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Genetic Relationship between *Rubus Parvifolius* and *R. Coreanus* (*Rubus*, Rosaceae) based on Simple Sequence Repeat Markers

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Abstract. *Rubus parvifolius* L. and *R. coreanus* Miq. are two morphologically distinct, endemic wild brambles and sympatric in China. Genetic diversity and genetic structure of the two species and their putative hybrids were investigated by using 10 SSR markers selected from 31 markers. A total of 98 alleles were amplified and 4 to 14 alleles per locus were obtained among 55 individuals. Genetic distance calculated from SSR data ranged from 0.7999 to 0.9347 and genetic identity from 0.0675 to 0.2233. Compared to the *R. parvifolius* (H_o = 0.4978) and *R. coreanus* (H_o = 0.5237), almost the same level of observed heterozygosity was observed in the putative hybrid populations (H_o = 0.5447). This was consistent with the low level of genetic differentiation (0.015 to 0.0309) within species and strong gene flow (N_m = 7.7254) among species. *Rubus parvifolius, R. coreanus* and their putative hybrids were categorized into three groups by structure analysis. The AMOVA analysis revealed low genetic differentiation among species (putative hybrids), with only 3.13% of total variability partitioned among them. Based on these results, impact of hybridization and introgression on genetic diversity of *R. parvifolius* and the relationship between *R. parvifolius* and *R. coreanus* were mainly discussed.

Introduction

Rubus parvifolius L. and *R. coreanus* Miq. are two morphologically distinct, wild brambles species in China. They are important fruit resources with potential breeding capabilities and useful for further breeding [1-4]. Based on significant morphological differences, the two species have been classified into two different subsections of section *Idaeobatus* in the genus *Rubus* (Rosaceae), with *R. parvifolius* assigned to subsection *Stimulantes* and *R. coreanus* to *Pungentes*[5]. Despite the morphological and palynological differences, these two species can be easily crossed [6, 7]. It had been reported that they also shared similar karyotypic features [6-8]. These similarities may have facilitated natural hybridization and formation of natural hybrids between the two species[9].

In the past ten years, we have collected, characterized and evaluated many promising wild *Rubus* germplasms. It is helpful for utilizing the germplasms effectively in

breeding program if we can identify and classify them reasonably. However, frequent hybridization and reproduction through apomixis has made the designation of some distinct species difficult in this genus[10, 11], such as *R. parvifolius* and *R. coreanus*.

Based on investigation and evaluation in China, *Rubus parvifolius* displays remarkable morphological diversity in traits. In contrast, *Rubus coreanus* is distributed widely in China and shows relatively little morphological variation. From field surveys, we have found that there is a morphological continuum exists between the two species in their sympatric region in southwestern China, especially in Xichong county, Sichuan province. Therefore, cytological and dominant markers (RAPD and ISSR) were used to analyze individuals of *R. parvifolius*, *R. coreanus* and their putative hybrids[8, 9]. Nevertheless, potential introgressive forms and hybrids make it difficult to clear cognition with these methods due to the limited amount of genetic variation within and among species at cytological characters and dominant markers. Simple sequence repeat (SSR), as a co-dominant marker, is an ideal molecular marker detection species interspecific hybridization[12]. In this study, our objectives are to enhance the understanding of the relationship between *R. parvifolius*, *R. coreanus* and putative hybrids by using molecular data and to accumulate information for the evolutionary process of wild brambles.

Materials and Methods

Plant Materials

Twenty-five *R. parvifolius*, twenty *R. coreanus* and ten putative hybrids were selected from Xichong County, Sichuan Province, China. Their main morphological characters and accession numbers were shown in Table 1. The voucher specimens were deposited in the College of Horticulture, Sichuan Agricultural University, China.

Table 1. Sources and morphological characteristics of the accessions used in this study

