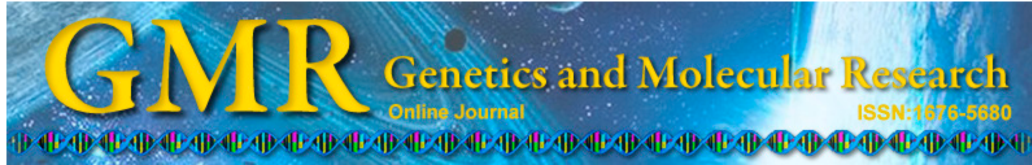

Genetic diversity of wild *Prunus cerasifera* Ehrhart (wild cherry plum) in China revealed by simple-sequence repeat markers

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ABSTRACT. Simple-sequence repeat (SSR) markers were employed to assess the genetic diversity of wild *Prunus cerasifera* Ehrhart (wild cherry plum) in China. Fourteen SSR primer pairs generated a total of 94 alleles (90 were polymorphic, accounting for 95.74%), with a mean of 6.71 alleles per locus. The number of alleles detected at each locus ranged from 2 at BPPCT 028 to 13 at BPPCT 002, with an average of 6.71 alleles per locus. Nei's genetic diversity ranged from 0.0938 to 0.4951 and Shannon's information index ranged from 0.1706 to 0.6882, with averages of 0.3295 and 0.4899, respectively. The SSR data indicated moderate genetic diversity of *P. cerasifera* in China. In the unweighted pair group method with arithmetic mean phylogenetic tree, the 40 forms of *P. cerasifera* were divided into 3 genetic clusters.

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However, the 3 clades determined using SSR data were not consistent with the classification based on morphological characters, such as fruit color. Because of the endangered status and the moderate genetic diversity of *P. cerasifera* in China, both *in situ* and *ex situ* conservation strategies should be adopted.

Key words: Conservation; Endangered species; Genetic diversity; Simple-sequence repeat; Wild *Prunus cerasifera*

INTRODUCTION

Morphological data are useful for botanical classification and identification, but they are easily influenced by environmental factors (Wünsch and Hormaza, 2002). Thus, molecular markers have been used extensively to study genetic relationships within and between plant species. Genetic diversity of a species is the results of long-term evolution and plays an important role in plant survival and evolution. Rich genetic variation generally indicates stronger adaptability and potentiality in evolution, particularly in the case of sharply changing climates or environments (Hamrick and Godt, 1996). Estimation of genetic variation and structure using molecular markers has become a common approach (Groom et al., 2006).

Simple-sequence repeat (SSR) markers are powerful tools for investigating the population genetics of wild species (Yuan et al., 2012; Zhai et al., 2012). Within the genus *Prunus*, numerous SSR markers have been developed, including markers for peach (Cipriani et al., 1999; Testolin et al., 2000; Dirlewanger et al., 2002), cherry (Downey and Iezzoni, 2000; Struss et al., 2003), and Japanese plum (Mnejja et al., 2004), and are transferable between species for genetic diversity analyses (Downey and Iezzoni, 2000; Dirlewanger et al., 2002).

Wild *Prunus cerasifera* is a morphologically diverse species with several subspecies described according to morphological characteristics and ecological or geographical criteria, such as flower (diameter, color of anthers) and fruit characteristics (color, size, shape), and quantitative data, such as flowering and fruit maturity dates, fruit weight, sugar content, or acidity (Faust and Suranyi, 1999; Bernhard and Claverie, 2005). The genetic diversity of *P. cerasifera* clones was previously analyzed using European accessions (Horvath, 2008). However, *P. cerasifera* from Asia has not been examined in genetic studies and the genetic diversity in this region has not been described. In China, wild *P. cerasifera* are distributed only in a narrow area in Huocheng, Xinjiang. *P. cerasifera* in this area show high morphological diversity, and 40 forms have been identified (Wang et al., 2007).

The objective of the present study was to analyze the genetic diversity among the 40 *P. cerasifera* forms in China using SSR molecular markers.

