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Natural hybridization origin of *Rhododendron agastum* (Ericaceae) in Yunnan, China: inferred from morphological and molecular evidence

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Abstract The natural hybridization that occurs between two sympatric species of *Rhododendron* subgenus *Hymenanthus* in Yunnan, China, was investigated. The assumed parents, *Rhododendron delavayi* Franch. and *R. decorum* Franch., are morphologically distinct, and the putative hybrid species, *R. agastum* Balf. f. et W. W. Smith, has an intermediate morphology. We used the main morphological characters, sequences of the nuclear ribosomal DNA ITS region, and the chloroplast DNA *trnL-F* intronspace to analyze the three species, and compared these morphological and molecular data with an artificial hybrid between *R. decorum* (♀) × *R. delavayi* (♂). From the results, we conclude that *R. agastum* is a natural hybrid between a female *R. delavayi* and a male *R. decorum*.

Keywords ITS · Morphology · Natural hybridization · *Rhododendron* · *trnL-F*

Introduction

Hybridization is an important factor in the evolution of many plant taxa (Yang et al. 2000; Tsukaya 2002; Collins

et al. 2003; Gonzalez-Rodriguez et al. 2004). It can create evolutionary novelties that promote adaptive evolution and speciation (Arnold 1996; Barton 2001). Hybrid speciation can stabilize adaptive gene combinations by an increase in ploidy (allopolyploidy) or chromosomal rearrangements at the same ploidy (homoploid hybrid speciation) (Rieseberg 2001). Homoploid hybrid speciation has been reported in both diploids (Rieseberg 1997) and tetraploids (Ferguson and Sang 2001; Nagamitsu et al. 2006). Homoploid hybrid speciation is less common than polyploidy speciation because it involves hybridization between taxa at the same ploidal level. It is also a type of sympatric speciation because the parental species must co-occur geographically to produce hybrids (Rieseberg 1997; Nagamitsu et al. 2006). In addition, a number of well-documented cases of diploid hybrid speciations suggest that natural hybridization may play an important role in evolutionary diversification (Schwarzbach et al. 2001; Wang et al. 2001; Ma et al. 2006).

Rhododendron (Ericaceae) is an example of diploid hybrid speciation, which may have played an important role in the genus's evolution and speciation. *Rhododendron* subgenus *Hymenanthus* has 24 subsections with 225 species, all of which are diploids ($2n = 26$) (Ming and Fang 1990). The relationships among these subsections are complex, and the distinctions between them may be obscured by hybridization. Cultivated species from different subsections will cross freely, and hybrids also occur in the wild (Chamberlain 1982; Wu 1986). There are more than 1,000 horticultural hybrids (Bean 1976), and Milne et al. (1999) and Milne (2000) reported on diploid hybridization between *Rhododendrons* in Turkey using morphological and molecular evidence. However, natural hybridization in *Rhododendron* has not been reported so far from the eastern Himalayas, and this present study will investigate

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the extent of natural hybridization within *Rhododendron* in Yunnan province, southwestern China.

Rhododendron delavayi, *R. decorum*, and *R. agastum* are included in subsection *Fortunea* Sleumer, subsect. *Arborea* Sleumer, and subsect. *Irrorata* Sleumer, respectively, all within the subg. *Hymenanthus* (Chamberlain 1982; Hu and Fang 1994). Cox (1994) first suggested a possible hybrid status of *R. agastum* between *R. delavayi* and *R. decorum*, but Chamberlain (2003) argued that *R. agastum* was a rather rare chance hybrid between two dissimilar parents of *R. arboreum* ssp. *delavayi* Franch and *R. decorum* Franch. *R. delavayi* and *R. decorum* are both diploid ($2n = 26$) and are easily crossed by hand pollination (Zhang et al. 1998). *R. delavayi*, *R. decorum*, and *R. agastum* are often sympatric in nature (Zhang 2003), but it has not been determined whether *R. agastum* is indeed a natural hybrid between *R. delavayi* and *R. decorum*.

