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REGULAR PAPER

Morphological and molecular evidence for natural hybridization in sympatric population of *Roscoea humeana* and *R. cautleoides* (Zingiberaceae)

Guang-Hui Du · Zhi-Qiang Zhang · Qing-Jun Li

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Abstract The sympatric occurrence of some species in *Roscoea* is very common, but little information is available on natural hybridization. However, some intermediate individuals were found on the sympatric population of *Roscoea humeana* and *R. cautleoides* at Ganhaizi population in northwestern Yunnan Province, China. We suspected that these intermediate individuals were the hybrids of *R. humeana* and *R. cautleoides* from the previous evidence, but could not confirm them. In this study, morphometric analysis was followed by examination of HAT-RAPD polymorphisms to determine the occurrence of natural hybridization between sympatric *R. humeana* and *R. cautleoides*. The results showed that most morphological characters of the putative hybrids were found to be intermediate between those of *R. humeana* and *R. cautleoides*. Meanwhile, molecular analysis confirmed that the morphological intermediates were derived from hybridization between the two species. From the analysis of the NewHybrids, the hybridization individuals were mainly F_1s. These results indicated that interspecific hybridization between *R. humeana* and *R. cautleoides* indeed occurred in sympatric population.

Keywords *Roscoea* · Sympatric occurrence · Hybrid · HAT-RAPD · Reproductive barrier

Introduction

Natural hybridization is very common in plants due to an incomplete reproductive isolation among closely related species (Stebbins 1959; Grant 1981). Recent study suggested that at least 25% of plant species were involved in hybridization with at least one other species (Mallet 2007). And natural hybridization is important in plant evolution as a mechanism of speciation (Arnold 1997; Rieseberg and Carney 1998; Arnold et al. 2003). So researches on hybridization, hybrids and hybrid zone in sympatric distributions of different species have become hotspots in plant phylogeny and evolution over the past few years (Ellstrand et al. 1996; Rieseberg et al. 2003).

Morphologically, hybrids typically display the intermediate of parental characters, but it should be noted that a few of morphological intermediates may form through convergent evolution or environmental selection, not by hybridization. (Rieseberg 1995; Rieseberg et al. 1999; Schwarzbach et al. 2001; Lexer et al. 2003). So it is very difficult to confirm the hybrids only by morphological evidence. Recently, with the using of molecular methods, a large number of researches on natural hybridization or hybrid speciation have been confirmed (Allan et al. 1997; Feliner et al. 2002; Wu et al. 2010).

G.-H. Du and Z.-Q. Zhang are the first two authors contributed equally to this paper.

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G.-H. Du · Z.-Q. Zhang · Q.-J. Li (✉)
Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun Town, Mengla County, Yunnan 666303, China
e-mail: qjli@xtbg.ac.cn

G.-H. Du
Graduate University of Chinese Academy of Sciences, Beijing 100039, China

Z.-Q. Zhang
Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

Molecular markers detecting variation at the DNA level offer numerous advantages over morphological and biochemical techniques as they are stable and detectable in all tissues and not affected by environmental or developmental stage (Tingey and Del Tufo 1993). Randomly amplified polymorphic DNA (RAPD) using single short arbitrary primers can quickly scan an entire genome without prior knowledge of the sequence (Williams et al. 1990). However, the major drawback of the method is that the profiling is sensitive to changes in the reaction conditions and the band reproducibility is very low, mainly attributed to the low annealing temperatures (Agarwal et al. 2008). Anuntalabhochai et al. (2000) reported a high annealing temperature RAPD (HAT-RAPD) technique, which had been shown greater polymorphism, reproducibility, and high resolution by increasing the annealing temperature to over 46°C. This technique has already been successfully used in the tropical fruits (Cutler et al. 2006, 2007; Ruangsuttapha et al. 2007) and the cut flower curcuma (Anuntalabhochai et al. 2007).

Roscoea J. E. Smith, the truly alpine genus of Zingiberaceae, only comprises 18 species (Cowley 2007). Most species of *Roscoea* occur in the eastern Himalayas (Nepal to north India) and the Hengduan Mountains of southwest China, between 1,200 and 4,880 m (Cowley 2007). These areas were widely regarded as global biodiversity hotspots (Wilson 1992; Myers et al. 2000). And many cases about natural hybridization had been reported in these areas (Zha et al. 2008, 2010; Zhu et al. 2009). Although the sympatric phenomenon in the genus of *Roscoea* is very common, little information is available on natural hybridization. It may be a reason why the origin and relationship of some *Roscoea* species are not clear now.

