Across all 15 non-allopatric populations examined, F_1s appeared to be rare or absent, and a majority of hybrids possessed more *R. spiciferum* than *R. spinuliferum* germplasm, indicating that hybridization is asymmetrical, with more frequent backcrossing towards *R. spiciferum*. However, when these populations are analysed separately the magnitude and direction of hybridization between *R. spiciferum* and *R. spinuliferum* varies.

Magnitude and direction of hybridization

When combining data from all 15 non-allopatric populations, both nuclear microsatellite and chloroplast *trnL-F* analyses consistently indicated that hybridization in *R. spiciferum* and *R. spinuliferum* is bidirectional but asymmetrical (Table 2; Fig. 3). Overall, there were significantly more hybrids with the nuclear germplasm and *trnL-F* haplotype of *R. spiciferum* than *R. spinuliferum* (Table 2). In three of the 15 populations (P11, P23 and P24) there was a significantly greater number of hybrid individuals with >50 % *R. spiciferum* nuclear germplasm compared with those with >50 % *R. spinuliferum*, indicating more backcrossing towards *R. spiciferum*. Among the remaining populations, eight had a majority of hybrid individuals containing >50 % *R. spiciferum* nuclear germplasm, but differences were not significant (Table 2). Similarly, among the 15 populations containing hybrids, four (P10, P17, P21 and P24) had significantly more hybrids with the *R. spiciferum* than with the *R. spinuliferum trnL-F* haplotype (Table 2; Fig. 4) and six showed similar trends, but the differences were not significant. This strong asymmetry indicates that *R. spiciferum* is favoured as the maternal (cpDNA) parent in these populations, which could be because hybrids possessing this cpDNA haplotype descended from a single F_1 possessing *R. spiciferum* cpDNA.

Collectively, our results indicated that a total of six populations showed significant patterns of asymmetrical hybridization for (1) both cpDNA and nuclear DNA (nDNA) (P24), (2) only nDNA (P11 and P23) and (3) only cpDNA (P10, P17 and P21). In these cases, asymmetrical hybridization may be due to three possible mechanisms. First, differences in flowering phenology may be playing an important role in determining the direction of hybridization as well as the extent of introgression in hybridizing *Rhododendron* populations (Milne and Abbott, 2008). Based on field observations, *R. spinuliferum* nears the end of flowering as *R. spiciliferum* reaches peak anthesis (L. J. Yan, unpubl. res.). This may provide more opportunities for bees to move pollen from the anthers of *R. spinuliferum* to the stigmas of *R. spiciferum* than vice versa, which should favour *R. spiciferum* as the maternal parent of F_1s. Given that cpDNA appears to be maternally inherited in *Rhododendron* (Zhang *et al.*, 2007), this could account for a bias in favour of *R. spiciferum* cpDNA in hybrids. In addition, the flowering time of hybrids overlaps longer with that of *R. spiciferum* (L. J. Yan, unpubl. res.), which might lead to more frequent backcrossing with this parent than the other, which would also account for the greater proportion of hybrids approaching this species in genotype.

A second possible mechanism driving asymmetrical hybridization and gene flow is pollinator behaviour. Based on pollinator observations in population 17, where cpDNA analysis also showed patterns reflecting asymmetrical hybridization (Table 2), bumblebees (*Bombus* spp.) and honey bees (*Apis cerana*) are common pollinators to both species and hybrids, but hornets (*Vespa* spp.) and solitary bees (*Osmia* spp.) typically only pollinate *R. spiciferum* and hybrids (L. J. Yan, unpubl. res.). This might be because *R. spinuliferum* has red tubular flowers, where nectar is less accessible than in the more open funnel-form flowers of *R. spiciferum*. Therefore, in some populations bumblebee and honeybee visitation may facilitate bidirectional hybridization, whereas asymmetrical patterns of hybridization may be caused by other pollinators transferring pollen solely between hybrids and one of the parent species. Similar patterns have been found in *Iris* hybrid zones (Emms and Arnold, 2000; Wesselingh and Arnold, 2000; Martin *et al.*, 2008). For instance, hummingbirds prefer *Iris fulva* and tend to transfer pollen between F_1 hybrids and this parental species, whereas bumblebees tend to transfer pollen between F_1 hybrids and *Iris hexagona* (Emms and Arnold, 2000). The extent of pollinator specialization in *Rhododendron* hybrid swarms remains to be tested.

