Indian J. Genet., 73(1): 72-81 (2013)
DOI : 10.5958/j.0019-5200.73.1.010

Genetic relationships between *Rubus parvifolius* and *R. coreanus* (Rosaceae), and preliminary identification one of their putative hybrids

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(Received: November 2012; Revised: January 2013; Accepted: January 2013)

Abstract

***Rubus parvifolius* L. and *R. coreanus* Miq. are two morphologically distinct, endemic wild bramble species in East Asia. The two species are sympatric in southwest of China. Natural hybrids between *R. parvifolius* and *R. coreanus* do generally occur. In this study, chromosomal, palynological, RAPD and ISSR markers were developed to identify *R. parvifolius*, *R. coreanus* and their putative hybrids. UPGMA model-based clustering of the genetic data indicated that two genetic units existed in these populations. One contained twenty-one accessions, mainly characterized by *R. parvifolius* and *R. coreanus* with typical morphological features ("pure"). The other was composed of sixteen accessions, which shared an intermediate morphology with the *R. parvifolius* and *R. coreanus* ("hybrid"). These results showed obvious genetic differentiation between their potential parents and among individuals of hybrids themselves in the long evolutionary history by several hybridization events. A triploid accession *R03-10* (2*n*=3*x*=21), was identified as a probable natural hybrid of *R. parvifolius* and *R. coreanus* based on morphological, chromosomal and palynological identifications. The species *R. parvifolius*, an important resource involved in the hybridization, multi-polyploidization and speciation of the genus *Rubus*, is discussed.**

Key words: Genetic relationship, *R. parvifolius* L., *R. coreanus* Miq., putative hybrid, RAPD, ISSR

Introduction

Rubus parvifolius L. and *R. coreanus* Miq. are two endemic wild brambles species in East Asia. They are important fruits with great commercial values [1-4]. Based on the morphological differences, the two species have been classified into two subsections of section *Idaeobatus* in the genus *Rubus* (Rosaceae), with *R. parvifolius* assigned to subsection *Stimulantes* and *R. coreanus* to *Pungentes* [5]. Their palynological features also exhibited significant differences [6].

Despite the significant morphological and palynological differences, the two species can be easily crossed [7, 8]. It had been reported that, to a large extent, *R. parvifolius* and *R. coreanus* from Korea, Japan and China shared similar karyotypic features [7-9]. These similarities may have facilitated natural hybridization and formation of natural hybrids between the two species.

In the past ten years, we found that *R. parvifolius* and *R. coreanus* were sympatric throughout the region in southwestern China, especially, in Xichong county, Sichuan province. A morphological continuum exist between the two species making it difficult to reliably identify some specimens. Their typical morphological features are shown in Table 1 and Fig. 1. To date, it is not known whether this morphological continuum is due to high variability within species or hybridization between species.

We assumed *R. parvifolius* and *R. coreanus* with typical morphologies as "pure" and individuals with intermediate morphologies as "hybrid" plants. In this paper, we described and compared the patterns of morphological and genetic variations of the "pure" type and "hybrid" type, evaluated the genetic distances of the two groups and documented the chromosomal and

*Corresponding author's e-mail: wangxrtj@163.com
Published by Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
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pollen morphological characteristics of the accession *R03-10*, as a representative of the putative hybrids.

Materials and methods

Plant materials

Thirteen *R. parvifolius*, twelve *R. coreanus* and twelve putative hybrids were used in this study (Table 1). Two weeks old leaves were directed harvested in the field and stored at –20^{o}C for DNA isolation. For root tip regeneration, live plants of these accessions were collected and planted in the scientific research center of Sichuan Agricultural University. Voucher specimens