R. humeana Balf. f. & W. W. Sm and *R. cautleoides* Gagnep. are closely related based on the ITS region (Ngamriabsakul et al. 2000). However, their floral characters and vegetative features are quite different. *R. humeana* has ovate leaves, short or no peduncles and purple flowers with a longer floral tube. While *R. cautleoides* has narrowly lanceolate leaves, long peduncles and bright yellow flowers (Fig. 1). From the previous work (Zhang et al. 2011), we knew that *R. humeana* and *R. cautleoides* were sympatric on the Mt. Yulong (Lijiang city, Yunnan Province, China). Moreover, the flowering seasons were completely overlapped and always lasted from early May to late June. The occasional pollinator (*Bombus* sp.) was the common pollinator of the two species. In addition, reciprocal hand pollinations could produce fruit (unpublished data). All of the above made the possibility of natural hybridization occur. In fact, at the overlapped zone of the sympatric population of *R. humeana* and *R. cautleoides*, there were a few individuals which had short peduncle and long tubed flower as *R. humeana*, but had lanceolate leaves and yellow flower like *R. cautleoides* (Fig. 1). We suspected that these individuals were the hybrids of *R. humeana* and *R. cautleoides*. If so, it would help us to study the gene flow and gene introgression between *R. humeana* and *R. cautleoides*, so morphological and molecular examinations were urgently required.

In this study, morphological characters and HAT-RAPD genetic markers were used to investigate the putative

Fig. 1 Floral and foliar morphology of *R. humeana*, putative hybrid and *R. cautleoides*, respectively. **a** The peduncle and bract of *R. humeana*, putative hybrid and *R. cautleoides* from *left* to *right*. **b** The leaf of *R. humeana*, putative hybrid and *R. cautleoides* from *top* to *down*. *Bar* 10 mm

hybrids as a cross between *R. humeana* and *R. cautleoides*. Once the hybrid status was confirmed, and then each sample was divided into different genotype classes to examine the population structure and the stability of hybrids.

Materials and methods

Study site and species

The distributions *R. humeana* and *R. cautleoides* are similar mainly on the Hengduan Mountains of southwest China. Lijiang is located in the core area of the Hengduan Mountains and is considered to own the highest diversity of *Roscoea* species (Ngamriabsakul et al. 2000; Cowley 2007; Zhang and Li 2008; Zhang et al. 2011). In this study, the research site was located at Ganhaizi (GHZ), the core of the distributional ranges of *R. humeana* and *R. cautleoides*, 25 km north of Lijiang city, in Yunnan, China, 27°05′N, 100°16′E, 3,120 m above sea level where *R. humeana* and *R. cautleoides* occurred together. At GHZ, a grassy and rocky slope on Mt. Yulong, *R. humeana* is concentrated in the flat grassland, while *R. cautleoides* is dominant on the rocky slop. The putative hybrids can only be found at the overlapped area mixed with *R. humeana* and *R. cautleoides*.

Plant sampling

Morphological characters such as flower color, leaf morphology, length of peduncle were used to distinguish *R. humeana*, *R. cautleoides* and their putative hybrids in the field. Because of their distinct floral and vegetative differences, it was very easy to discriminate two putative parental species in field. We chose randomly 30 individuals with ovate leaves, short or no peduncles and long-tubed purple flowers as the samples of *R. humeana* in the flat grassland, where *R. humeana* dominated. We also selected randomly 30 individuals with lanceolate leaves, long peduncles and short-tubed yellow flowers as the samples of *R. cautleoides* at rocky slope, where *R. cautleoides* dominated. A total of 38 individuals with wide lanceolate leaves, short peduncle, long tubed and yellow flower were selected as the putative hybrids at the overlapped area of *R. humeana* and *R. cautleoides*. All the individuals were always at least 10 m apart to minimize the possibility of sampling the same genets twice. Floral and vegetative traits were measured for each 30 individuals of *R. humeana*, *R. cautleoides* and putative hybrids. After making morphological measurements, the leaves of each individual (about 1 g fresh weight) were dried with silica gels for DNA analyses. All 38 putative hybrids and 20 individuals of each putative parental species were used for molecular analyses. All voucher specimens were deposited at the Xishuangbanna Tropical Botanical Garden (HITBC).

Morphological analysis

Thirteen morphological traits were measured: corolla tube length, peduncle length, dorsal petal length, dorsal petal width, labellum length, labellum width, lateral petal length, lateral petal width, distance of staminodes, leaf length, leaf width, leaf length: width ratio and leaf thickness. Finally, 30 flowers and leaves from each of the three groups (the result was 29 for *R. cautleoides*) were recorded using a vernier caliper. Mean value, standard deviation and significant differences were calculated for the morphological statistic analyses by the method of Tovar-sánchez and Oyama (2004), and principal components analysis (PCA) distinguished putative hybrids from their putative parental individuals. All statistical analyses were used by R (http://www.r-project.org/).