Morphologically, hybrids typically display a mosaic of parental and intermediate characters, although extreme and novel characters appear quite often in the hybrid phenotype. Morphological characters alone are of limited value when identifying natural hybrids, but molecular studies have greatly enhanced our knowledge in this field (Marhold et al. 2002). A species with morphological characters intermediate between two recognized species has always been considered to be a hybrid (Grant 1981). Interspecific hybrids are most commonly identified by incongruences between nuclear and chloroplast DNA phylogenies that may indicate different parental contributions to the hybrid genome (Yang et al. 2000; Tsukaya et al. 2003; Tsukaya 2004). In this study, we use morphological characters, nuclear ribosomal DNA (nrDNA) (ITS region), and chloroplast DNA (cpDNA) (*trnL-F* intron-spacer) to investigate the putative natural hybridization origin of *R. agastum* as a cross between *R. delavayi* and *R. decorum*.

Materials and methods

Plant materials

Healthy young leaves of 17 samples (five *R. delavayi*, four *R. decorum*, and eight *R. agastum*) were collected from five natural populations in Yunnan Province, southwestern China (Table 1). In addition, a leaf sample was also taken from an artificial hybrid between *R. decorum* (♀) × *R. delavayi* (♂) that has been in cultivation at the Kunming Botanical Garden since the 1980s (Zhang et al. 1998). The five natural populations were collected at Junzishan and Maxiongshan in eastern Yunnan, and at Dapingdi, Shibaoshan, and Dazhuping in northwestern Yunnan. The putative hybrid *R. agastum* occurred sympatrically with

R. delavayi and *R. decorum* in all five sites. These samples covered the natural distribution range of *R. agastum*. Voucher specimens were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

Morphological diagnostic characters

Fifteen large plants (taller than 3 m) of each species were collected from five natural populations in 2005 and 2006. Fifteen flowers were selected from each individual and floral characteristics measured using vernier calipers. Seven quantitative and five qualitative morphological traits were measured for each individual: (1) number of corolla lobes, (2) number of stamens, (3) stamen length, (4) scape length, (5) calyx length, (6) corolla length, (7) pistil length, (8) corolla shape, (9) corolla color, (10) spots in the corolla tube, (11) hairs on the lower leaf surface, and (12) hairs on the fruit. For each species, means and standard deviations were calculated for all quantitative variables using SPSS 11.5 for Windows (SPSS, Chicago, IL, USA). Character means were compared among the three species by analysis of variance (ANOVA) and were evaluated for significant differences using the Tukey HSD post hoc test (Padgett et al. 1998).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from leaves following a modified CTAB protocol (Doyle and Doyle 1987). The universal primers *trn-c* and *trn-f* (Taberlet et al. 1991) were used to PCR amplify the *trnL-F* intron-spacer region. The nrDNA ITS region was amplified using the primers ITS4 and ITS5 (White et al. 1990). The PCR reactions were carried out in 25-μl volumes. The reaction mix contained 0.625 U AmpliTaq DNA polymerase, 1× PCR buffer, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP, 0.3 μmol/l primer and 20–60 ng genomic DNA. PCR reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norfolk, CT). The PCR conditions included an initial denaturation at 94°C for 4 min, followed by 30 cycles of 1 min at 94°C for template denaturation, 1 min at 50°C for primer annealing, 1.5 min at 72°C for extension, and finished with an extension step of 10 min at 72°C. The PCR products were purified using a Sangon Purification kit according to the manufacturer's protocol for sequencing PCR reactions. Purified PCR products were cloned into Promega's pGEM-T System I vector according to the manufacturer. Ten clones of ITS from two individuals of *R. agastum* were obtained, and plasmid preparations were carried out following Sangon's protocols. Sequencing reactions were performed using PRISM Dye Terminator

Table 1 Plant materials and accession numbers for sequences of the ITS and *trnL-F* loci in GenBank