Table 1. Description of the accessions used in the study

Taxon and general morphology	Accession No.	Locality	Potentiality in breeding and ploidy level
R. parvifolius L.	*R04-1*	N 31°47' E 105°46', —	Very high yield, diploid[9]
Subshrub, stolon, leaflet 3-5, corymb with 3-30 flowers: calyx; unfolding when flower opens, has more or less setosi. Mature fruits red with diameter 0.5-1.5 cm, weight 0.5-1.2 g and drupelet 1-45. Seed has one per drupelet, economic traits varied greatly among populations[34]	*R04-2*	N 31°47' E 105°46', —	Drupelet 1-4 per fruit, triploid[9]
	R03-3	N 30°55' E 105°54', 520 m	mixed diploid-tetraploid[9]
	R03-4	N 30°55' E 105°54', 550 m	No fruits, tetraploid[9]
	R03-12	N 30°55' E 105°54', 413 m	
	R03-16	N 31°00' E 105°49', 380 m	
	R03-25	N 30°58' E 105°40', 415 m	
	R03-34	N 30°55' E 105°54', 478 m	
	R03-42	N 30°55' E 105°54', 382 m	
	R03-44	N 30°55' E 105°54', 394 m	
	R03-48	N 31°00' E 105°49', 380 m	
	R03-60	N 31°00' E 105°48', 387 m	
	R03-96	N 31°00' E 105°48', 388 m	Mature fruits red, very high yield
R. coreanus Miq.	*R02-3*	N 29°36' E 103°29', 750 m	Mature fruits turn black from bright red with average weight 0.55 g, very high yield, diploid[9]
Shrub, semi erect, leaflet 5-7, corymb with several to 50 flowers; calyx, rolling up when flower opens, has no setosi. Mature fruit is black or dark red with a diameter 0.6-1.4 cm, weight 0.35-1.0 g, most drupelet 19-31, and seed has one per drupelet and is more light than *R. parvifolius*[34]	*R01-4*	N 29°54' E 103°01', 680 m	Mature fruits turn dark red from light red with average weight 0.57 g, very high yield, diploid[9]
	R01-5	N 29°54' E 103°01', 700 m	Very high yield
	R01-14	N 29°54' E 103°01', 680 m	Very high yield
	R03-11	N 30°55' E 105°54', 415 m	
	R03-14	N 31°00' E 105°48', 399 m	
	R03-37	N 31°00' E 105°48', 401 m	
	R03-46	N 31°00' E 105°48', 420 m	
	R03-66	N 31°01' E 105°47', 429 m	
	R03-81	N 31°01' E 105°47', 436 m	
	R03-82	N 31°01' E 105°47', 451 m	Very high yield, drupelet > 20
	R03-83	N 31°01' E 105°47', 460 m	Very high yield, drupelet compact
Putative hybrid	*R03-10*	N 30°55' E 105°54', 414 m	Many flowers did not set fruits, triploid
Subshrub-shrub, stolon- semi erect, leaflet 3-7, large and broad, canes and calyx have long and dense prickles; inflorescences ten to more than twenty flowers, and most flowers did not set fruits.	*R03-24*	N 31°01' E 105°47', 477 m	
	R03-29	N 31°01' E 105°47', 471 m	
	R03-31	N 30°55' E 105°54', 467 m	
	R03-43	N 31°01' E 105°47', 485 m	
	R03-45	N 30°55' E 105°54', 476 m	
	R03-59	N 31°01' E 105°47', 428 m	
	R03-64	N 31°01' E 105°47', 439 m	
	R03-65	N 30°55' E 105°54', 429 m	
	R03-79	N 30°58' E 105°40', 417 m	
	R03-93	N 31°58' E 105°40', 417 m	
	R03-94	N 31°58' E 105°40', 445 m	Mature fruits red, higher yield than other transitional accessions.

Note: The morphological features of typical *R. parvifolius* and *R. coreanus* were from Wang *et al*. [34].

Fig. 1. Comparison of morphological features between putative hybrid *R03-10* and its parents. A, B-putative hybrid; C, D - *R. parvifolius* (Wang *et al*., [34]); E *R. coreanus* (Wang *et al*., [34])

of cited plants were made and housed at the college of horticulture.

DNA extraction

Genomic DNA was isolated from leaves following the protocol described by Zhou [10]. Briefly, five grams leaf material was ground to a fine powder in liquid nitrogen and placed in a microcentrifuge tube with 600 µl of extraction buffer I (100 mM pH 8.0 Tris-HCl, 50 mM pH 8.0 EDTA and 250 mM NaCl) at 0^{o}C for 10 min. The extracted mixture was centrifuged at 12000 rpm 4^{o}C for 10 min and the precipitation was transferred to a new tube and dissolved with 600 µl preheated extraction buffer II (50 mM pH 8.0 Tris-HCl, 20 mM pH 8.0 EDTA, 1.4 mM NaCl, 2% CTAB and 0.05% the β-mercaptoethanol) at 65^{o}C for 60 min. The homogenate was mixed with equal volume mixture of chloroform/isoamyl alcohol (24:1, v/v) and centrifuged at 12000 rpm 4^{o}C for 10 min. The upper aqueous phase was recovered and the DNA precipitation was obtained using equal volume of isopropyl alcohol at 4^{o}C for 10 min. The pellet was washed once each with 70 and

100% ethanol. The air-dried DNA was dissolved in 100 µl TE buffer with 10 µg•ml^{-1} RNase A (10 ng•ml^{-1}) and incubated at 37^{o}C for 30 min. Integrity and purity of the total DNA were verified on 1% (w/v) ethidium bromide (EtBr) stained agarose gel in addition to the absorbance spectrum at wavelength from 220 to 300 nm by utilizing Nanodrop 1000. The sample DNA was diluted as 50 ng•µl^{-1} for RAPD and ISSR analysis.