Finally, asymmetries in the composition of the pollen pool may be another mechanism driving asymmetrical hybridization and gene flow in our hybrid zones. Typically, *R. spinuliferum* plants are larger, have more flowering branches and have more pollen grains in their anthers (L. J. Yan, unpubl. res.). Extreme differences in the pollen pool composition resulting in asymmetry in the pollen pool have been shown in mulberry hybrid zones, where 92 % of the pollen pool was from hybrids and *Morus alba*, while only 8 % was from *Morus rubra*. This, in turn, facilitates asymmetrical gene introgression via interspecific ovule discounting (Burgess *et al.*, 2008). Although such pollen asymmetries in our *Rhododendron* hybrid swarms have not been fully evaluated, their potential contribution to hybrid swarm structure, coupled with asymmetries in flowering time and pollinator preference, provide an additional mechanistic explanation for the asymmetrical patterns of hybrid swarm structure found in this study.

The presence of asymmetrical patterns of hybridization and gene flow in some populations but little to none in others may also reflect the age of a hybrid swarm, and how it formed. If the hybrids in a hybrid swarm originated from a single F_1, most of them would have that F_1 as a maternal ancestor, because the mechanisms that restrict hybrid formation between the parent species would also limit backcrossing to them, by restricting the deposition, germination or growth of dissimilar pollen, including F_1 pollen (Campbell *et al.*, 2003). No such limits would apply to F_1 mothers, which can only self, breed with other hybrids, or be pollinated by a parental individual. However, the occasional exception to this would gradually introduce the cpDNA of the other species into a long-lived hybrid swarm. Therefore, hybrid swarms with a strong cpDNA bias might have originated recently, with most cpDNA coming from the founding F_1 hybrids, whereas those with a more even mix are either older or originated from multiple F_1s, including at least one with the cpDNA of each parent species.

Do cpDNA and nuclear population structure vary across the 15 non-allopatric populations?

Hybrids in most of the non-allopatric populations showed concordant patterns in their nuclear composition and cpDNA haplotypes. Specifically, hybrids with *R. spiciferum* cpDNA tended to have >50 % *R. spiciferum* germplasm. However, population 10 and population 21 displayed the reverse pattern, in which a significantly higher number of hybrids had the *R. spiciferum* cpDNA haplotype, whereas the majority of hybrids had >50 % *R. spinuliferum* germplasm (Table 2). This could result from an original F_1 with *R. spiciferum* cpDNA that mostly backcrossed towards *R. spinuliferum*. Otherwise, it could reflect chance, or the age of contact between parent

species either promoting or restricting hybridization. All except one of the non-allopatric populations examined in this study included both parental species and hybrids, although the number of hybrids varied among populations (Table 2).

While most populations contained a spectrum of nuclear genotypes, from intermediates to backcrosses approaching each parent, there were exceptions to this pattern. In population 10, 18 of the 20 hybrid-like individuals had between 25 and 75 % nDNA of *R. spinuliferum*, whereas only two individuals appeared to be second-generation backcrosses. While this could be the result of hybrids breeding with one another, it may simply be a young hybrid swarm, where most hybrids are second generation, and later-generation backcrosses have not had time to form.

