Genetic Relationships among 10 *Prunus* Rootstock Species from China, Based on Simple Sequence Repeat Markers

Qijing Zhang and Dajun Gu¹

Liaoning Institute of Pomology, Yingkou, Liaoning 115009, People's Republic of China

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ABSTRACT. To improve the efficiency of breeding programs for *Prunus* rootstock hybrids in China, we analyzed the subgenus status and relationship of 10 Chinese rootstock species, by using 24 sets of simple sequence repeat (SSR) primers. The SSR banding patterns and phylogenetic analysis indicated that subgenus *Cerasus* is more closely related to subgenus *Prunophora* than to subgenus *Amygdalus*, and that subgenus *Lithocerasus* is more closely related to subgenus *Prunophora* and subgenus *Amygdalus* than to subgenus *Cerasus*. In addition, *Prunus triloba* was more closely related to *Prunus tomentosa* than to the members of subgenus *Amygdalus*. Therefore, we suggest that *P. tomentosa* and *P. triloba* should be assigned to the same group, either to subgenus *Lithocerasus* or *Prunophora*, and we also propose potential parent combinations for future *Prunus* rootstock breeding.

Prunus (Rosaceae) is a large genus with significant economic importance, since it includes a variety of popular stone fruit species [e.g., peach (*Prunus persica*), apricot (*Prunus armeniaca*), almond (*Prunus dulcis*), and sweet cherry (*Prunus avium*)]. The genus comprises a polyploid series, of which the basic chromosome number is $x = 8$, and is characterized morphologically by flowers with five sepals, five petals, and a solitary carpel with a terminal style; fruit that are fleshy drupes; and branches with solid pith (Rehder, 1990). Many *Prunus* species are also important as sources of rootstocks. Furthermore, the genetic resources within the genus are abundant, since the genus comprises ≈ 200 species, and ≈ 100 *Prunus* species are indigenous to China (Yü and Li, 1986). However, the classification of *Prunus* species is sometimes controversial, partly owing to the ease of interspecific hybridization (Dosba et al., 1994), which results in the frequent occurrence of intermediate morphotypes. Previously, Rehder (1990) divided *Prunus* into five subgenera: *Prunophora* (plum and apricot), *Amygdalus* (peach, nectarine, and almond), *Cerasus* (cherry), *Padus* (cluster cherry), and *Laurocerasus* (laurel cherry), and later, Ingram (1948) added a sixth subgenus, *Lithocerasus*, by splitting the bush cherry and rock cherry.

Species that originated in China include *P. tomentosa*, *P. triloba*, *Prunus japonica*, *Prunus glandulosa*, and *Prunus humilis*, which are among the important dwarf rootstock species used for peach, plum, and apricot (Ding, 1997). Of these species, *P. tomentosa* has been cultivated for fruit production in China for over 2000 years, owing to its cold resistance and good fruiting habit (Yü and Li, 1986), and *P. humilis* has been gradually increasing in popularity as a promising Chinese fruit tree, owing to the high calcium content of its fruit. Meanwhile, *Prunus pseudocerasus* and *Prunus serrulata* (subgenus *Cerasus*) have long been used as rootstocks for sweet cherry, ever since sweet cherry was introduced to China, and *Prunus davidiana* can be used as a rootstock for plum, apricot, and

peach, whereas *Prunus salicina* and *Prunus sibirica* can be used as a rootstock for cherry, plum, apricot, and peach. Hybridization among *Prunus* species has been successful and has been taken up as an effective strategy for improving rootstock cultivars (Felipe, 2009; Jones, 1928; Shoferistov, 1989; Zhang and Gu, 2015). However, using such a strategy to improve the efficiency of breeding programs requires a sufficient understanding of the groups' phylogeny and of the degree of homology between the genomes of different species.