RAPD and ISSR analysis

Primers were selected out from a set of primers (85 RAPD primers and 50 ISSR primers) through pre-optimization tests. Those primers exhibiting high polymorphism and good reproducibility were further used to screen the full set of accessions (Table 3). PCR amplification reactions were performed in a PTC-200 cycler (MJ Research, Waltham, USA). 20 ng of genomic DNA were amplified in a volume of 25 µl containing 1 × PCR buffer, 2.0 mM $MgCl_2$, 0.24 mM dNTPs, 0.6 µM primer and 1.5 U of Taq DNA polymerase (Sangon, Shanghai, China). The cycling programme began with an initial pre-denaturation for 5 min at 94^{o}C, followed by 45 cycles at 94^{o}C for 30s, 36^{o}C (for RAPD, 55^{o}C for ISSR) for 30 s and 72^{o}C for 2 min plus a final extension at 72^{o}C for 10 min. The PCR products were separated in a 1.5% agarose gel containing 0.5 µg•ml^{-1} EtBr. The bands were then visualized under UV light and photographed.

Data analysis

The electropherogram results were presented as a binary matrix, consisting of '1' for the presence and '0' for the absence of the amplicons at the same locus. A dendrogram was constructed by following UPGMA (unweighted paired group method using arithmetic averages) option of SAHN (sequential, agglomerative, hierarchical and nested) module in NTSYS software package (Version 2.20) [11-13]. Genetic distances of Euclidean's simple matching coefficient measure (GD_{SM}) was estimated as: $GD_{SM} = 1 - [(N_{11} + N_{00})/(N_{11} + N_{10} + N_{01} + N_{00})]$, where N_{11}, N_{00}, N_{10} and N_{01} were the number of band alleles common to both individuals, absence in both individuals; specific to individuals *i* or *j*, respectively. N represented the total number of band alleles [14].

Morphology comparison, chromosome analysis and pollen spectrum for the accession R03-10

Morphology description and comparison was followed as Yü *et al*. [5].Chromosome preparation and description were followed procedures of Wang *et al*. [9, 15]. For pollen spectrum analysis, scanning electron microscopy (SEM) was applied referring to Wang *et al*. [6, 16]. At least 20 pollen grains were screened in pollen spectrum analysis.

Results and discussion

Genetic diversity among the 37 accessions based on RAPD and ISSR markers

Among 135 primers tested, 12 RAPD and 8 ISSR primers selected for the analysis generated polymorphic allelic patterns. RAPD analysis of 37 genotypes yielded 224 fragments, 96.97% (217) of which were polymorphic with an average of 18.1 polymorphic fragments per primer. The size of the amplicons ranged from 200 bp to 3891 bp. As for ISSR fingerprinting, the 8 primers detected 134 polymorphic loci, 93.48% in 143 gross amplified fragments, with an average of 16.8 fragments per primer. Maximum polymorphism was observed in the amplification pattern by primer 2 and 835 (shown in Fig. 2A and B respectively, and in Table 2). In addition, 18 primers (except primers 2 and 4) amplified one to three genotype-specific bands and could accurately differentiate 23 *Rubus* accessions (Table 3).

On the basis of molecular data, a matrix of genetic similarity (GS) coefficient was computed following the procedure of Nei [17]. The similarity coefficient based on 12 RAPD and 8 ISSR amplicons ranged from 0.429 to 0.831, with an average of 0.613. The maximum GS coefficient index was observed between *R. coreanus R01-14* and *R02-3*, which were collected from Ya'an and Mt. Emei, respectively. The minimum GS coefficient was shown in *R. parvifolius R04-2* and the putative hybrid *R03-29*. Within the "pure" group of *R. coreanus*, GS coefficients varied from 0.507 to 0.831, with an average of 0.675. The 13 "pure" *R. parvifolius* accessions had lower values of coefficient index, ranging from 0.443 to 0.799, with an average of 0.643.

A dendrogram of the genetic relationships among both "pure" and "hybrid" types was drawn using GS values according to the UPGMA method. The results indicated that these accessions were divided into two major groups (Fig. 3). One was consisted of 21 accessions mainly characterized by 'pure' *R. parvifolius* and *R. coreanus*, except *R03-34*, *R03-42*, *R03-44*, *R03-11* and *R03-14*. The other group contained 16 accessions characterized by "hybrid" with

Table 2. RAPD and ISSR primers used in this study and the number of polymorphic bands amplified