Potential effects of hybridization

Based on the present genetic evidence, the outcome of natural hybridization in this study could be multifold. According to our 45 classes NEWHYBRIDS analyses, F_1 individuals are rare, and possibly absent among all hybrid individuals detected here (Supplementary Data Table S8). This finding differs from previous hybridization studies on *Rhododendron*, in which hybrid zones tend to be dominated by F_1-generation hybrids (Milne *et al*., 2003; Milne and Abbott, 2008; Zha *et al*., 2010), although one example is known to contain eight F_2 hybrids and two F_1s but no detectable backcrosses (Ma *et al*., 2010). In populations where F_1s dominate, it is presumed to be due to their habitat-mediated superiority, which could prevent gene flow between their parental species by F_1s outcompeting other hybrid classes (Milne *et al*., 2003; Christe *et al*., 2016). Our results, however, are similar to previous reports of the structure of hybrid swarms/zones formed by many other naturally hybridizing plant taxa (e.g. Cruzan and Arnold, 1993; Arnold, 2006; Arnold *et al*., 2010; Zeng *et al*., 2011), in which F_1s are rare to absent. Rarity of F_1s in hybrid populations may in some degree be due to fairly strong prezygotic reproductive isolation (Arnold *et al*., 2010), minimizing initial F_1 formation events. Conversely, once an F_1 is formed, all of its descendants will be hybrid derivatives, and these can hence rapidly multiply in number, producing later-generation hybrids, from which the transfer of novel alleles between hybridizing lineages via repeated backcrossing (introgression) becomes possible (Arnold and Hodges, 1995; Rieseberg, 1995; Arnold *et al*., 1999, 2001, 2003). In *Rhododendron*, seeds are formed in such massive numbers that even a tiny proportion of F_1 seed can lead to F_1-dominated hybrid zones, where a strong fitness advantage exists for F_1 seeds (Milne *et al*., 2003). In the current study, therefore, there appears to be no such advantage for F_1s, although we have not tested this directly.

Certain population genetic parameters examined indicated that hybrids are more variable than their parents (Table 1), but this result is likely an artefact of sample size whereby the detection of rare alleles, for example, may be greater for hybrid classes compared with the parental populations. The fact that observed heterozygosity (Table 1) in hybrids was more comparable to *R. spiciferum* than *R. spinuliferum* indicates a plausible outcome of the magnitude of asymmetrical hybridization found in our study. Furthermore, the presence of second- and possibly third-generation backcrosses among the individuals examined indicates a strong potential for introgression in both directions. The detection of introgressed individuals requires markers that are strongly species-specific, such that the detection of one such marker in individuals resembling the other species would reveal interspecific gene flow. In the current study, only cpDNA haplotypes were species-specific, with none present in the other species. The lack of species-specific nuclear markers might indicate recent divergence between these species or the occurrence of ongoing gene flow between them on a broad scale, according to Wu's (2001) genic species concept.

Although some hybrids have formed a single population in our study (population 10) and are separate from other parental populations to some degree, the contribution of *R. spinuliferum* to their nDNA composition varies between 25 and 85 %, indicating that most of these individuals belong to F_1/F_2 hybrids or BC_1 hybrid classes. Thus, even in this extreme case, where the potential for hybrid speciation through reproductive isolation of this hybrid population is plausible, collectively our results indicate that hybridization between *R. spiciferum* and *R. spinuliferum* is more likely to lead to introgression than to hybrid speciation.