MATERIAL AND METHODS

Plant materials

A total of 40 different forms covering the entire distribution range were sampled based on previous morphological studies (Wang et al., 2007) (Table 1). Fresh leaves were collected from adult trees and stored at -20°C. All voucher specimens were deposited at the Yining Normal University, Yining, China.

Table 1. Information of plant materials used in the present study.

Forms of *Prunus cerasifera*	Fruit color	Fruit shape	Origin
f. armeniaca N. R. Cui et L. Wang	Yellow	Round	Huocheng, China
f. suconea Z. Xu	Yellow	Round	Huocheng, China
f. luteola N. R. Cui	Yellow	Round	Huocheng, China
f. diluteocitrea Z. Xu et L. Wang	Yellow	Round	Huocheng, China
f. erythrocardata N. R. Cui et L. Wang	Red	Cordate	Huocheng, China
f. purunicolora Z. Xu et L. Wang	Red	Round	Huocheng, China
f. phoenicea Z. Xu	Red	Round	Huocheng, China
f. arbiculata N. R. Cui et L. Wang	Red	Round	Huocheng, China
f. ziziphicolora Z. Xu et L. Wang	Red	Elliptic	Huocheng, China
f. puniceocarpa N. R. Cui et L. Wang	Red	Round or elliptic	Huocheng, China
f. opseflorecena Z. Xu et L. Wang	Red	Round or elliptic	Huocheng, China
f. calorcarpa N. R. Cui et L. Wang	Red	Round or elliptic	Huocheng, China
f. elliptica Z. Xu et L. Wang	Red	Elliptic	Huocheng, China
f. rubra Z. Xu et L. Wang	Red	Oblate	Huocheng, China
f. rosea Z. Xu et L. Wang	Red	Ovate	Huocheng, China
f. erythrocarpa Z. Xu et L. Wang	Red	Ovate	Huocheng, China
f. ovata N. R. Cui et L. Wang	Red	Ovate	Huocheng, China
f. erythroorbiculata Z. Xu et L. f. Wang	Red	Round	Huocheng, China
f. huochengensis Z. Xu et L. Wang	Red	Round	Huocheng, China
f. macrocarpa Z. Xu et L. Wang	Dark purple	Ovate	Huocheng, China
f. macroovoida Z. Xu et L. Wang	Dark purple	Ovate	Huocheng, China
f. morecarpa Z. Xu et L. Wang	Dark purple	Round	Huocheng, China
f. macronigraocarpa Z. Xu et L. Wang	Dark purple	Oblate	Huocheng, China
f. praecooflora N. R. Cui et L. Wang	Dark purple	Oblate or round	Huocheng, China
f. pendula bailey N. R. Cui et L. Wang	Dark purple	Oblate or round	Huocheng, China
f. humila N. R. Cui et L. Wang	Dark purple	Oblate or round	Huocheng, China
f. biflora Z. Xu et L. Wang	Dark purple	Oblate or round	Huocheng, China
f. mucronata N. R. Cui et L. Wang	Dark purple	Ovate	Huocheng, China
f. cardata N. R. Cui et L. Wang	Dark purple	Ovate	Huocheng, China
f. auraria Z. Xu et L. Wang	Dark purple	Round	Huocheng, China
f. glabana Z. Xu et L. Wang	Dark purple	Round	Huocheng, China
f. nigrooarpa N. R. Cui et L. Wang	Dark purple	Elliptic	Huocheng, China
f. grandiflora N. R. Cui et L. Wang	Dark purple	Round	Huocheng, China
f. longistigna N. R. Cui et L. Wang	Dark purple	Ovate	Huocheng, China
f. macrophylla N. R. Cui et L. Wang	Dark purple	Ovate	Huocheng, China
f. paniculata N. R. Cui et L. Wang	Dark purple	Round	Huocheng, China
f. micrantha N. R. Cui et L. Wang	Dark purple	Round	Huocheng, China
f. moltoflora N. R. Cui et L. Wang	Dark purple	Ovate	Huocheng, China
f. nigroovata Z. Xu et L. Wang	Dark purple	Ovate	Huocheng, China
f. microcarpa Z. Xu et L. Wang	Dark purple	Ovate	Huocheng, China

Isolation of genomic DNA, polymerase chain reaction (PCR) amplification, and microsatellite genotyping

Total genomic DNA was extracted from fresh leaf tissue from all individuals using the CTAB method, with minor modifications (Doyle and Doyle, 1990). The quantity and quality of DNA was detected by 1% agarose gel electrophoresis, and adjusted to a final concentration of approximately 30 to 50 ng/μL.