Method	Primer	Sequence (5'-3')	Polymorphic bands	Method	Primer[b]	Sequence (5'-3')	polymorphic bands
RAPD	SBS-G-08[a]	TCACGTCCAC	18	ISSR	UBC807	$(AG)_8T$	15
	SBS-X-08[a]	CAGGGGTGGA	15		UBC810	$(GA)_8T$	20
	SBS-A-08[a]	GTGACGTAGG	20		UBC825	$(AC)_8T$	14
	SBS-A-10[a]	GTGATCGCAG	23		UBC834	$(AG)_8YT$	12
	SBS-A-12[a]	TCGGCGATAG	20		UBC835	$(AG)_8YC$	19
	1	AGCCAGACGA	20		UBC889	$DBD(AC)_7$	14
	SBS-G-09[a]	CTGACGTCAC	20		UBC890	$VHV(GT)_7$	20
	SBS-A-04[a]	AATCGGGCTG	20		UBC891	$HVH(TG)_7$	20
	SBS-G-02[a]	GGCACTGAGG	22				
	2	GGTCCTCAGG	10				
	3	TCGGAGTGGC	18				
	4	ACTCAGGAGC	11				
	Total		217		Total		134
	Average		18.1		Average		16.8

Note: [a]Primers from SBS Genetech (Beijing, China), and Primers 1-4 from reference by Graham and McNicol [20]; [b]Primers numbers follow those in UBC set # 9 (807-891); Y=C/T, R=A/G, D=A/G/T, B=C/G/T, V=A/C/G, H=A/C/T.