ID	Taxon	Voucher numbers	ITS sequences	<i>trnL-F</i> sequences
DL1	<i>R. delavayi</i>	Junzishan, 2005-5-10-1	DQ677622	DQ784109
DL2	<i>R. delavayi</i>	Maxiongshan, 2005-5-11-4	EF0208347	EF0208370
DL3	<i>R. delavayi</i>	Dapingdi, 2005-5-25-4	EF0208350	EF0208373
DL4	<i>R. delavayi</i>	Shibaoshan, 2005-6-24-4	EF0208349	EF0208372
DL5	<i>R. delavayi</i>	Dazhuping, 2005-6-24-8	EF0208348	EF0208371
DC1	<i>R. decorum</i>	Junzishan, 2005-5-10-3	DQ677623	DQ784106
DC2	<i>R. decorum</i>	Maxiongshan, 2005-5-11-5	EF0208351	EF0208374
DC3	<i>R. decorum</i>	Dapingdi, 2005-5-25-5	EF0208353	EF0208376
DC4	<i>R. decorum</i>	Shibaoshan, 2005-6-24-5	EF0208352	EF0208375
A1	<i>R. agastum</i>	Junzishan, 2005-5-10-18	DQ677625	DQ784107
A2	<i>R. agastum</i>	Junzishan, 2005-5-10-16	EF0208361	–
A3	<i>R. agastum</i>	Junzishan, 2005-5-10-13	EF0208360	–
A4	<i>R. agastum</i>	Dapingdi, 2005-5-25-3	DQ677627	EF0208381
A5	<i>R. agastum</i>	Shibaoshan, 2005-6-24-3	DQ677626	DQ784108
A5-1	Clone		EF0208369	–
A5-2	Clone		EF0208368	–
A5-3	Clone		EF0208367	–
A5-4	Clone		EF0208366	–
A5-5	Clone		EF0208365	–
A5-6	Clone		EF0208364	–
A6	<i>R. agastum</i>	Shibaoshan, 2005-6-24-5	EF0208363	EF0208380
A7	<i>R. agastum</i>	Dazhuping, 2005-6-24-8	DQ677624	EF0208379
A7-1	Clone		EF0208359	–
A7-3	Clone		EF0208358	–
A7-4	Clone		EF0208357	–
A7-5	Clone		EF0208356	–
A8	<i>R. agastum</i>	Maxiongshan, 2005-5-11-3	EF0208355	EF0208378
DD	<i>R. decorum</i> (♀) × <i>R. delavayi</i> (♂)	Kunming, 2006-4-24-1	EF0208354	EF0208377

Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). DNA sequences were obtained with an ABI 3700 automated sequencer (Perkin Elmer).

Sequence alignment

Contiguous DNA sequences were edited using SeqMan (DNASTAR package) and sequences aligned using Clustal X (Thompson et al. 1997). Primers ITS4 and ITS5 were used for all samples to double-check nucleotide site polymorphisms and accuracy of the sequence. All sequences obtained in this study were deposited in GenBank (Table 1).

Results

Morphological identification

The most prominent difference between the putative parents and their hybrid is the variation in qualitative

characters (Fig. 1; Table 2). The corolla of *R. delavayi* is red, *R. decorum* is white, and *R. agastum* is pink. Likewise the shape of the corolla in *R. agastum* is intermediate between the two putative parents. The hairs on the lower leaf surface and on the fruit of *R. delavayi* are very thick but they are absent on *R. decorum*; *R. agastum* has fewer hairs than *R. delavayi*. The spots in the corolla tube, the number of corolla lobes, and the number of stamens in the putative hybrid are similar to those in *R. delavayi*. Only one of the quantitative morphological characters of the putative parents, *R. delavayi* and *R. decorum*, is significantly different, namely, scape length, with that of *R. agastum* being similar to *R. delavayi*. Stamen length and pistil length are much longer in *R. agastum* than in *R. delavayi*, but are not significantly different from *R. decorum*. The calyx length and corolla length are not significantly different between the putative hybrid and its parents. Some morphological characters of *R. agastum* and the artificial hybrid *R. decorum* (♀) × *R. delavayi* (♂), such as the corolla shape, the number of corolla lobes, the number of stamens, corolla



Fig. 1 Differences in floral coloration and floral shape in the *Rhododendron* species. **a, e** *R. delavayi*, **b, f** *R. agastum*, **c, g** the artificial hybrid of *R. decorum* and *R. delavayi*, **d, h** *R. decorum*