DNA extraction and HAT-RAPD reaction

DNA was extracted from silica gel dried leaf using a modified CTAB method (Doyle and Doyle 1987). DNA quality and concentration were assessed by 1% (m/v) agarose gel electrophoresis with uncut λDNA (Takara) and NanoDrop 1000 spectrophotometer (Thermo company).

PCR amplification using random decamer primers (synthesized by Shanghai Sangon company) was performed following HAT-RAPD protocol (Anuntalabhochai et al. 2007). PCR reactions were run in a total volume of 25 μL containing 10–40 ng template DNA, 2.5 μL 10× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs mix, 4 mmol/L of primer, and 1.5 U Taq polymerase (Takara). These 25 μl solutions were then amplified in a DNA Programmable Thermal Cycler (ABI company) using the following cycling profile: 95°C for 2 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 46°C for 30 s, and extension at 72°C for 45 s, followed by a final 5 min at 72°C, hold at 4°C. After the thermal cycling program had been completed, the amplification products were electrophoresed in 1.5% (m/v) agarose gels with DL-2000 DNA marker (Takara).

Photographs from the nucleic acid dye stained agarose gels were used to score the data for the subsequent analyses. Comigrating 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 (Zha et al. 2008, 2010). Ideally, species-specific marker bands are present in all individuals of one species and none of the other. However, it is frequently not possible to find such bands, possibly due to the close relationship of the parental

Table 1 Morphological characters of *R. cautleoides*, *R. humeana* and putative hybrid (units: mm)

Character	*R. cautleoides* (mean ± SD)	Putative hybrid (mean ± SD)	*R. humeana* (mean ± SD)	The putative hybrid is different from ($P < 0.05$)	Is the putative hybrid intermediate?
Corolla tube length	33.3 ± 1.7	62.4 ± 1.7	89.6 ± 1.7	Both	Yes
Peduncle length	163.3 ± 5.7	82.3 ± 5.7	23.0 ± 5.7	Both	Yes
Dorsal petal length	24.3 ± 0.7	32.2 ± 0.7	34.8 ± 0.7	Both	Yes
Dorsal petal width	17.0 ± 0.6	22.0 ± 0.6	29.0 ± 0.6	Both	Yes
Labellum length	27.2 ± 0.8	25.1 ± 0.8	28.2 ± 0.8	*R. humeana*	No
Labellum width	32.5 ± 0.9	29.7 ± 1.0	27.5 ± 0.7	*R. cautleoides*	Yes
Lateral petal length	27.6 ± 0.6	31.4 ± 0.6	35.6 ± 0.6	Both	Yes
Lateral petal width	8.3 ± 0.9	11.4 ± 0.9	14.0 ± 0.9	Both	Yes
Distance of staminodes	2.5 ± 0.4	–[a]	–[a]	*R. cautleoides*	Yes
Leaf length	112.8 ± 3.6	81.6 ± 3.6	86.5 ± 3.6	*R. cautleoides*	Yes
Leaf width	14.4 ± 0.7	24.2 ± 0.7	35.4 ± 0.7	Both	Yes
Leaf length:width ratio	8.2 ± 0.2	3.4 ± 0.2	2.5 ± 0.2	Both	Yes
Leaf thickness	0.23 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	Both	Yes
Scores of intermediate character					12 : 1

[a] No value for this character

Table 2 The first and second eigenvalues for morphological characters (the variance explained by first and second principal component included)

Character	Component	
	PC1	PC2
Corolla tube length	0.943	−0.042
Peduncle length	−0.856	0.233
Dorsal petal length	0.831	−0.124
Dorsal petal width	0.887	−0.062
Labellum length	0.268	0.834
Labellum width	−0.245	0.742
Lateral petal length	0.822	0.211
Lateral petal width	0.527	0.043
Distance of staminodes	−0.833	0.375
Leaf length	−0.514	0.549
Leaf width	0.876	−0.179
Leaf length:width ratio	−0.844	0.402
Leaf thickness	0.824	−0.348
Eigenvalue	7.72	1.70
Contribution (%)	59.40	13.09
Accumulated contribution (%)	59.40	72.49

species or the introgression. In this study, less stringent methods for defining marker bands that are more common in one taxon than the other had been used based on several studies (Allan et al. 1997; Neuffer et al. 1999; Feliner et al. 2002; Archibald et al. 2004). The fragments were scored as either present (1) or absent (0) for each of the primer–accession combinations and the presence or absence of each band was scored in a binary data matrix (see Table S1 in Supplementary Data, available online).