Fourteen SSR primer pairs with high PCR success and polymorphism were selected from loci developed in relative *Prunus* species (Dirlewanger, 2002; Mnejja, 2004) (Table 2). DNA amplifications were performed in 15-μL reaction volumes containing 1.5 μL 10X PCR buffer, 0.2 μM of each primer, and approximately 50 ng genomic DNA. The reactions were conducted in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 5 min following by 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension step at 60°C for 30 min. The amplified products

were separated using an ABI 3730xl DNA Analyzer with GeneScan™600 LIZ (Applied Biosystems) as an internal size standard, and sizes were determined using GeneMapper ver. 3.2 (Applied Biosystems).

Table 2. Information for 14 SSR loci.

SSR name	SSR sequences	Repeat motif	Origin
BPPCT 002	TCG ACA GCT TGA TCT TGA CC CAA TGC CTA CGG AGA TAA AAG AC	$(AG)_{25}$☆	Dirlewanger, 2002
BPPCT 004	CTG AGT GAT CCA TTT GCA GG AGG GCA TCT AGA CCT CAT TGT T	$(CT)_{22}$☆	Dirlewanger, 2002
BPPCT 007	TCA TTG CTC GTC ATC AGC CAG ATT TCT GAA GTT AGC GGT A	$(AG)_{22}$ $(CG)_2$ $(AG)_4$☆	Dirlewanger, 2002
BPPCT 008	ATG GTG TGT ATG GAC ATG ATG A CCT CAA CCT AAG ACA CCT TCA CT	$(GA)_{36}$☆	Dirlewanger, 2002
BPPCT 028	TCA AGT TAG CTG AGG ATC GC GAG CTT GCC TAT GAG AAG ACC	$(TC)_{15}$☆	Dirlewanger, 2002
BPPCT 030	AAT TGT ACT TGC CAA TGC TAT GA CTG CCT TCT GCT CAC AC C	$(AG)_{25}$☆	Dirlewanger, 2002
BPPCT 040	ATG AGG ACG TGT CTG AAT GG AGC CAA ACC CCT CTT ATA CG	$(GA)_{14}$☆	Dirlewanger, 2002
CPSCT007+/AY426195	GTG GCC GGA CGA GAG AAC CGA TCG AAT GAA GCT CAG TG	$(GA)_3GC(GA)_3$… $(GA)_7$$^{\Delta}$	Mnejja, 2004
CPSCT012/AY426200	ACG GGA GAC TTT CCC AGA AG CTT CTC GTT TCC TCC CTC CT	$(GA)_{16}$$^{\Delta}$	Mnejja, 2004
CPSCT017+/AY426203	CAA CTC CAA GCT CTGC TCC T AGA GCT ACA CCA GCC AAA GG	$(CT)_{16}$$^{\Delta}$	Mnejja, 2004
CPSCT018/AY426204	AGG ACA TGT GGT CCA ACC TC GGG TTC CCC GTT ACT TTC AT	$(CA)_5(CT)_{20}$$^{\Delta}$	Mnejja, 2004
CPSCT021/AY426206	GCC ACT TCG GCT AAA AGA GA TCC ATA TCT CCT CCT GCT TGA	$(GA)_{15}$$^{\Delta}$	Mnejja, 2004
CPSCT042/AY426226	TGG CTC AAA AGC TCG TAG TG CCA ACC TTT CGT TTC GTC TC	$(GA)_{10}$$^{\Delta}$	Mnejja, 2004
CPSCT044/AY426228	CCA GCA CAG AGA AAA CGA TG GAG CTC CTA CTC TGA GTC TGT AAA A	$(GT)_8(AT)_4$…$(GA)_7CA(GA)_4$$^{\Delta}$	Mnejja, 2004

Stars: developed from *Prunus persica* cultivar ‘Merrill O’Henry (Dirlewanger et al., 2002); triangles: developed from *Prunus salicina* Lindl. (Japanese plum) (Mnejja et al., 2004).