Table 2 Variation (mean \pm 1 SD) in morphological characters of *R. delavayi*, *R. agastum*, *R. decorum*, and an artificial hybrid

Morphological character	<i>R. delavayi</i>	<i>R. agastum</i>	Artificial hybrid <i>R. decorum</i> (♀) \times <i>R. delavayi</i> (♂)	<i>R. decorum</i>
Number of corolla lobes	5	5	6–9	6–9
Number of stamens	10	10	11–16	11–16
Stamen length (mm)	24.10 \pm 3.42 ^a	31.20 \pm 4.32 ^b	–	25 \pm 3.82 ^{a,b}
Scape length (mm)	8.66 \pm 2.25 ^a	8.75 \pm 1.21 ^a	–	33.64 \pm 7.76 ^b
Calyx length (mm)	2.00 \pm 0.16 ^a	2.60 \pm 0.38 ^a	–	2.88 \pm 0.83 ^a
Corolla length (mm)	44.17 \pm 6.49 ^a	40.9 \pm 2.01 ^a	–	46.88 \pm 3.15 ^a
Pistil length (mm)	29.41 \pm 5.02 ^a	38.8 \pm 1.92 ^b	–	36.75 \pm 2.47 ^{a,b}
Corolla shape	Bell	Between bell and bugle	Between bell and bugle	Bugle
Corolla color	Red	Pink	Pink	White
Spots in the corolla tube	Less	More or less	None	None
Hairs on the lower leaf surface	Thick	Less	Less	None
Hairs on the fruit	Thick	Less	Less	None

Species with the *same letters* do not differ significantly for that character ($P < 0.05$)

color, spots in the corolla tube, and hairs on the fruit are intermediate between those of *R. delavayi* and *R. decorum*. The morphological characters of *R. agastum* and the artificial hybrid are similar but can be clearly distinguished.

Nuclear ribosomal DNA ITS sequences

Eighteen sequences of nrDNA ITS region were directly sequenced from the 17 samples of *R. delavayi*, *R. decorum*, *R. agastum*, and the artificial hybrid (Table 1). Sequences of ten clones from two individuals of *R. agastum* (A5 and A7) were also obtained. The length of each ITS region was 654 bp, which is the same for all the samples. Six sites in the ITS region were polymorphic, distinguishing the two haplotypes that represented *R. decorum* and *R. delavayi* (Table 3; Fig. 2). The eight ITS sequences of *R. agastum* and the artificial hybrid could be distinguished from the

sequences of *R. delavayi* and *R. decorum*. Two sequence types were found among the ten ITS clones of *R. agastum*. Four ITS clones matched sequences from *R. delavayi*, and five sequences were identical to *R. decorum*. One ITS clone possessed *R. delavayi* nucleotides at five sites while the other site matched *R. decorum* (Table 3).

Chloroplast DNA *trnL-F* sequences

Sequences of approximately 950 bp of the chloroplast *trnL-F* region were obtained from 16 individuals of *R. delavayi*, *R. decorum*, *R. agastum*, and the artificial hybrid. Twelve sites were variable, distinguishing the two haplotypes of *R. delavayi* and *R. decorum* (Table 4). The *trnL-F* sequences of the eight *R. agastum* samples were identical with the *R. delavayi* sequence, and the sequence of the artificial hybrid was the same as that of *R. decorum*.

Table 3 Alignment of ITS sequences obtained from examined *R. delavayi*, *R. decorum*, *R. agastum*, *R. decorum* (♀) × *R. delavayi* (♂), and clone sequences of *R. agastum*

Specimens	ITS sequence region (bp)					
	94	101	114	203	494	505
DL1, DL2, DL3, DL4, DL5	C	C	T	G	C	T
DC1, DC2, DC3, DC4	T	G	G	T	T	C
A1, A2, A3, A4, A5, A6, A7, A8, DD	Y	S	K	K	Y	Y
A5-1, A5-4, A5-5, A7-1, A7-5	T	G	G	T	T	C
A5-2	T	C	T	G	C	T
A5-3, A5-6, A7-3, A7-4	C	C	T	G	C	T