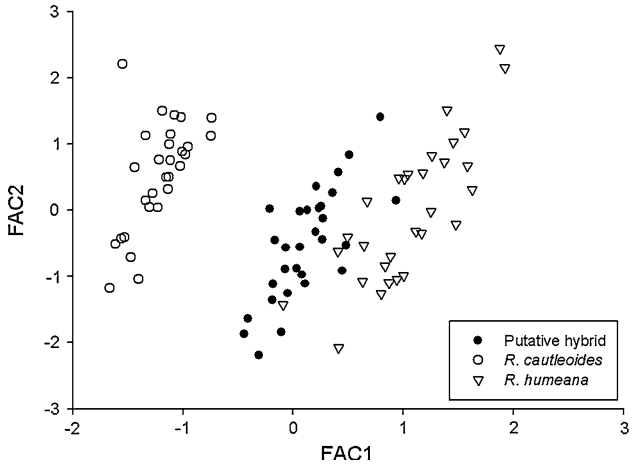

Fig. 2 Plot of *Roscoea* samples by first and second factor scores derived from PCA

Analytical methods of HAT-RAPD data

Based on the polymorphic HAT-RAPD markers, two different methods were used for analyses. Firstly, principal co-ordinate analysis (PCOA) using GenAlEx 6.4 (Peakall and Smouse 2006). This analysis determined the genetic relationship of the species, and allowed us to distinguish hybrids from the parental samples. In this application, intermediate individuals well separated from the distinct *R. humeana* and *R. cautleoides* were assumed to be hybrids. Second, we estimated the posterior probability using a Bayesian method. For each individual, the posterior probability was used to divide it to *R. humeana*, *R. cautleoides*, or to early generation hybrid classes (F_1, F_2, or backcross). This procedure was run in the program 'NewHybrids' using a

Markov Chain Monte Carlo (MCMC) method (Anderson and Thompson 2002). Using the default settings of this program which assign posterior probabilities for six possible classes (parents, F_1, F_2, backcross to each parent), posterior distributions were calculated with 10^5 iterations of the Monte Carlo Markov Chains, after a 10^5 iterations' burn-ins, without using any prior information of individual or allele frequency. Individuals were assigned to one of the six genotypic classes if posterior probability ≥ 0.95. If the hybrid classes were beyond the second generation, the NewHybrids program did not normally attempt to identify them (Anderson and Thompson 2002). Therefore, individuals with the posterior probability <0.95 might be later generation hybrid derivatives.

Table 3 The size and amplification ratio of specific RAPD fragments in three *Roscoea* specimens

Primer	Sequence (5′-3′)	Fragment size	*R. humeana*	Putative hybrid	*R. cautleoides*
B1046	GTCGGAGCGG	500	1.00	1.00	0.05
OPW 08	GACTGCCTCT	400	0.05	0.63	0.90
		800	0.95	0.76	0.00
OPW 16	CAGCCTACCA	800	1.00	1.00	0.20
OPR 16	CTCTGCGCGT	1,000	0.90	0.53	0.00
		1,200	0.60	0.53	0.00
OPT 16	GGTGAACGCT	250	1.00	0.84	0.05
OPB 01	GTTTCGCTCC	500	0.65	0.63	0.10
		1,600	0.20	0.92	0.90
OPG 13	CTCTCCGCCA	400	1.00	0.95	0.05
OPD 03	GTCGCCGTCA	450	0.15	0.53	0.85
		600	1.00	1.00	0.00
		1,100	0.80	0.39	0.30
S2077	GTTCGCTCCC	650	1.00	1.00	0.05
S2100	CAAAGGCGTG	1,100	0.05	0.76	0.70
S2159	GTCGTGCGGA	400	0.05	0.58	0.90
OPA 04	AATCGGGCTG	900	1.00	0.82	0.05
OPD 18	GAGAGCCAAC	650	1.00	0.97	0.20
OPN 06	GAGACGCACA	550	1.00	1.00	0.05
OPA 07	GAAACGGGTG	1,700	1.00	0.63	0.20
OPA 08	GTGACGTAGG	400	1.00	0.95	0.05
OPA 15	TTCCGAACCC	550	0.05	0.24	0.75
		600	1.00	0.95	0.05
		650	0.05	0.29	0.70
OPA 17	GACCGCTTGT	550	0.90	0.89	0.00
		750	0.15	0.84	1.00
OPB 12	CCTTGACGCA	400	1.00	1.00	0.00
		1,100	1.00	0.74	0.00
OPJ 10	AAGCCCGAGG	1,200	0.85	0.42	0.00
OPX 03	TGGCGCAGTG	400	0.40	0.79	1.00
		500	0.00	0.47	0.80
		550	0.95	0.53	0.10
		600	0.95	0.84	0.00
OPX 11	GGAGCCTCAG	450	0.00	0.61	0.85
		1,400	0.00	0.84	0.80
		1,600	0.60	0.63	0.00
OPE 02	GGTGCGGGAA	850	0.95	0.79	0.05
OPC 01	TTCGAGCCAG	260	1.00	1.00	0.10
		400	0.00	0.45	0.95
OPI 16	TCTCCGCCCT	800	1.00	0.92	0.10
OPO 11	GACAGGAGGT	900	0.05	0.71	0.90
OPO 13	GTCAGAGTCC	600	0.20	0.82	0.95