Genetic diversity analyses

Amplified fragments for all loci were scored as alleles based on peak sizes on the peak. The number of alleles per locus, effective number of alleles per locus, Nei’s genetic diversity index (H), and Shannon’s information index (I) were estimated using the POPGENE software (Yeh et al., 1999). SSR alleles were also coded to generate a 1/0 matrix and Nei’s genetic distance was calculated using POPGENE 32 (Nei, 1978; Yeh et al., 1999). Unweighted pair group method with arithmetic mean tree was produced using the Phylip program package (Felsenstein, 2004).

RESULTS

Fourteen SSR loci generated a total of 94 alleles, of which 90 (95.74%) were polymorphic. The number of alleles detected at each locus ranged from 2 in BPPCT 028 to 13 in BPPCT 002, with an average of 6.71 alleles per locus. H ranged from 0.0938 to 0.4951 and I ranged from 0.1706 to 0.6882, with averages of 0.3295 and 0.4899, respectively (Table 3).

Table 3. Characteristics of 14 SSR loci detected in *Prunus cerasifera*.

SSR names	Size range (bp)	N_A	N_E	H	I
BPPCT 002	188-210	13	10.6836	0.3709	0.5241
BPPCT 004	176-188	3	1.677	0.0938	0.1706
BPPCT 007	121-147	5	4.7805	0.4764	0.6693
BPPCT 008	88-125	9	7.44405	0.3301	0.4589
BPPCT 028	158-162	2	1.98075	0.4951	0.6882
BPPCT 030	131-155	9	6.1019	0.2175	0.3443
BPPCT 040	132-144	7	5.40045	0.3316	0.5001
CPSCT007+/AY426195	160-221	12	10.155	0.3835	0.5624
CPSCT012/AY426200	151-162	4	3.05225	0.3001	0.4524
CPSCT017+/AY426203	173-207	8	5.8473	0.2806	0.4332
CPSCT018/AY426204	137-167	6	4.0813	0.2344	0.3822
CPSCT021/AY426206	133-168	6	5.5696	0.4606	0.6531
CPSCT042/AY426226	128-177	7	5.15125	0.2927	0.4522
CPSCT044/AY426228	183-199	3	2.23965	0.3268	0.5070
Total		94			
Mean		6.71		0.3295	0.4899

Allele number per locus (N_A), the effective number of alleles per locus (N_E), Nei's genetic diversity (H), and Shannon information index (I).

In the unweighted pair group method with arithmetic mean phylogenetic tree, the 40 *P. cerasifera* forms were divided into 3 clusters (Figure 1). Cluster I included 4 forms, of which 3 forms were with dark purple fruits and 1 had red fruits. Cluster II comprised 18 forms, 3 of which had yellow fruits, 4 had red fruits, and 11 had dark purple fruits. Cluster III also included 18 forms, including 1 form with yellow fruits, 10 with red fruits, and 7 with dark purple fruits. Within each cluster, forms with the same colored fruits did not cluster into 1 single clade, but were mixed together.