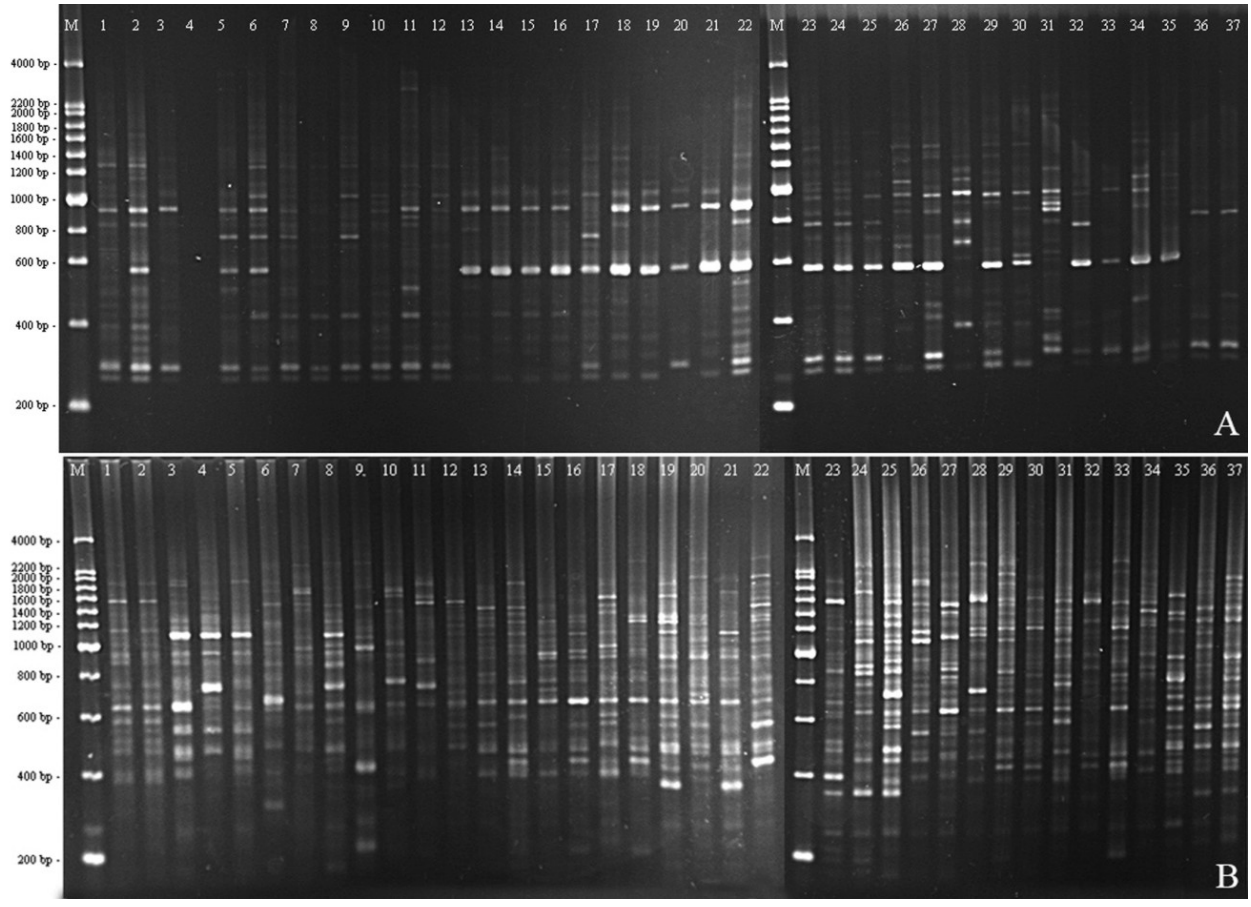

Fig. 2. Typical polymorphic RAPD (A) and ISSR (B) banding pattern from primer 2[20] and UBC 835 for the amplification of *R. parvifolius* (1-13), *R. coreanus* (14-25) and putative hybrids (26-37). Lanes 1-37 correspond to the following samples: 1 *R04-1*, 2 *R04-2*, 3 *R03-3*, 4 *R03-4*, 5 *R03-12*, 6 *R03-16*, 7 *R03-25*, 8 *R03-34*, 9 *R03-42*, 10 *R03-44*, 11 *R03-48*, 12 *R03-60*, 13 *R03-96*, 14 *R02-3*, 15 *R01-4*, 16 *R01-5*, 17 *R01-14*, 18 *R03-11*, 19 *R03-14*, 20 *R03-37*, 21 *R03-46*, 22 *R03-66*, 23 *R03-81*, 24 *R03-82*, 25 *R03-83*, 26 *R03-10*, 27 *R03-24*, 28 *R03-29*, 29 *R03-31*, 30 *R03-43*, 31 *R03-45*, 32 *R03-59*, 33 *R03-64*, 34 *R03-65*, 35 *R03-79*, 36 *R03-93*, 37 *R03-94*

Table 3. Genotype specific RAPD and ISSR markers among 23 bramble genotypes

Method	Accession No.	Primers revealing specific RAPDs (No. of base pairs of a band)	Method	Accession No.	Primers[d] revealing specific ISSRs (No. of base pairs of a band)
RAPD	*R04-1*	G-09[c] (227)	ISSR	*R04-2*	UBC807 (535)
	R03-34	A-08[c] (1888), X-08[c] (1722)		*R03-16*	UBC825 (1686)
	R03-43	A-12[c] (1948), G-02[c] (1708)		*R03-25*	UBC807 (222), UBC889 (1664)
	R03-45	G-09[c] (1513)		*R03-94*	UBC891 (1888)
	R03-46	3 (1697)		*R03-34*	UBC810 (1511)
	R03-82	A-10[c] (1142)		*R03-42*	UBC890 (1161)
	R03-14	G-08[c] (1900)		*R03-45*	UBC807 (1072)
	R03-11	G-02[c] (383)		*R01-4*	UBC834 (1600)
	R03-24	1 (1268)		*R03-46*	UBC889 (276)
	R03-29	A-04[c] (2904), A-12[c] (1513)		*R03-81*	UBC835 (2098, 2497)
	R03-31	A-04[c] (1517), A-10[c] (1888)		*R03-82*	UBC810 (2590)
	R03-10	A-12[c] (3218)		*R03-93*	UBC890 (1688)
	R03-59	G-09[c] (1296)			
	R03-79	3 (961)			

Note: [c]Primers from SBS Genetech (Beijing, China), and Primers 1 and 3 from reference by Graham and McNicol [20]; [d]Primers numbers follow those in UBC set # 9 (807-891).