Results

Morphological analysis

General statistics of measured traits for *R. humeana* and *R. cautleoides* and putative hybrids are listed in Table 1. All of the thirteen characters examined (except the labellum length) in the putative hybrids were between the two parents or close to one parent. Principal components analysis of the combined morphological data set indicated that the first and second extracted factors accounted for 72.49% of the total variances (59.40% for PC1 and 13.09% for PC2) (Table 2). According to the broken-stick model (Joliffe 1986; Jackson 1993), only the first factor, and possibly to a small extent the second factor, accounted for a significant amount of variation. In the first factor, all the corresponding eigenvalues exhibited in positive or negative directions, except labellum length and labellum width loaded highly. While in the second factor, except labellum length and labellum width, all variables had low loadings (Table 2). In the plot of individual component scores, the hybrids were distributed within the area between the two parental species but were more concentrated towards individuals of *R. humeana*. Along the first axis, *R. humeana* and *R. cautleoides* individuals showed a general separation, with putative hybrid individuals intermediate (Fig. 2). However, no notable differentiation between individuals was evident along the second axis (Fig. 2).

HAT-RAPD and PCO analysis

In the 216 RAPD primers, a total of 42 clear and reliable polymorphic RAPD markers (from 27 primers) were generated during the analysis. Among these, 27 RAPD markers were common in *R. humeana* and 15 in *R. cautleoides*. Additive profiles of parental specific bands were observed in the most putative hybrids (Table 3).

PCO analysis using the 42 polymorphic markers separated the putative hybrids from the distinct *R. humeana* and *R. cautleoides* (Fig. 3). The first two principal co-ordinates which accounted for 80.05% of the variance (66.64 and 13.41% for the first and second axes, respectively) clearly separated the 78 individuals into three clusters. The clusters matched exactly the three morphological categories assigned, except for accession 15, 17 and 20, which, from morphology, were classed as *R. humeana*, and were close to the putative hybrids (Fig. 3).

NewHybrids analysis

The NewHybrids analysis (Fig. 4) indicated that all individuals of *R. cautleoides* were confirmed to be the pure parental species with posterior probability ≥0.99, and most accessions of *R. humeana* were the pure parental species except the accession 15, 17 and 20. The individual 15, 17 and 20 might be the hybrids because they had the posterior probability of F_1 and F_2. Among the putative hybrids, twenty-five were determined to be F_1s with the posterior probability ≥0.95, and the remaining thirteen could not be assigned a class with 95% certainty. Of these, eight had the posterior probability ≥0.90 might being F_1s, whereas the other five had a possibility of being F_1s and a probability of being F_2s, there was no evident definition of the six genotype classes, but they might be later generation hybrid derivatives of *R. humeana* and *R. cautleoides*.

Discussion

Gottlieb (1972) presented several criteria for testing the status of putative hybrid: sympatric distribution, intermediate characters, interfertility, and biochemical additivity. Although single criterion could not offer an accurate means for testing the status of hybrid, all of these criteria that could be met provided a credible approach for confirming a particular taxon which had originated through hybridization (Gottlieb 1972; Padgett et al. 1998; Zhang et al. 2007). So these criteria can also be applied to our study.

Our field observations showed that the putative hybrids were located sympatrically with parental species. Moreover, the flowering seasons of putative parental species were completely overlapped, providing the possible occurrence of natural hybridization in floral phenology. Although the common pollinators were not the main pollinators of the two species, it provided the possibility of one's pollen touched the other's stigma occurred. In addition, reciprocal hand pollinations could produce fruits and seeds. Therefore, due to the preliminary evidence (Zhang et al. 2011) obtained in the field, it was reasonable to deduce the hybrid status, but not enough to confirm it. In this study, the intermediacy in several characters was also