Taxa	Typical morphology	Voucher
R. parvifolius L.	Shrubs 1-2 m tall, leaflet 3-5, Branchlets grayish brown or reddish brown to blackish brown, with soft hairs and sparse, curved prickles. Apex of leaflets obtuse, rarely acute; abaxial surface of calyx with needle-like prickles. Mature fruits red. Seed has one per drupelet, economic traits varied greatly among populations.	*R03-97, R03-98, R03-99, R03-100, R03-101, R03-102, R03-103, R03-104, R03-105, R03-106, R03-107, R03-108, R03-109, R03-110, R03-111, R03-112, R03-113, R03-114, R03-115, R03-116, R03-117, R03-118, R03-119, R03-120, R03-121*
R. coreanus Miq.	Shrubs 1-3 m tall, semi erect, leaflet 5-7, Branchlets reddish brown to purplish brown, cylindric, robust, glabrous. Terminal inflorescences corymbs; apex of sepals acuminate to caudate; abaxial surface of calyx pubescent. Mature fruit is black or dark red and seed has one per drupelet and is much lighter than *R. parvifolius*.	*R03-11, R03-14, R03-122, R03-123, R03-124, R03-125, R03-126, R03-127, R03-128, R03-129, R03-130, R03-131, R03-132, R03-133, R03-134, R03-135, R03-136, R03-137, R03-138, R03-139*
Putative hybrid	Shrub, stolon-semi erect leaflet3-7, 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.	*R03-10, R03-65, R03-79, R03-140, R03-141, R03-142, R03-143, R03-144, R03-145, R03-146*

Note. Materials were collected from Xichong County, the latitude from N30°55' to N31°01', longitude from E105°40' to E105°54' and the altitude from 363 m to 520 m.

DNA Extraction and PCR Amplification

Total DNA was isolated from silica-dried leaves using a modified CTAB protocol[13]. *Rubu*s SSR primer were selected from published reports in red raspberry[14] and blackberry[15-17]. Ten SSR markers with strong, unambiguous banding patterns were selected for use in this study (Table 2). PCR amplification reactions were performed in the thermal cycler PTC-200 (MJ Research, Waltham, USA). 10 ng of genomic DNA were amplified in a volume of 20 µl containing 0.4 µmol/L each primer and 1× Taq PCR Master Mix (Kangwei, China). The reaction was initially denatured at 94℃ for 4 min, and then subjected to 31 cycles of 94℃ for 1 min, 49℃ to 55℃ (annealing temperature) for 1 min, and 72℃ for 2 min, followed by a-10 min 72℃ final extension. The PCR products were separated in an 8% denaturing polyacrylamide gels. The bands were detected with silver staining contained 0.2% formaldehyde as described by Panaud[18] with some modifications. Their weights determined by Gelpro32 software with a 20 bp DNA ladder (Kangwei, China) as the standard.

Table 2. SSR primers used in this study

No.	Primer	Sequence (5'-3')	N_a	H_o	H_e	References
1	Rub1C6	F: GTTTAGGTAAGCAATGGGAAAGCTC R: TCTGCCTCTGCATTTTACACAG	11	0.6346	0.8850	[17]
2	Rubus r47	F: AAGCAGGACACCTCAGATGC R: CAGCCAACCATCATCAGCTA	9	0.4423	0.8396	[14]
3	Rubus75	F: CATTTCATCCAAATGCAACC R: CACAACCAGTCCCGAGAAAT	14	0.2449	0.8929	
4	Rubus98	F: GGCTTCTCAATTTGCTGTGTC R: TGATTTGAAATCGTGCGGTTA	4	0.5192	0.6787	
5	Rubus117	F: CCAACTGAAACCTCATGCAC R: ACTTGGTCCTGTTGGTCTGG	10	0.6226	0.8570	
6	Rubus123	F: CAGCAGCTAGCATTTTACTGGA R: GCACTCTCCACCCATTTCAT	10	0.4545	0.8806	
7	Rh_ME0013bG01	F: CCCTCCATCTCCACCATAAA R: GTAAGGCCACCCCATTGAG	7	0.7000	0.7774	[16]
8	Rh_ME0013cE02	F: AGGGTGGGTCTGAGATTGTG R: AACAGTGCACAGGGGCTAAT	8	0.3878	0.8430	
9	Rh_ME0015cH02	F: TGGATTTCCACACGCACATA R: TGTTGGATTTGCCTCCTTTC	13	0.5636	0.9063	
10	ssrRhCBA23	F: ATTGTGTGCATCACTCTGAGAACCG R: ATCGGGGATTTGGTGTGGGTTTAGG	12	0.5926	0.8892	[15]
	Total	—	98	5.162	8.45	—
	Mean	—	9.8	0.5162	0.845	—

Note. N_a, Observed no. of alleles; H_o, Observed heterozygosity; H_e, Expected heterozygosity.

Data Analyses

Fragments amplified with SSR primers were scored as presence (1) and absence (0). Genetic diversity was assessed by calculating N_a (number of alleles per locus), H_o (observed heterozygosity), H_e (expected heterozygosity under Hardy-Weinberg equilibrium), GD (genetic distance) and GI (genetic identity) using the programs POPGENE version 1.32[19].