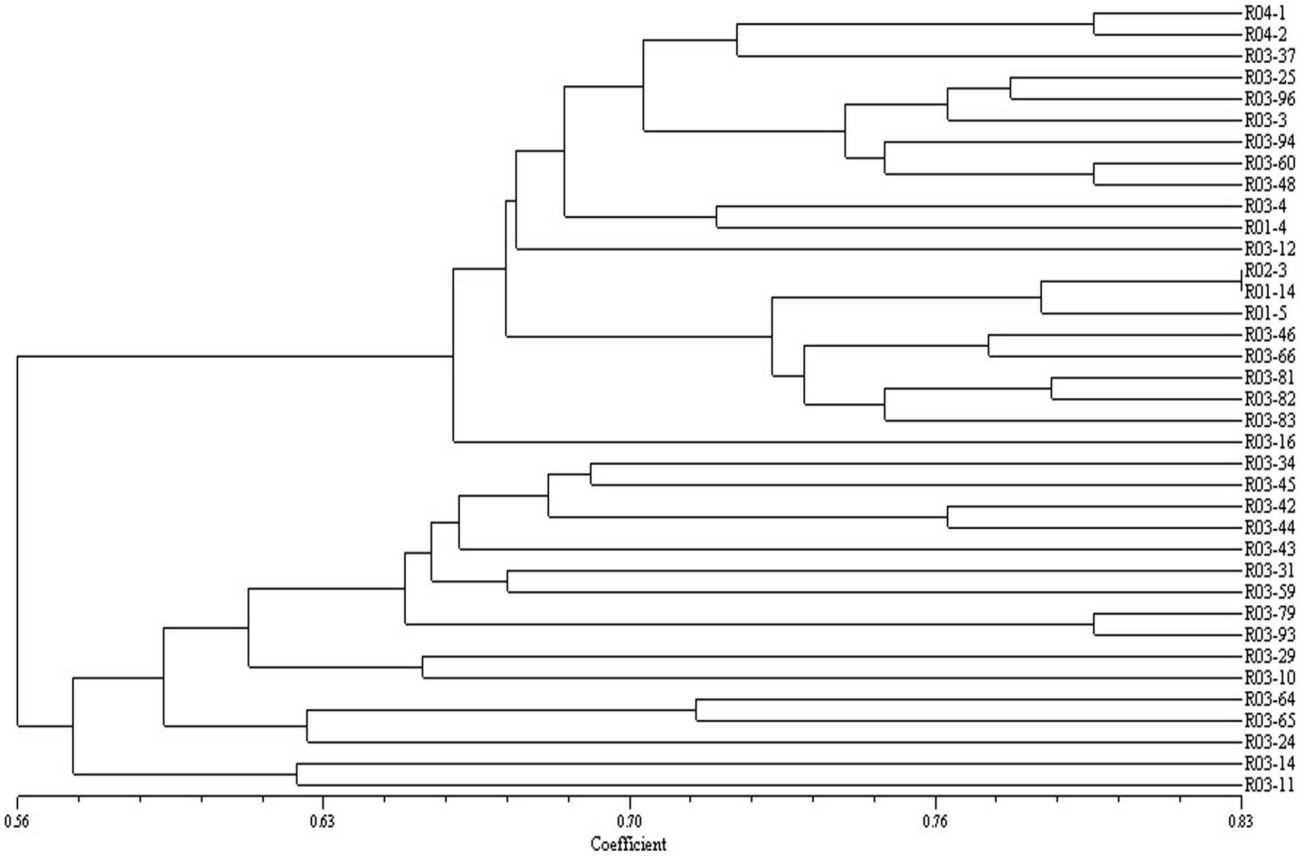

Fig. 3. Genetic relationship among 37 accessions based on RAPD and ISSR data using UPGMA cluster analysis

intermediate morphology, with an exception *R03-94*. The two groups were both consist of three subclusters, in which 13 *R. parvifolius* accessions dispersed without clear regularity and the 12 *R. coreanus* accessions clustered together yet.

Assessment by RAPD and ISSR markers

To identify the differences or relationships of these materials, both RAPD and ISSR molecular markers were used. In the literature, genetic diversity and genetic relationships among *Rubus* species had been elucidated by RAPD or ISSR techniques [18-23]. In the present investigation, at first both two types were used in combination. The amplification of large number of polymorphic bands revealed relatively high genetic variations among the accessions. These results might be capable to explain the observed morphological diversities. In addition, 32 genotype-specific markers were also isolated, which could be further used in identification and selection of specific excellent germplasm in *Rubus*. The 12 RAPD primers came up with 96.97% polymorphic loci, compared with 93.48% by 8 ISSR primers. As there was a good correspondence between the two markers, RAPD was relatively more effective in genetic diversity assessment and accurate differentiation of *Rubus* species.

Genetic relationship between R. parvifolius and R. coreanus

Previous studies had revealed that *R. parvifolius* and *R. coreanus* shared high similarity in karyotypic features [7-9]. In addition, *R. parvifolius* and *R. coreanus* could produce naturally mutual cross [7, 8]. In our investigations, morphologically intermediate accessions were sympatric with typical *R. parvifolius* and *R. coreanus*. It was very possible that these intermediates were hybrids from natural crossing of the two species. In this study, most accessions with intermediate features fell in a cluster showing obvious genetic differentiation between their potential parents and among individuals of themselves in the long evolutionary history. Regardless of the source of variation in the parental and hybrid populations, this molecular variation indicates that the hybrid population is the likely result from several hybridization events involving multiple parents.

In addition, 8 “pure” accessions in *R. coreanus* from different regions clustered together (Fig. 3), suggesting the high genetic stability and narrow genetic background. However, 13 “pure” *R. parvifolius* accessions distributed in all three subclusters. Actually, in the past karyotypic studies, various ploidy levels were existed in the *R. parvifolius* populations, yet merely diploid in *R. coreanus* populations [9]. Noteworthy, the majority of these *R. parvifolius* accessions in this study were collected only from one small region in Sichuan Province, with typical morphological features and high uniformity, showing intraspecific abundant genetic diversity. Therefore, that the molecular data showed the variance of the two species was consistent with the previous cytological data.