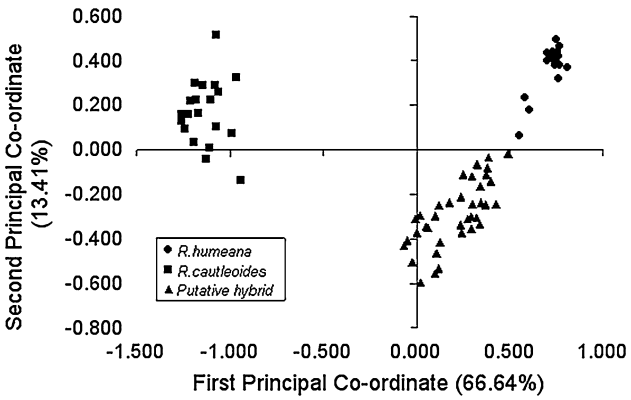

Fig. 3 Plot of *Roscoea* samples by PCO analysis based on HAT-RAPD bands

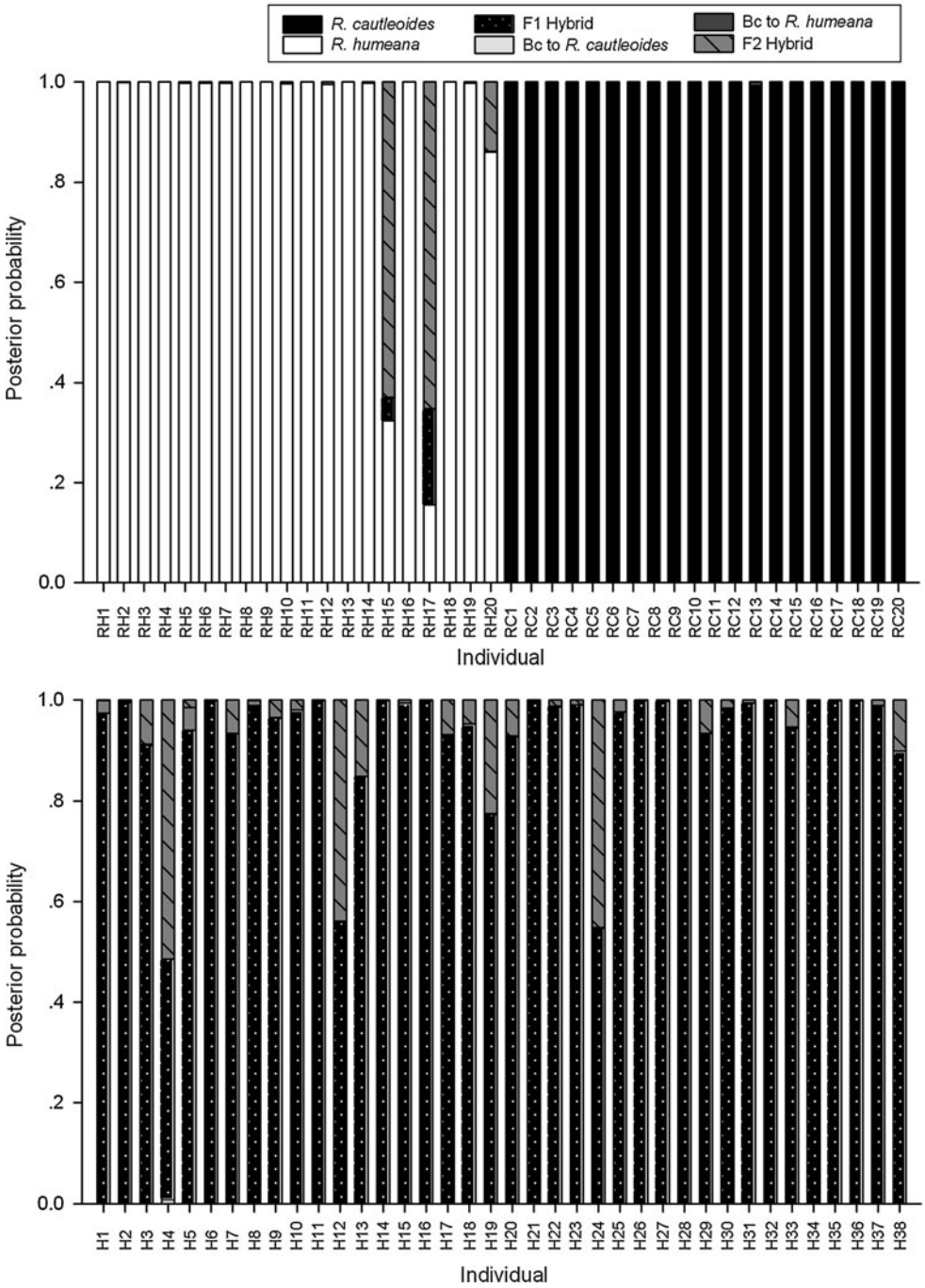

Fig. 4 Posterior probability of each individual belonging to parental or hybrid class. Rh1–Rh20, *R. humeana*; Rc1–Rc20, *R. cautleoides*; H1–H38, putative hybrids

the forceful evidence for the hybrid criteria. In addition, the molecular evidence confirmed the occurrence of natural hybridization from morphological presumption, because species-specific markers of *R. humeana* and *R. cautleoides* were detected in all the putative hybrids. The present results clearly indicated that the natural hybridization has occurred between *R. humeana* and *R. cautleoides*. This is the first report for the genus.