K G/T, Y C/T, S G/C

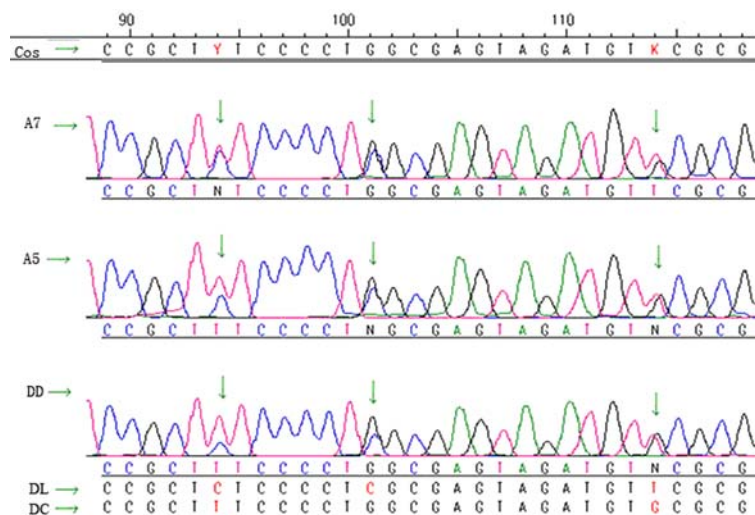
Discussion

Gottlieb (1972) discussed several criteria for testing whether a particular diploid taxon has originated through hybridization: geographic distribution in the region of parental sympatry, morphological intermediacy in several characters, partial fertility, and biochemical additivity. Although no single criterion can provide a clear means for testing a hypothesis of hybridization, each criterion that can be met provides a higher level of evidence of a hybrid origin (Gottlieb 1972; Padgett et al. 1998). In the present

study, geographic overlap, intermediate morphology, fertility and molecular data of *R. agastum* satisfy the four criteria of Gottlieb.

Rhododendron delavayi, *R. decorum*, and *R. agastum* are widespread in northwestern and eastern Yunnan and grow sympatrically in subalpine and alpine forests (Wu 1986; Hu and Fang 1994). We observed and measured 12 morphological traits of the three species and an artificial hybrid between *R. decorum* and *R. delavayi*. The characters of flower, leaf and fruit appeared distinct between the two parental species, and those of *R. agastum* displayed a mosaic of parental and intermediate characters. The artificial hybrid indicated gene flow between *R. delavayi* and *R. decorum* and that the *R. decorum* (♀) × *R. delavayi* (♂) cross is fertile (Zhang et al. 1998).

Furthermore we have performed interspecific crossing by hand between these species in the wild since March 2005, and the results reveal that *R. agastum* can backcross with *R. delavayi* and *R. decorum* and produce many viable seeds. Nuclear DNA is inherited biparentally and chloroplast DNA is inherited maternally in the majority of angiosperms (Yang et al. 2000; Tsukaya et al. 2003). The fact that our artificial hybrid of *R. decorum* (♀) × *R. delavayi* (♂) possessed the *R. decorum* cpDNA haplotype strongly suggests that this occurs in the case of *Rhododendron*. The

Fig. 2 Alignment of sequences of the ITS region of *R. decorum* (DC), *R. delavayi* (DL), *R. agastum* (A), and the artificial hybrid of *R. decorum* and *R. delavayi* (DD). An example of variation can be noted at sites 94, 101 and 114**Table 4** Alignment of *trnL-F* sequences obtained from examined *R. delavayi*, *R. decorum*, *R. agastum*, and *R. decorum* (♀) × *R. delavayi* (♂)

Specimens	<i>trnL-F</i> sequence region (bp)											
	124	299	326	328	329	331	332	333	336	808	938	939
DL1-DL5	G	T	T	T	T	T	T	T	G	A	G	T
A1-A8	G	T	T	T	T	T	T	T	G	A	G	T
DD	A	G	A	A	A	A	A	A	T	C	A	A
DC1-DC4	A	G	A	A	A	A	A	A	T	C	A	A

nuclear ITS data clearly indicate molecular evidence with polymorphic states in sequences obtained by direct sequencing in the hybrid at positions where the putative parents differed, supporting a hybrid origin of *R. agastum* (Table 3). The *trnL-F* sequences of these species indicate *R. delavayi* as the maternal parent of the hybrid species and *R. decorum* as the paternal parent. The morphological and molecular evidence presented in this study allows the postulation of a hybrid origin of *R. agastum*. Our results therefore support the hypothesis of Cox (1994).