CONCLUSIONS

Collectively, our cpDNA and nuclear microsatellite analyses confirm that *R.* ×*duclouxii* is not a hybrid species but rather comprises a range of natural hybrids between *R. spiciferum* and *R. spinuliferum*. Therefore, the 'hybrid species' concept of *R.* ×*duclouxii* is untenable. Our results further indicate that hybridization between *R. spiciferum* and *R. spinuliferum* is bidirectional but asymmetrical in some populations, and that the magnitude and direction of hybridization varies among hybrid swarms. Although our results indicate that hybridization between *R. spiciferum* and *R. spinuliferum* is more likely to lead to introgression than to hybrid speciation in our hybrid swarms, to fully evaluate the factors controlling gene flow and species barrier maintenance in this system, a thorough assessment of the breeding system and fitness attributes of this hybridizing group is necessary.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: principal coordinates analysis for 566 accessions of *R. spiciferum*, *R. spinuliferum* and their hybrids based on genetic distances, showing results for (a) the first and second principal coordinates and (b) the first and third principal coordinates. Figure S2: ΔK statistics ($K = 1$–10) based on STRUCTURE analysis of 566 individuals of *R. spiciferum*, *R. spinuliferum* and their hybrids with prior allopatric population information included in the analysis; the result indicated the optimal K value is 2. Table S1: collection information for nine allopatric and 15 non-allopatric populations in Yunnan Province, China. Table S2: morphological differences among *R. spiciferum*, hybrid and *R. spinuliferum*. Table S3: characteristics of 13 nuclear microsatellite loci used in this study. Table S4: genotypes of 566 accessions of *R. spiciferum*, *R. spinuliferum* and their hybrids with 13 nuclear

microsatellite loci arranged in Excel using GenALEx 6.501. Table S5: *P* values for Hardy–Weinberg equilibrium test based on Nei's G_{is} analysed in GenoDive 2.0b27. Table S6: test of linkage disequilibrium across all loci. Table S7: identification of 566 accessions of *Rhododendron* based on morphological, cpDNA (*trnL-F*) haplotype and nuclear (STRUCTURE, hybrid index, NEWHYBRIDS) analyses. Table S8: group assignment using the 45 classes method for NEWHYBRIDS.

ACKNOWLEDGEMENTS

We are grateful to Drs Jing-Li Zhang, Yong-Peng Ma and Jie Liu, Miss Miao-Miao Li, Mrs Xian-Liang Gao and Mr Hou-Chen Xi for their assistance in data analysis or field collection. We also thank the two anonymous reviewers for their constructive comments on the manuscript. This study was supported by the National Key Basic Research Program of China (2014CB954100), the National Natural Science Foundation of China (Y61F011211), the Key Research Program of the Chinese Academy of Sciences (KJZD-EW-L07, KSCX2-EW-Q-10) and the CAS President's International Fellowship Initiative (2017VBB0008). L.J.Y. was financially supported by the China Scholarship Council as a 1-year Visiting Scholar at Columbus State University, University System of Georgia, Georgia, USA.

LITERATURE CITED

Abbott R, Albach D, Ansell S, *et al.* 2013. Hybridization and speciation. *Journal of Evolutionary Biology* **26**: 229–246.

Anderson E. 1948. Hybridization of the habitat. *Evolution* **2**: 1–9.

Anderson E. 1949. *Introgressive hybridization*. New York: Wiley.

Anderson E, Thompson E. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**: 1217–1229.

Araya-Anchetta A, Scoles G, Giles J, Busch J, Wagner D. 2013. Hybridization in natural sympatric populations of *Dermacentor* ticks in northwestern North America. *Ecology and Evolution* **3**: 714–724.

Arnold ML. 1992. Natural hybridization as an evolutionary process. *Annual Review of Ecology and Systematics* **23**: 237–261.

Arnold ML. 1997. *Natural hybridization and evolution*. Oxford: Oxford University Press.

Arnold ML. 2006. *Evolution through genetic exchange*. New York: Oxford University Press.

Arnold ML, Hodges SA. 1995. Are natural hybrids fit or unfit relative to their parents? *Trends in Ecology and Evolution* **10**: 67–71.

Arnold ML, Bulger MR, Burke JM. 1999. Natural hybridization: how low can you go and still be important? *Ecology* **80**: 371–381.

Arnold ML, Kentner EK, Johnston JA, Cornman S, Bouck AC. 2001. Natural hybridisation and fitness. *Taxon* **50**: 93–104.

Arnold ML, Bouck AC, Cornman RS. 2003. Verne Grant and Louisiana Irises: is there anything new under the sun? *New Phytologist* **161**: 143–149.

Arnold ML, Tang S, Knapp SJ, Martin NH. 2010. Asymmetric introgressive hybridization among Louisiana *Iris* species. *Genes* **1**: 9–22.