Although we confirmed that the putative hybrids were the progeny of natural hybridization between *R. humeana* and *R. cautleoides*, the number of the putative hybrids was rare in the wild and they occurred only in a few positions. Another observation for the putative hybrids was that there was no obvious habitat differentiation from their parental species, unlike many other stabilized hybrids which occupied novel or extreme habitats (Rieseberg 1997). The putative hybrids always occurred intermixed with the parents or in intermediate habitat, suggesting that they were not stabilized, but perhaps they only infrequently produced F_1 hybrids (Wu et al. 2010). In fact, a Bayesian analysis in the program NewHybrids indicated that the population structure of the hybrids were mainly F_1s.

Except most hybrids was the F_1s, we found that the hybrids were close to *R. humeana* from the PCA and PCOA analyses. There were two possible reasons for the hybrids close to one of the parental species. One possible was that the hybrids were back-crosses with one of the parents (Archibald et al. 2004), the other was one of the parental species to be the main maternal donor (González-Rodríguez et al. 2004). While the first reason was impossible, from the result of the program NewHybrids, most of the hybrids were the F_1s, no indication of the back-cross to *R. humeana*. We suspected that *R. humeana* was the maternal parent of the hybrids, so some plastid genes especially the chloroplast genes are needed to work out this question, because of the maternal inheritance of the chloroplast genomes in most angiosperms (Harris and Ingram 1991; Olmstead and Palmer 1994).

The possibility of hybridization occurred between species depends on the strength of interspecific reproductive barriers (Ellstrand et al. 1996; Campbell et al. 2002). Prezygotic barriers limited the transfer of pollen from individuals of one species to stigmas of other species. Even if pollen were transferred between species, formation of hybrids could still be prevented by postzygotic reproductive barriers (Campbell et al. 2002). Sympatric occurrence, flowering seasons overlap and common pollinators indicated the prezygotic barriers existed but not very stringent. Moreover, reciprocal hand pollinations between *R. humeana* and *R. cautleoides* could produce fruits and seeds. Although the fertility of hand-cross seeds was not detected, some reported that the postzygotic barriers of *Roscoea* were relatively weak (Cowley 1982, 2007). So the permeable reproductive barrier provided the possibility of natural hybridization between *R. humeana* and *R. cautleoides*.

Although the not stringent interspecific reproductive barriers made *R. humeana* and *R. cautleoides* produce the hybrids, the impact of natural hybridization between *R. humeana* and *R. cautleoides* on the evolution and speciation was still not clear. Future work should concentrate on comparing the fitness of hybrids and their parental species, and considering the effect of natural hybridization.

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References

Agarwal M, Shrivastava N, Padh H (2008) Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Rep 27:617–631

Allan GJ, Clark C, Rieseberg LH (1997) Distribution of parental DNA markers in *Encelia virginensis* (*Asteraceae*: *Heliantheae*), a diploid species of putative hybrid origin. Plant Syst Evol 205:205–221

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

Anuntalabhochai S, Chiangda J, Chandet R, Apawat P (2000) Genetic diversity within lychee (*Litchi chinensis* Sonn.) based on RAPD analysis. Acta Hortic 575:253–259

Anuntalabhochai S, Sitthiphrom S, Thongtaksin W, Sanguansermsri M, Cutler RW (2007) Hybrid detection and characterization of *Curcuma* spp. using sequence characterized DNA markers. Sci Hortic 111:389–393

Archibald JK, Wolfe AD, Johnson SD (2004) Hybridization and gene flow between a day and night flowering species of *Zaluzianskya* (Scrophulariaceae S.S., tribe *Manuleeae*). Am J Bot 91:1333–1344

Arnold ML (1997) Natural hybridization and evolution. Oxford University Press, New York

Arnold ML, Bouck AC, Cornman RS (2003) Verne Grant and Louisiana irises: is there anything new under the sun? New Phytol 161:143–149

Campbell DR, Waser NM, Pederson GT (2002) Predicting patterns of mating and potential hybridization from pollinator behavior. Am Nat 159:438–450

Cowley EJ (1982) A revision of *Roscoea* (Zingiberaceae). Kew Bull 36:747–777

Cowley EJ (2007) The genus *Roscoea*. Royal Botanic Gardens, Kew

Cutler RW, Chundet R, Handa T, Anuntalabhochai S (2006) Development of sequence characterized DNA markers linked to a temperature dependence for flower induction in lychee (*Litchi chinensis* Sonn.) cultivars. Sci Hortic 107:264–270

Cutler RW, Sitthiphrom S, Marha J, Anuntalabhochai S (2007) Development of sequence characterized DNA markers linked to a temperature insensitivity for fruit production in Longan (*Dimocarpus longan* Lour.) cultivars. Agr Crop Sci 193:74–78

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Ellstrand NC, Whitkus RW, Rieseberg LH (1996) Distribution of spontaneous plant hybrids. Proc Natl Acad Sci USA 93:5090–5093