We have not observed any evidence to indicate whether *R. agastum* represents the F₁ generation or a later backcross. The morphological characters confirm that *R. agastum* represents a stabilized hybrid. The *trnL-F* sequences show the direction of gene flow between *R. delavayi* and *R. decorum*, but the data of the ITS clone from *R. agastum* indicate that gene exchange may have occurred between *R. agastum* and its parental species. Moreover, our artificial interspecific crossings between these species reveal that *R. agastum* can backcross with the two parental species. However, the results presented here do not suggest whether the hybrid between *R. delavayi* and *R. decorum* can occur repeatedly. Thus, more molecular population-level analyses and data on the reproductive biology are needed.

It should be pointed out that *R. agastum* has two varieties: var. *agastum* Balf. f. et W. W. Smith and var. *pennivenium* (Balf. f. et Forrest) T. L. Ming (Chamberlain 1982; Hu and Fang 1994). The two varieties are dissimilar in many morphological traits such as the shape of their leaf tips, the hairs on the calyx, filament and style. They also have different geographic distributions: *R. var. agastum* is distributed in eastern and northwestern Yunnan and *R. var. pennivenium* is distributed in southwestern Yunnan; thus they do not occur sympatrically in the wild (Chamberlain 1982; Wu 1986). The hybridization origin of *R. var. agastum* was investigated here, and the status of *R. var. pennivenium* with view to its origin is the logical subject for further studies.

Although our study confirms that *R. agastum* is of hybrid origin, derived from *R. delavayi* and *R. decorum*, it is difficult to determine its phylogenetic position in subg. *Hymenanthes*. Furthermore, the taxonomic significance of the morphological differences on which the classification is based is not always obvious. We realize that the proposed affinities between the subsections of this subgenus are speculative, but it is hoped that this may stimulate further research. In our study, these species demonstrate that diploid hybridization may be frequent among sympatric species of subsect. *Arborea*, subsect. *Fortunea*, and subsect. *Irrorata*. More work on the phylogeny is therefore necessary to verify the relationship of the three subsections within *Rhododendron* subg. *Hymenanthes*.

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References

- Arnold ML (1996) Natural hybridization and introgression. Princeton University Press, Princeton
- Barton NH (2001) The role of hybridization in evolution. *Mol Ecol* 10:551–568
- Bean WJ (1976) Trees and shrubs hardy in the British Isles III (NRh), 8th edn. John Murray, London
- Chamberlain DF (1982) A revision of *Rhododendron* II. Subgenus *Hymenanthes*. *Notes R Bot Gard Edinb* 39:209–486
- Chamberlain DF (2003) *Rhododendrons* in the wild: a taxonomist's view. In: Argent G, Mcfarlane M (eds) *Rhododendrons* in horticulture and science. The Royal Botanic Garden, Edinburgh, pp 42–52
- Collins D, Mill RR, Moller M (2003) Species separation of *Taxus baccata*, *T. canadensis*, and *T. cuspidate* (taxaceae) and origins of their reputed hybrids inferred from RAPD and cpDNA data. *Am J Bot* 90:175–182
- Cox PA (1994) Note of natural hybrids and intraspecific variation of *Rhododendron* in China. In: Duan CZ, Liao SC, Li QL, Li XW (eds) Scientific investigation of the plant on Cangshan Mountain. Yunnan Science and Technology Press, Kunming
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochem Bull* 19:11–15
- Ferguson D, Sang T (2001) Speciation through homoploid hybridization between allotetraploids in peonies (*Paeonia*). *Proc Natl Acad Sci USA* 98:3915–3919
- Gonzalez-Rodriguez 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
- Hu LC, Fang MY (1994) Flora of China. Tomus 57(2). Science, Beijing
- Ma XF, Szmidt AE, Wang XR (2006) Genetic structure and evolutionary history of a diploid hybrid pine *Pinus densata* inferred from the nucleotide variation at seven gene loci. *Mol Biol Evol* 23:807–816
- Marhold K, Lihova J, Perny M, Grupe R, Neuffer B (2002) Natural hybridization in *Cardamine* (Brassicaceae) in the Pyrenees: evidence from morphological and molecular data. *Bot J Linn Soc* 139:275–294
- Milne RI, Abbott RJ (2000) Origin and evolution of invasive naturalized material of *Rhododendron ponticum* L. in the British Isles. *Mol Ecol* 9:541–556
- Milne RI, Abbott RJ, Wolff K, Chamberlain DF (1999) Hybridization among sympatric species of *Rhododendron* (Ericaceae) in Turkey: morphological and molecular evidence. *Am J Bot* 86:1776–1785