Therefore, the analysis of genetic relationships among *Prunus* species can provide basic information for broadening the genetic basis of rootstock breeding programs. Molecular systematic analyses have been performed on *Prunus* rootstock species using isozymes (Mowrey and Werner, 1990), restriction-fragment length polymorphisms [RFLPs (Uematsu et al., 1991)], random amplified polymorphic DNA [RAPD (Shimada et al., 2001)], internal transcribed spacer (ITS) sequences (Sangtae and Wen, 2001), and the combination of nuclear ribosomal ITS and plastid markers (Chin et al., 2014). In addition, genomic SSR markers have also been used for genotyping, germplasm characterization, and fingerprinting in many plant species, since they are polymerase chain reaction (PCR)-based, highly polymorphic, codominant, abundant, and highly reproducible (Powell et al., 1996), and moreover, genomic SSR markers have been developed in almost every cultivated *Prunus* species. However, previous studies of rootstock species from China have not used these SSR markers. Accordingly, in the present study, we aimed to determine the relationships and genetic diversity of 17 *Prunus* species and two hybrids using SSR analysis, with particular emphasis on the genetic relationships of 10 rootstock species that originated in China.

Materials and Methods

PLANT MATERIALS. We sampled 40 accessions from the Xiongyue Arboretum (Yingkou, China) of which five accessions

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¹Corresponding author. E-mail: dajungu@163.com.

(three species) belonged to subgenus *Prunophora*, five accessions (three species) to subgenus *Amygdalus*, eight accessions (four species) to subgenus *Lithocerasus*, and 22 accessions (seven species and two hybrids) to subgenus *Cerasus* (Table 1).

GENOMIC DNA EXTRACTION. Total genomic DNA was extracted from young expanded terminal leaves using the citrimonium bromide method (Doyle and Doyle, 1990) with minor modifications. Then, the concentration and quality of the DNA were assessed using ultraviolet–visible spectrophotometry (DU 800; Beckman Coulter, Fullerton, CA) and electrophoresis on 1% TAE agarose gels, and the samples were diluted to a final concentration of 250 ng·μL⁻¹.

Table 1. Forty *Prunus* accessions used in genetic relationship analysis among 10 rootstock species from China by simple sequence repeat.