Feliner NG, Aguilar JF, Rossello JA (2002) Reticulation or divergence: the origin of a rare serpentine endemic assessed with chloroplast, nuclear, and RAPD markers. Plant Syst Evol 231:19–38

González-Rodríguez A, Arias DM, Valencia S, Oyama K (2004) Morphological and RAPD analysis of hybridization between *Quercus affinis* and *Q. laurina* (Fagaceae), two Mexican red oaks. Am J Bot 91:401–409

Gottlieb LD (1972) Leaves of confidence in the analysis of hybridization in plants. Ann Mo Bot Gard 59:435–446

Grant V (1981) Plant speciation. Columbia University Press, New York

Harris SA, Ingram R (1991) Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. Taxon 40:393–412

Jackson DA (1993) Stopping rules in principal components analysis: a comparison of heuristical and statistical approaches. Ecology 74:2204–2214

Joliffe IT (1986) Principal component analysis. Springer, New York

Lexer C, Welch M, Raymond O, Rieseberg LH (2003) The origins of ecological divergence in *Helianthus paradoxus* (Asteraceae): selection on transgressive characters in a novel hybrid habitat. Evolution 57:1989–2000

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

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Neuffer B, Auge H, Mesch H, Amarell U, Brandl R (1999) Spread of violets in polluted pine forests: morphological and molecular evidence for the ecological importance of interspecific hybridization. Mol Ecol 8:365–377

Ngamriabsakul C, Newman MF, Cronk CB (2000) Phylogeny and disjunction in *Roscoea* (Zingiberaceae). Edinb J Bot 57:39–61

Olmstead RG, Palmer JD (1994) Chloroplast DNA systematics: a review of methods and data analysis. Am J Bot 81:1205–1224

Padgett DJ, Les DH, Crow GE (1998) Evidence for the hybrid origin of *Nuphar* × *rubrodisca* (Nymphacaceae). Am J Bot 85:1468–1476

Peakall R, Smouse PE (2006) GenAlEx 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Rieseberg LH (1995) The role of hybridization in evolution: old wine in new skins. Am J Bot 82:944–953

Rieseberg LH (1997) Hybrid origins of plant species. Annu Rev Ecol Syst 28:359–389

Rieseberg LH, Carney SE (1998) Plant hybridization. New Phytol 140:599–624

Rieseberg LH, Archer MA, Wayne RK (1999) Transgressive segregation, adaptation, and speciation. Heredity 83:363–372

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Lexer C (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. Science 301:1211–1216

Ruangsuttapha S, Eimert K, Schröder MB, Silayoi B, Denduangboripant J, Kanchanapoom K (2007) Molecular phylogeny of banana cultivars from Thailand based on HAT-RAPD markers. Genet Resour Crop Evol 54:1565–1572

Schwarzbach AE, Donovan LA, Rieseberg LH (2001) Transgressive character expression in a hybrid sunflower species. Am J Bot 88:270–277

Stebbins GL (1959) The role of hybridization in evolution. Proc Am Philos Soc 103:231–251

Tingey SV, del Tufo JP (1993) Genetic analysis with random amplified polymorphic DNA markers. Plant Physiol 101:349–352

Tovar-Sánchez E, Oyama K (2004) Natural hybridization and hybrid zones between *Quercus crassifolia* and *Quercus crassipes* (Fagaceae) in Mexico: morphological and molecular evidence. Am J Bot 91:1352–1363

Williams JGK, Kubelik AR, Livak KJ, Rafalski A, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers useful as genetic markers. Nucleic Acids Res 18:6531–6535

Wilson EO (1992) The diversity of life. Belknap Press of Harvard University Press, Cambridge

Wu W, Zhou RC, Huang YL, Boufford DE, Shi SH (2010) Molecular evidence for natural intergeneric hybridization between *Liquidambar* and *Altingia*. J Plant Res 123:231–239

Zha HG, Milne RI, Sun H (2008) Morphological and molecular evidence of natural hybridization between two distantly related *Rhododendron* species from the Sino-Himalaya. Biol J Linn Soc 156:119–129

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

Zhang ZQ, Li QJ (2008) Autonomous selfing provides reproductive assurance in an alpine ginger *Roscoea schneideriana* (Zingiberaceae). Ann Bot 102:531–538

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. J Plant Res 120:457–463

Zhang ZQ, Kress WJ, Xie WJ, Ren PY, Gao JY, Li QJ (2011) Reproductive biology of two Himalayan alpine gingers (*Roscoea* spp., Zingiberaceae) in China: pollination syndrome and compensatory floral mechanisms. Plant Biol 13:582–589

Zhu XF, Li Y, Wu GL, Fang ZD, Li QJ, Liu JQ (2009) Molecular and morphological evidence for natural hybridization between *Primula secundiflora* Franchet and *P. Poissonii* Franchet (Primulaceae). Acta Biol Cracov Bot 51:29–36