Morphology, chromosome and pollen characteristics of the putative hybrid R03-10

Morphological characters of the accession *R03-10,* such as semi-erect, 5-7 leaflets, long and recurved prickles and black mature fruit were similar to morphological characters of *R. coreanus.* However, some morphological features such as calyx with less setosi, short, wide, unfolding and also unrolling up when flower opens were similar to *R. parvifolius* (Fig. 1). From the above main morphology data, we speculate that the accession *R03-10* is probably a hybrid from natural crossing of *R. parvifolius* and

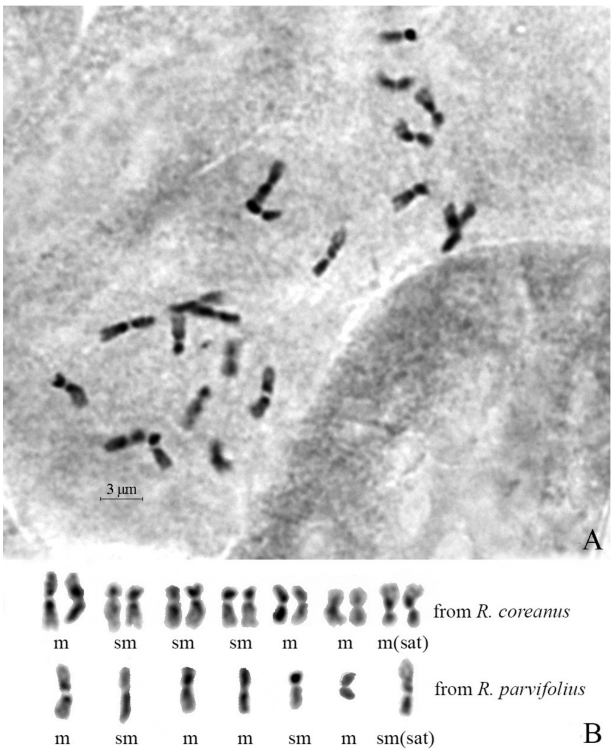

Fig. 4. The chromosome of putative hybrid *R03-10* (2*n*=3*x*=21=10m (2SAT) + 11sm (1SAT)). A. Chromosome number; B. Karyotype. Scale bar represents 3 μm

R. coreanus. By further cytological examination, the putative hybrid were triploid with chromosome number 2*n*=*3x*=21=10m (2SAT) + 11sm (1SAT) (Fig. 4), and chromosomes comprised 10 metacentric chromosomes with two satellites and 11 submetacentric chromosomes with a satellite on the short arms, varying in length from 1.6 to 2.3 μm, and in arm ratio from 1.0 to 3.2, were similar to those of *R. parvifolius* and *R. coreanus* [15]. Based on karyotype analysis, three sets of chromosomes in the putative hybrid *R03-10*, two were from *R. coreanus,* and the other one was from *R. parvifolius* (Fig. 4, B).

Pollen analysis also revealed that *R03-10* was a triploid putative hybrid, and that its potential parents were *R. parvifolius* and *R. coreanus*. There were two types of pollens existed in *R03-10*, one designated as normal type, and the others abnormal one (Fig. 5, D). The former type, characterized by features of prolate (P/E=1.60), was similar to *R. parvifolius* [6]. Pollen features, medium in size (polar axis × equatorial diameter = 23.9 × 11.3 μm - 29.8 × 18.5 μm), striate-perforate in exine ornamentation, and 2.63 perforates per square micron, were much alike *R. coreanus* [6]. While the abnormal ones, which counted for most part of the pollen constitutions, were shrunken and small (one half or less the size of normal types) (Fig. 5, D).

Identification of triploid putative hybrid R03-10

One particular example, the triploid accession *R03-10* (2*n*=3*x*=21) (Fig. 4), whose morphological characters were similar to that of *R. parvifolius* and *R. coreanus* (Fig. 1), was probably a hybrid from natural crossing as illustrated by the preliminary morphology, cytology and palynology data. These data from karyotypic structures, RAPD and ISSR markers and field investigation indicated that the triploid accession probably arose through the fusion of an unreduced gamete from *R. coreanus* and a reduced gamete from