Buerkle CA, Wolf DE, Rieseberg LH. 2003. The origin and extinction of species through hybridization. In: Bringham CA, Swartz MW, eds. *Population viability in plants: conservation, management, and modeling of rare plants*. New York: Springer, 117–141.

Burgess KS, Morgan M, Husband BC. 2008. Interspecific seed discounting and the fertility cost of hybridization in an endangered species. *New Phytologist* **177**: 276–284.

Burke JM, Arnold ML. 2001. Genetics and the fitness of hybrids. *Annual Review of Genetics* **35**: 31–52.

Campbell DR, Alarcon R, Wu CA. 2003. Reproductive isolation and hybrid pollen disadvantage in *Ipomopsis*. *Journal of Evolutionary Biology* **16**: 536–540.

Cristescu ME, Constantin A, Bock DG, Cáceres CE, Crease TJ. 2012. Speciation with gene flow and the genetics of habitat transitions. *Molecular Ecology* **21**: 1411–1422.

Christe C, Stölting KN, Paris M, Fraïsse C, Birerne N, Lexer C. 2016. Adaptive evolution and segregating load contribute to the genomic landscape of divergence in two tree species connected by episodic gene flow. *Molecular Ecology* **25**: 2482–2498.

Cruzan MB, Arnold ML. 1993. Ecological and genetic associations in an *Iris* hybrid zone. *Evolution* **47**: 1432–1445.

De hert K, Jacquemyn H, Van Glabeke S, *et al.* 2012. Reproductive isolation and hybridization in sympatric populations of three *Dactylorhiza* species (Orchidaceae) with different ploidy levels. *Annals of Botany* **109**: 709–720.

Dieringer D, Schlötterer C. 2003. Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes* **3**: 167–169.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Earl DA. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**: 359–361.

Ellstrand NC. 2003. Current knowledge of gene flow in plants: implications for transgene flow. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **358**: 1163–1170.

Ellstrand NC, Prentice HC, Hancock JF. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Systematics* **30**: 539–563.

Emms SK, Arnold ML. 2000. Site-to-site differences in pollinator visitation patterns in a Louisiana *Iris* hybrid zone. *Oikos* **91**: 568–578.

Escaravage N, Pornon A, Doche B, *et al.* 1997. Breeding system in an alpine species: *Rhododendron ferrugineum* L. (Ericaceae) in the French northern Alps. *Canadian Journal of Botany* **75**: 736–743.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611–2620.

Excoffier L, Lischer HE. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564–567.

Fogelqvist J, Verkhozina AV, Katyshev AI, *et al.* 2015. Genetic and morphological evidence for introgression between three species of willows. *BMC Evolutionary Biology* **15**: 1–10.

Goetsch LA, Craven LA, Hall BD. 2011. Major speciation accompanied the dispersal of Vireya rhododendrons (Ericaceae, *Rhododendron* sect. *Schistanthe*) through the Malayan archipelago: evidence from nuclear gene sequences. *Taxon* **60**: 1015–1028.

Gompert Z, Buerkle CA. 2010. INTROGRESS: a software package for mapping components of isolation in hybrids. *Molecular Ecology Resources* **10**: 378–384.

Goudet J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). http://www2.unil.ch/popgen/softwares/fstat.htm (last accessed 28 July 2016).

Gourbière S, Mallet J. 2010. Are species real? The shape of the species boundary with exponential failure, reinforcement, and the "missing snowball". *Evolution* **64**: 1–24.

Grant PR, Grant BR, Petren K. 2005. Hybridization in the recent past. *American Naturalist* **166**: 56–67.

Grant VR 1981. *Plant speciation*, 2nd edn. New York: Columbia University Press.

Handel-Mazzetti H. 1936. Die Abnahme eines Teiles verpflichtet zur Abnanme des ganzen Werkes. *Symbolae Sinicae* **7**: 775.