- Ming TL (1984) The revision of subgenus *Hymenanthus* in Yunnan and Tibet. *Acta Bot Yunnanica* 6:141–171
- Ming TL, Fang RZ (1990) The phylogeny and evolution of genus *Rhododendron*. *Acta Bot Yunnanica* 12:353–365
- Nagamitsu T, Kawahara T, Kanazashi A (2006) Endemic dwarf birch *Betula apoiensis* (Betulaceae) is a hybrid that originated from *Betula ermanii* and *Betula ovalifolia*. *Plant Species Biol* 21:19–29
- Padgett DJ, Les DH, Crow GE (1998) Evidence for the hybrid origin of *nuphar* × *rubrodisca* (Nymphaeaceae). *Am J Bot* 85:1468–1476
- Rieseberg LH (1997) Hybrid origins of plant species. *Annu Rev Ecol Syst* 28:359–389
- Rieseberg LH (2001) Chromosomal rearrangements and speciation. *Trends Ecol Evol* 16:351–358
- Schwarzbach AE, Donova LA, Rieseberg LH (2001) Transgressive character expression in a hybrid sunflower species. *Am J Bot* 88:270–277
- Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of the three non-coding regions of chloroplast DNA. *Plant Mol Biol* 17:1105–1109
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins GD (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Tsukaya H (2002) Leaf anatomy of a rheophyte, *Dendranthema yoshinaganthum* (Asteraceae), and of hybrids between *D. yoshinaganthum* and a closely related non-rheophyte, *D. indicum*. *J Plant Res* 115:329–333
- Tsukaya H (2004) Gene flow between *Impatiens radicans* and *I. javerensis* (Balsaminaceae) in Gunung Pangrango, Central Java, Indonesia. *Am J Bot* 91:2119–2123
- Tsukaya H, Fukuda T, Yokoyama J (2003) Hybridization and introgression between *Callicarpa japonica* and *C. mollis* (Verbenaceae) in central Japan, as inferred from nuclear and chloroplast DNA sequence. *Mol Ecol* 12:3003–3011
- Wang XR, Szmidt AE, Savolainen O (2001) Genetic composition and diploid hybrid speciation of a high mountain pine, *Pinus densata*, native to the Tibetan Plateau. *Genetics* 159:337–346
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T (eds) *PCR protocols: a guide to methods and applications*. Academic, San Diego, pp 315–322
- Wu CY (1986) *Flora of Yunnanica*. Tomus 4. Science, Beijing
- Yang TW, Yang YA, Xiong ZG (2000) Paternal inheritance of chloroplast DNA in the genus *Larrea* (Zygophyllaceae). *Am J Bot* 87:1452–1458
- Zhang CQ (2003) *Rhododendron*. Chinese Architecture Press, Beijing
- Zhang CQ, Feng BJ, Lu YL (1998) The study of hybridization of *Rhododendron*. *Acta Bot Yunnanica* 20:94–96