Genetic structure was investigated using a Bayesian clustering approach without information on the accession origin and assuming the admixture model and correlated

allele frequencies (STRUCTURE 2.2.3)[20]. The species (Putative hybrid) structure was assessed with analysis of molecular variance (AMOVA) using the ARLEQUIN version 3.0 software[21].

Results

Genetic Diversity based on SSR Markers

Among 31 primers tested, 10 primers selected for the analysis generated polymorphic allelic patterns. A total number of 98 alleles were obtained for 55 *Rubus* individuals (Table 2). The estimated values of the expected heterozigosity (H_e) of the studied loci ranged from 0.6787 at locus Rubus98 to 0.9063 at locus Rh_ME0015cH02 with a mean value of 0.8450. Correspondingly the estimated value of the observed heterozigosity (H_o) varied between 0.2449 at loci Rubus75 to 0.7000 at loci Rh_ME0013bG01 with a mean value of 0.5162. The observed heterozigosity is lower than the expected one in all studied SSR loci. An example of SSR pattern, obtained with the primers of Rh_ME0015cH02, is shown in Fig. 1.

Simple sequence repeat analysis revealed a high level of heterozygosity between *R. parvifolius* and *R. coreanus* populations (Table 3). The genetic differentiation (F_{st}) level at 0.0123 (putative hybrid) to 0.0309 (*R. coreanus*) and the values of the Shannon's information index ranged from 1.6685 (putative hybrid) to 1.9624 (*R. parvifolius*). The lowest Nei's expected heterozygosity was calculated for putative hybrid with 0.7782 and highest for *R. parvifolius* with 0.8298. Compared to the *R. parvifolius* (H_o = 0.4978) and *R. coreanus* (H_o = 0.5237), almost the same level of observed heterozygosity was observed in the putative hybrid population (H_o = 0.5447).

Table 4 summarizes the genetic distance (GD) and genetic identity (GI) statistics based on Nei's unbiased estimate[22]. Putative hybrid population shows the relatively farther genetic distance compared with *R. parvifolius* and *R. coreanus*. The AMOVA analysis revealed low genetic differentiation among species (putative hybrids), with only 3.13% of total variability partitioned among them. In addition, the gene flow between *R. parvifolius*, *R. coreanus* and putative hybrids was very strong (N_m = 7.7254), indicating possible introgression.

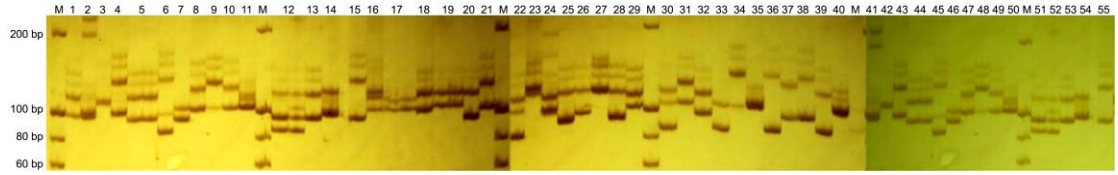

Figure 1. SSR amplification pattern of *R. parvifolius*, *R. coreanus* and putative hybrids genomic DNA by primer Rh_ME0015cH02

Note. Lanes 1-55 corresponded to following samples: 1 *R03-141*, 2 *R03-97*, 3 *R03-98*, 4 *R03-123*, 5 *R03-142*, 6 *R03-99*, 7 *R03-100*, 8 *R03-124*, 9 *R03-101*, 10 *R03-79*, 11 *R03-102*, 12 *R03-143*, 13 *R03-103*, 14 *R03-104*, 15 *R03-147*, 16 *R03-144*, 17 *R03-10*, 18 *R03-11*, 19 *R03-105*, 20 *R03-65*, 21 *R03-106*, 22 *R03-125*, 23 *R03-107*, 24 *R03-108*, 25 *R03-126*, 26 *R03-128*, 27 *R03-110*, 28 *R03-111*, 29 *R03-127*, 30 *R03-129*, 31 *R03-112*, 32 *R03-130*, 33 *R03-113*, 34 *R03-114*, 35 *R03-131*, 36 *R03-132*, 37 *R03-115*, 38 *R03-133*, 39 *R03-134*, 40 *R03-145*, 41 *R03-116*, 42 *R03-14*, 43 *R03-117*, 44 *R03-146*, 45 *R03-135*, 46 *R03-136*, 47 *R03-118*, 48 *R03-119*, 49 *R03-137*, 50 *R03-138*, 51 *R03-139*, 52 *R03-120*, 53 *R03-121*, 54 *R03-140*, 55 *R03-122*.