Harrison RG. 1990. Hybrid zones: windows on evolutionary process. In: Futuyma D, Antonovics J, eds. *Oxford surveys in evolutionary biology*. New York: Oxford University Press, 69–128.

Haygood R, Ives A, Andow D. 2003. Consequences of recurrent gene flow from crops to wild relatives. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **270**: 1879–1886.

Huxel GR. 1999. Rapid displacement of native species by invasive species: effects of hybridization. *Biological Conservation* **89**: 143–152.

Kameyama Y, Kasagi T, Kudo G. 2008. A hybrid zone dominated by fertile F_1s of two alpine shrub species, *Phyllodoce caerulea* and *Phyllodoce aleutica*, along a snowmelt gradient. *Journal of Evolutionary Biology* **21**: 588–597.

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.

Levin DA, Francisco-Ortega J, Jansen RK. 1996. Hybridization and the extinction of rare plant species. *Conservation Biology* **10**: 10–16.

Lexer C, Buerkle C, Joseph J, Heinze B, Fay M. 2007. Admixture in European *Populus* hybrid zones makes feasible the mapping of loci that contribute to reproductive isolation and trait differences. *Heredity* **98**: 74–84.

Liu BB, Abbott RJ, Lu ZQ, Tian B, Liu JQ. 2014. Diploid hybrid origin of *Ostryopsis intermedia* (Betulaceae) in the Qinghai-Tibet Plateau triggered by Quaternary climate change. *Molecular Ecology* **23**: 3013–3027.

Ma YP, Milne RI, Zhang C, Yang J. 2010. Unusual patterns of hybridization involving a narrow endemic *Rhododendron* species (Ericaceae) in Yunnan, China. *American Journal of Botany* **97**: 1749–1757.

Ma YP, Xie WJ, Sun WB, Marczewski T. 2016. Strong reproductive isolation despite occasional hybridization between a widely distributed and a narrow endemic *Rhododendron* species. *Scientific Reports* **6**: 19146.

Mallet J. 2007. Hybrid speciation. *Nature* **446**: 279–283.

Martin NH, Sapir Y, Arnold ML. 2008. The genetic architecture of reproductive isolation in Louisiana irises: pollination syndromes and pollinator preferences. *Evolution* **62**: 740–752.

Marczewski T, Chamberlain DF, Milne RI. 2015. Hybridization in closely related *Rhododendron* species: half of all species-differentiating markers experience serious transmission ratio distortion. *Ecology and Evolution* **5**: 3003–3022.

Minder AM, Rothenbuehler C, Widmer A. 2007. Genetic structure of hybrid zones between *Silene latifolia* and *Silene dioica* (Caryophyllaceae): evidence for introgressive hybridization. *Molecular Ecology* **16**: 2504–2516.

Meirmans PG, Van Tienderen PH. 2004. genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* **4**: 792–794.

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 among two interfertile *Rhododendron* species: low frequency of post-F1 hybrid genotypes in alpine hybrid zones. *Molecular Ecology* **17**: 1108–1121.

Milne RI, Abbott RJ, Wolff K, Chamberlain DF. 1999. Hybridization among sympatric species of *Rhododendron* (Ericaceae) in Turkey: morphological and molecular evidence. *American Journal of Botany* **86**: 1776–1785.

Milne RI, Terzioglu S, Abbott RJ. 2003. A hybrid zone dominated by fertile F_1s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology* **12**: 2719–2729.

Milne RI, Davies C, Prickett R, Inns LH, Chamberlain DF. 2010. Phylogeny of *Rhododendron* subgenus *Hymenanthes* based on chloroplast DNA markers: between–lineage hybridisation during adaptive radiation? *Plant Systematics and Evolution* **285**: 233–244.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a number of individuals. *Genetics* **89**: 538–590.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* **28**: 2537–2539.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Rhymer JM, Simberloff D. 1996. Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics* **27**: 83–109.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223–225.

Rieseberg LH. 1995. The role of hybridization in evolution: old wine in new skins. *American Journal of Botany* **82**: 944–953.