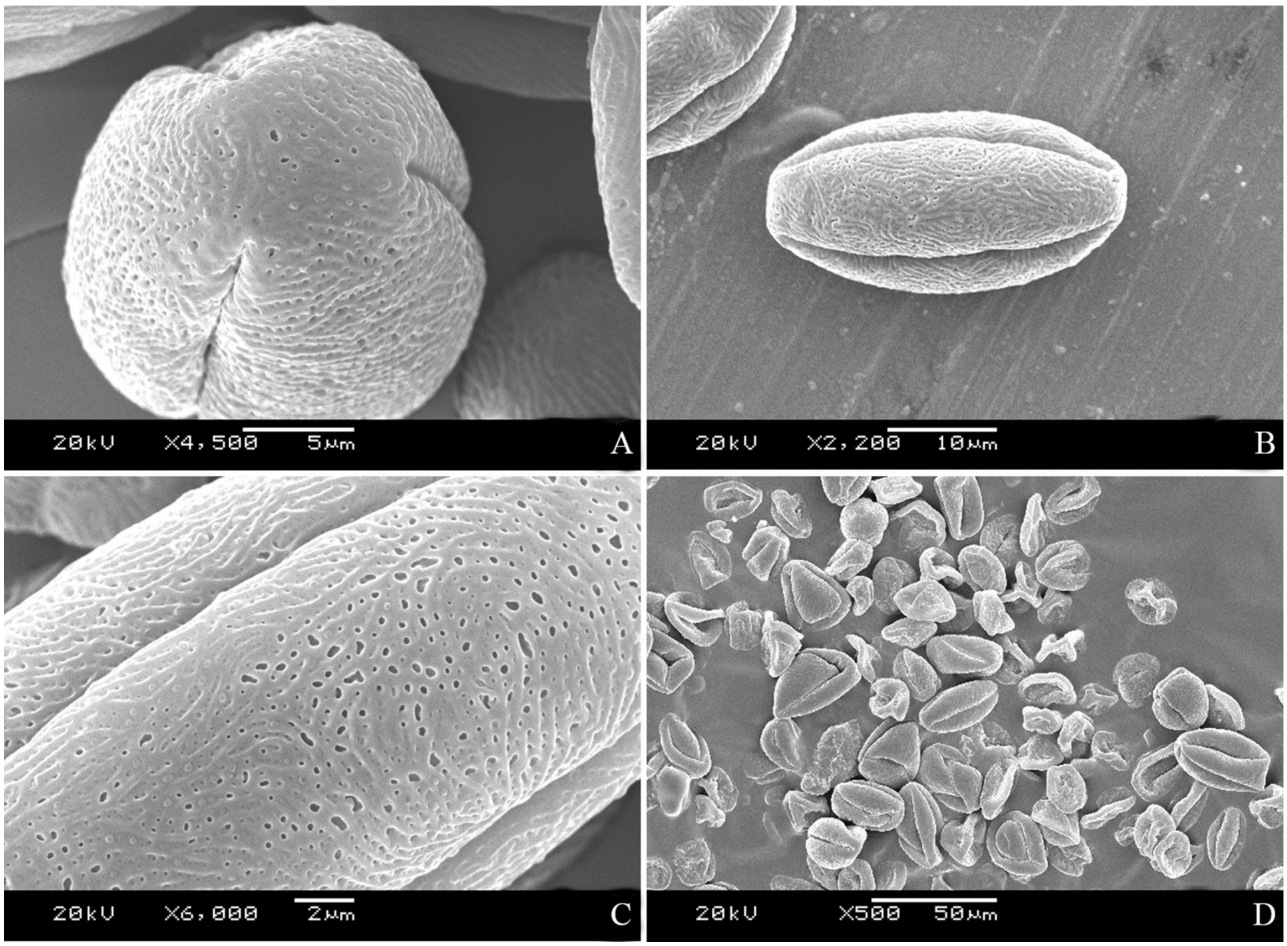

Fig. 5. Pollen morphology of putative hybrid *R03-10*. A. Pollen in polar view (×4500); B. Pollen in equatorial view (×2200); C. Pollen exine ornamentation (×6000); D. Abortive pollen (×500)

R. parvifolius. This hypothesis should be further confirmed by investigations of the chromosome pairing, synapsis and meiotic pollen mother cells of *R03-10*. Moreover, the origins of this triploid hybrid might be commendably explained by these results, accumulating useful information for the crossability of *R. parvifolius*, even for the phylogenetic evolution of the genus *Rubus*.

Possible role of R. parvifolius in hybridization, multi-polyploidization and speciation of the genus Rubus

As observed in many other fruit crops, strong apomixis, asexual propagations, hybridization and polyploidization played important role in the evolution and speciation of the *Rubus* [24-27]. It was reported that the species *R. parvifolius* was one of the parents of many *Rubus* hybrids, *viz*., *R.* × *hiraseanus* (*R. parvifolius* × *R. coreanus*, 2*x*) [7-8, 28], *R.* × *nikaii* (*R. parvifolius* × *R. phoenicolasius*, 2*x*) [29-30] and *R.* × *tawadanus* (*R. parvifolius* × *R. sieboldii* (4*x*), 3*x*) [31-33]. However, the observations that *R. coreanus* could hybridize with *Rubus* species other than *R. parvifolius* had not yet been reported. It was just the crossability of *R. parvifolius* with other species and other natural crossings alike that account for the frequent gene exchanges in *Rubus* species. Combining the various ploidy levels and abundant intraspecific genetic diversity revealed in this study, these circumstances highlighted the possible indispensable role of *R. parvifolius* in hybridization, multi-polyploidization and speciation of the genus *Rubus*, partly causing their rich diversity of morphology. For instance, the triploid *R. parvifolius* with reduced seed production may be very useful for seedless raspberry breeding, if the trait is not just the result of a cytological abnormality and can be transferred to commercial cultivars. Molecular genealogy research is to be applied to the genus *Rubus* in the future.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (Grant No. 30971990).

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