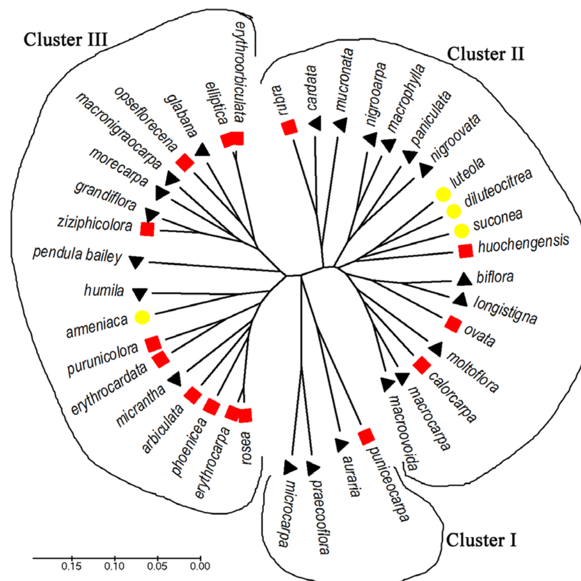

Figure 1. Unrooted UPGMA phylogenetic tree based on Nei's genetic distances of SSR data. Only the forms' names are shown. Yellow circles, red squares, and dark triangles beside taxa names represent forms with yellow-, red-, and dark purple-colored fruits, respectively.

DISCUSSION

The amount of genetic diversity within populations is a fundamental parameter in evolutionary and conservation biology. High levels of genetic variation are expected to increase the potential of populations to respond to selection and to maintain the health of individuals. The 14 SSR markers in the present study displayed relatively high polymorphism levels, except for BPPCT 004. Our results showed that the genetic diversity of *P. cerasifera* in China is moderate (6.71 alleles per locus, H = 0.3295, and I = 0.4899), lower than that revealed in European populations (10.6 alleles per locus, H = 0.75 and I = 0.75) (Horvath et al., 2008). This may be related to the different markers used. Additionally, the habitats of *P. cerasifera* are limited and homogenous, and species within homogenous habitats typically possess relatively low genetic diversity, as demonstrated in *Typha latifolia* (Tsyusko et al., 2005) and *Dayaoshania cotinifolia* (Wang et al., 2013).

Prunus cerasifera is a morphologically diverse species, particularly in its flower and fruit characteristics (flowers' diameter, color of anthers, fruits' color, size, shape), some quantitative traits (such as flowering and fruit maturity dates, fruit weight, plant height, leaf size), and vary among different geographic ranges and populations (Kovalev, 1939, 1941; Faust and Suranyi, 1999). In the narrow distribution area in China, 40 forms were recognized based on fruit, flower, leaf, and other morphological traits (Wang et al., 2007). The 40 forms were divided into 3 lineages based on fruit colors, which were yellow, red, and dark purple. However, the 3 lineages divided by fruit color was not supported by the SSR data. The unweighted pair group method with arithmetic mean phylogenetic tree showed that the 40 forms clustered into 3 clades, but each clade included more than 1 colored fruit form. This indicates that classification based on morphological characteristics, such as fruit color, does not reflect the actual phylogenetic relationships of the morphological complicate species. Similarly, subspecies classification using morphological characteristics of European species was not confirmed by chloroplast DNA data (Horvath, 2008). The inconsistency between morphological and molecular data may have been observed because morphological traits are easily influenced by environmental factors (Wünsch and Hormaza, 2002). Furthermore, hybridizations (Horvath, 2008) and polyploidization (Faust and Suranyi, 1999; Zohary and Hopf, 2000) may have contributed to morphological diversification. Various levels of ploidy, with di-, tetra-, and hexaploid forms, were observed (Beridze and Kvatchadze, 1981; Bajashvili, 1991). Polyploidization was found to cause morphological character differentiation in *P. cerasifera* (Hancock, 2003; Horvath et al., 2008).

P. cerasifera is only distributed in some valleys of the Boro Jolo Mountain in Huocheng, China, and is listed as a national key protected species. Over the past 20 years, human activities have posed serious threats to the wild fruit forest in this region. The areas of wild fruit forest are reduced and their habitats are under degradation. *P. cerasifera* resources have been severely damaged. Thus, conservation of this endangered species is important. The endangered status of this species resulted from habitat damage from human activity. Thus, *in situ* conservation strategies should be implemented, such as restriction on fruit picking and overstocking as well as protection of the entire environment in which *P. cerasifera* grows. Additionally, the sizes of the populations should be increased through artificial breeding. Moreover, in this study, 14 polymorphic microsatellite loci revealed moderate level of genetic diversity in *P. cerasifera*. Thus, *ex situ* conservation strategies can be adopted to establish new populations in other areas.

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