Table 3. Statistics analysis of genetic diversity for *R. parvifolius*, *R. coreanus* and putative hybrids based on SSR markers

Taxa	N_a	H_o	H_e	Nei	I	F_{st}
Putative hybrid	6.5	0.5447	0.8237	0.7782	1.6685	0.01229
R. parvifolius L.	9.1	0.4978	0.8477	0.8298	1.9624	0.015
R. coreanus Miq.	8.5	0.5237	0.8392	0.8174	1.8957	0.0309
Species (Putative hybrid) level	9.8	0.5162	0.8450	0.8368	2.0076	0.0313

Note. N_a, Observed number of alleles; H_o, Observed heterozygosity; H_e, Expected heterozygosity; Nei, Nei's expected heterozygosity; I, Shannon's Information index; F_{st}, genetic differentiation.

Table 4. Nei's genetic distance and genetic identity between *R. parvifolius*, *R. coreanus* and the putative hybrids

Taxa	Putative hybrid	*R. parvifolius*	*R. coreanus*
Putative hybrid	-	0.8539	0.7999
R. parvifolius	0.1579	-	0.9347
R. coreanus	0.2233	0.0675	-

Note. Nei's genetic distance (below diagonal) and genetic identity (above diagonal).

Genetic Structure of *R. parvifolius* and *R. coreanus* and Putative Hybrids

The program STRUCTURE is used to assign individuals to populations, study hybrid zones, identify migrants and admixed individuals, and estimate population allele frequencies in situations where many individuals are migrants or admixed. In the STRUCTURE analysis for 55 individuals, the number of clusters (*K*) was varied from two to ten, with 10 replicate runs performed for all K values. There was no clear genetic structure according to the Log probability of data, *LnP* (D) (Fig. 2a). The true structure of *R. parvifolius* and *R. coreanus* and their putative hybrids existed at *K* equaling to 3 by calculating Δ*K* (Fig. 2b).

In Fig. 2c, based on SSR data, the three groups were marked as group I, II and III, respectively. *R. parvifolius* and *R. coreanus* and their putative hybrids were found in the group II, indicating hybridization between the two species. Eleven *R. coreanus* belonged to the group II and fifteen *R. parvifolius* belonged to the group III. There were two putative hybrids in the group III may due to highly variability within *R. parvifolius*. In addition, eleven *R. coreanus* clustered together suggesting the high genetic stability. However, twenty-five *R. parvifolius* accessions were distributed in all three groups, showing intraspecific abundant genetic diversity. Therefore, the molecular data are congruent with the previous cytological data [8, 9].

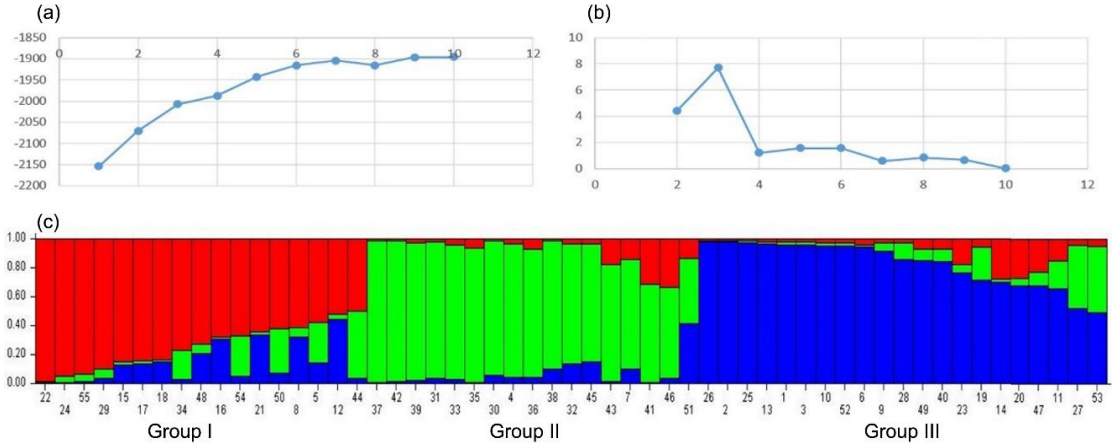

Figure 2. Genetic structure of *R. parvifolius*, *R. coreanus* and their putative hybrids

Note. (a): Plot of the *Ln* probability of data, *LnP (D)*, over 10 runs for each *K* value; (b): Magnitude of

ΔK as a function of K; (c): Structure of *R. parvifolius*, *R. coreanus* and putative hybrids. Lanes 1-55 are the same as Fig. 1.