Rieseberg LH. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**: 359–389.

Rieseberg LH, Carney SE. 1998. Plant hybridization. *New Phytologist* **140**: 599–624.

Rieseberg LH, Wendel JF. 1993. Introgression and its consequences in plants. In: Harrison RG, ed. *Hybrid zones and the evolutionary process*. New York: Oxford University Press, 70–109.

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**: 103–106.

Slatkin M. 1985. Gene flow in natural populations. *Annual Review of Ecology and Systematics* **16**: 393–430.

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. *Science* **236**: 787–792.

Smulders MJM, Beringen R, Volosyanchuk R, *et al.* 2008. Natural hybridization between *Populus nigra* L. and *P. x canadensis* Moench. Hybrid offspring competes for niches along the Rhine river in the Netherlands. *Tree Genetics & Genomes* **4**: 663–675.

Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* **60**: 561–588.

Starr TN, Gadek KE, Yoder JB, Flatz R, Smith CI. 2013. Asymmetric hybridization and gene flow between Joshua trees (Agavaceae: *Yucca*) reflect differences in pollinator host specificity. *Molecular Ecology* **22**: 437–449.

Sun Y, Abbott RJ, Li L, Li L, Zou J, Liu J. 2014. Evolutionary history of purple cone spruce (*Picea purpurea*) in the Qinghai–Tibet Plateau: homoploid hybrid origin and Pleistocene expansion. *Molecular Ecology* **23**: 343–359.

Tagane S, Hiramatsu M, Okubo H. 2008. Hybridization and asymmetric introgression between *Rhododendron eriocarpum* and *R. indicum* on Yakushima Island, southwest Japan. *Journal of Plant Research* **121**: 387–395.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Tiffin P, Olson MO, Moyle LC. 2001. Asymmetrical crossing barriers in angiosperms. *Proceedings of the Royal Society of London-Series B, Biological Sciences* **268**: 861–867.

Vähä JP, Primmer CR. 2006. Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology* **15**: 63–72.

Wesselingh R, Arnold M. 2000. Pollinator behaviour and the evolution of Louisiana *Iris* hybrid zones. *Journal of Evolutionary Biology* **13**: 171–180.

Wolf DE, Takebayashi N, Rieseberg LH. 2001. Predicting the risks of extinction through hybridization. *Conservation Biology* **15**: 1039–1053.

Wu CI. 2001. The genic view of the process of speciation. *Journal of Evolutionary Biology* **14**:851–865.

Yan LJ, Gao LM, Li DZ. 2013. Molecular evidence for natural hybridization between *Rhododendron spiciferum* and *R. spinuliferum* (Ericaceae). *Journal of Systematics and Evolution* **51**: 426–434.

Yan LJ, Zhang ZR, Li DZ, Gao LM. 2014. Isolation and characterization of microsatellite markers for the Chinese endemic species *Rhododendron spinuliferum*. *Plant Diversity and Resources* **36**: 41–46.

Yan LJ, Liu J, Möller M, *et al.* 2015. DNA barcoding of *Rhododendron* (Ericaceae), the largest Chinese plant genus in biodiversity hotspots of the Himalaya-Hengduan Mountains. *Molecular Ecology Resources* **15**: 932–944.

Zeng YF, Liao WJ, Petit RJ, Zhang DY. 2011. Geographic variation in the structure of oak hybrid zones provides insights into the dynamics of speciation. *Molecular Ecology* **20**: 4995–5011.

Zha HG, Milne RI, Sun H. 2010. Asymmetric hybridization in *Rhododendron agastum*: a hybrid taxon comprising mainly F_1s in Yunnan, China. *Annals of Botany* **105**: 89–100.

Zhang JL, Zhang CQ, Gao LM, Yang JB, Li HT. 2007. Natural hybridization origin of *Rhododendron agastum* (Ericaceae) in Yunnan, China: inferred from morphological and molecular evidence. *Journal of Plant Research* **120**: 457–463.