Discussion

High Level of Genetic Diversity by SSR Markers

SSR markers have been developed from expressed sequence tag and genomic libraries in both red raspberry (subgenus *Idaeobat*us)[14] and blackberry (subgenus *Rubus*)[15-17], while have almost not yet been reported in wild Rubus species in China. In this study, SSR analysis of 55 individual's yield 9.8 observed number of alleles (N_a) with a total of mean observed heterozygosity (H_o) is 0.5162. The results displayed same levels of genetic diversity (H_o = 0.54) and higher in number (except primers Rub1C6 and ssrCBA23) to those obtained in wild black raspberry and high levels of genetic diversity compare with Cultivars black raspberry (H_o = 0.36) by using the same SSRs[17]. This result is in accordance with morphological classification of the varieties, further demonstrating that SSR markers are effective to identify wild *Rubus* species. These results also support that primers designed from blackberry and raspberry species generally can be used to wild *Rubus* germplasms in China.

Genetic Relationship between *R. parvifolius* and *R. coreanus*

Spontaneous hybridization between *R. parvifolius* and *R. coreanus* has been commonly found in their sympatric distribution areas [6-8]. Introgressive hybridization produces new genotypes, increasing genetic diversity and may lead to the establishment of novel species that are adapted to particular environments[23, 24]. Based on the morphological features and SSRs results of the high level of observed number of alleles (N_a = 9.1) and Shannon's Information index (I = 1.96) of *R. parvifolius* showing intraspecific abundant genetic diversity. The high crossability between *R. parvifolius* and *R. coreanus* in the present study may shows that the genomic components of *R. parvifolius* could be directly introgressed into *R. coreanus* without complex operation processes. It may be caused that *R. coreanus* populations have a high level of observed heterozygosity (H_o = 0.5237). The observed combination with molecular markers in these morphologically continuum individuals implies that they are probably the descendants of cross and backcross between *R. parvifolius* and *R. coreanus*.

Genetic structure reflects the interactions among species' long-term evolutionary, genetic drift, gene flow and natural selection[25, 26]. In the present study, low level of genetic distance (GD = 0.0675) and genetic differentiation (F_{st} = 0.0313), high level of genetic identity and strong gene flow (N_m = 7.7254) indicated that *R. parvifolius* and *R. coreanus* have very close relationship. Strong evidence has been found for contemporary hybridization between *R. coreanus* and *R. parvifolius*. But *R. coreanus* could hybridize with *Rubus* species other than *R. parvifolius* had not yet been reported before. This may indicate that members of the genus can be difficult to classify into distinct species for many reasons, including hybridization between species and apomixes[27, 28]. In such a background, *R. parvifolius* and *R. coreanus* should do further study by combined with other polyploids and hybrids in sect. *Idaeobatus* and sect. *Malachobatus*. Putative hybrid populations have low level of observed number of alleles (N_a = 6.5). The result shows that sympatric distribution of *R. parvifolius*, *R. coreanus* and their hybrids can transfer genetic information between species and probably facilitate their adaptive evolution[29].

The Role of *R. parvifolius* in Speciation within *Rubus*

In this study, natural hybridization and introgression between *R. parvifolius* and *R. coreanus* was ascribed to vary levels of importance with regard to the genetic makeup of species and the evolutionary history of species complex. It was reported that the species *R. parvifolius* can produce hybrids with many genus Rubus[6, 7, 30-33]. Species circumscription is complicated by polyploidy, agamospermy, lack of a universal species concept, and frequent hybridization[34]. Hybridization has long been recognized as a potential pathway for gene flow between species. Plant hybridization zones have recently been studied because they are perceived to be the dynamic centers of ecological and evolutionary processes for plants and their associated communities. Combining with the present results and the previous research[8, 9], we suspect that natural hybridization between *R. parvifolius* and *R. coreanus*, as well as with other kinds of hybridization may be the cause of *R. parvifolius* populations had undergone inter-subspecies hybridization and introgression. Therefore, we speculate that *R. parvifolius* might participate in the formation of some species in genus *Rubus*.

Natural hybridization occurs widely in plants and is an important mechanism of speciation in flowering plants[35, 36]. *Rubus parvifolius* populations contain rich genetic diversity that may play a key role in *Rubus* breeding. Thus, the genetic information should be further studied by chloroplast and nuclear DNAs, shedding new lights on the origin of hybrids within *Rubus*.

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