Code	Accession	Type	Species	Subgenus	Origin
1	Bing	Cherry	<i>Prunus avium</i>	<i>Cerasus</i>	America
2	Hongdeng	Cherry	<i>P. avium</i>	<i>Cerasus</i>	China
3	Lapins	Cherry	<i>P. avium</i>	<i>Cerasus</i>	Canada
4	Early Ruby	Cherry	<i>P. avium</i>	<i>Cerasus</i>	Russia
5	Gisela 5	Cherry	<i>Prunus cerasus</i> × <i>Prunus canescens</i>	<i>Cerasus</i>	German
6	Colt	Cherry	<i>P. avium</i> × <i>Prunus</i> <i>pseudocerasus</i>	<i>Cerasus</i>	UK
7	Xinxing	Cherry	<i>Prunus fruticosa</i>	<i>Cerasus</i>	Russia
8	Askaya	Cherry	<i>P. fruticosa</i>	<i>Cerasus</i>	Russia
9	Xiwang	Cherry	<i>P. fruticosa</i>	<i>Cerasus</i>	Russia
10	Early Richmond	Cherry	<i>P. cerasus</i>	<i>Cerasus</i>	Unknown
11	Shuanyingtao A	Cherry	<i>P. cerasus</i>	<i>Cerasus</i>	Unknown
12	Shuanyingtao B	Cherry	<i>P. cerasus</i>	<i>Cerasus</i>	Unknown
13 ^z	Daqingye	Cherry	<i>P. pseudocerasus</i>	<i>Cerasus</i>	China
14 ^z	Xiaoqingye	Cherry	<i>P. pseudocerasus</i>	<i>Cerasus</i>	China
15 ^z	Laiyangaiying	Cherry	<i>P. pseudocerasus</i>	<i>Cerasus</i>	China
16 ^z	Dawolouye	Cherry	<i>P. pseudocerasus</i>	<i>Cerasus</i>	China
17 ^z	Shanying A	Cherry	<i>Prunus serrulata</i>	<i>Cerasus</i>	China
18 ^z	Shanying B	Cherry	<i>P. serrulata</i>	<i>Cerasus</i>	China
19 ^z	Shanying C	Cherry	<i>P. serrulata</i>	<i>Cerasus</i>	China
20 ^z	Shanying D	Cherry	<i>P. serrulata</i>	<i>Cerasus</i>	China
21	Mahaleb	Cherry	<i>Prunus mahaleb</i>	<i>Cerasus</i>	Unknown
22 ^z	Maoyingtao A	Cherry	<i>Prunus tomentosa</i>	<i>Lithocerasus</i>	China
23 ^z	Maoyingtao B	Cherry	<i>P. tomentosa</i>	<i>Lithocerasus</i>	China
24 ^z	Maoyingtao C (whipping)	Cherry	<i>P. tomentosa</i>	<i>Lithocerasus</i>	China
25 ^z	Yuyemei A	Almond	<i>Prunus triloba</i>	<i>Amygdalus</i>	China
26 ^z	Yuyemei B	Almond	<i>P. triloba</i>	<i>Amygdalus</i>	China
27 ^z	Yuyemei C	Almond	<i>P. triloba</i>	<i>Amygdalus</i>	China
28 ^z	Fuli	Plum	<i>Prunus salicina</i>	<i>Prunophora</i>	China
29	Gaixiandali	Plum	<i>P. salicina</i>	<i>Prunophora</i>	China
30 ^z	Xiaohuangli	Plum	<i>P. salicina</i>	<i>Prunophora</i>	China
31 ^z	Gaiguo 3	Cherry	<i>Prunus humilis</i>	<i>Lithocerasus</i>	China
32 ^z	Gaiguo 4	Cherry	<i>P. humilis</i>	<i>Lithocerasus</i>	China
33 ^z	Yuli	Cherry	<i>Prunus japonica</i>	<i>Lithocerasus</i>	China
34 ^z	Mailiwhite	Cherry	<i>Prunus glandulosa</i>	<i>Lithocerasus</i>	China
35 ^z	MailiRed	Cherry	<i>P. glandulosa</i>	<i>Lithocerasus</i>	China
36	Yinghua	Cherry	<i>Prunus yedoensis</i>	<i>Cerasus</i>	Japan
37 ^z	Shantao	Peach	<i>Prunus davidiana</i>	<i>Amygdalus</i>	China
38	Chongyanghong	Peach	<i>Prunus persica</i>	<i>Amygdalus</i>	China
39	Katy	Apricot	<i>Prunus armeniaca</i>	<i>Prunophora</i>	America
40 ^z	Shanxing	Apricot	<i>Prunus sibirica</i>	<i>Prunophora</i>	China

^z*Prunus* rootstock species that originated in China.

PCR AMPLIFICATION AND PRODUCT ELECTROPHORESIS. The diluted genomic DNA was PCR amplified using 24 sets of SSR primers that were previously developed in peach, apricot, and cherry (Table 2). The 20-μL reaction mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM MgCl₂, 0.1 mM each dNTP, 0.2 M each primer, 40 ng genomic DNA, and 0.45 U Taq polymerase and were amplified using a thermocycler (PTC-200; MJ Research, Waltham, MA) with the following program: an initial denaturation of 4 min at 94 °C; 28–31 cycles of 45 s at 94 °C, 45 s at 57.5–62 °C, and 45 s at 72 °C; and a final extension of 8 min at 72 °C. The amplification products were electrophoresed on 8% polyacrylamide gels in 1× TBE buffer with a constant current of 40 mA, and the gels were stained with silver nitrate (Promega, Madison, WI), according to the manufacturer's instructions. In addition, amplification with each primer combination was performed twice to verify the reproducibility of our results.

SSR DATA ANALYSIS. Each SSR band was considered an individual character, and the presence or absence of the band was scored using binary code (present = 1, absent = 0). Subsequently, a data matrix was assembled and analyzed using PAUP* (Swofford, 1998), and a pairwise distance matrix was generated based on total character differences. The genetic relatedness of the *Prunus* species was analyzed using the unweighted pair group method with arithmetic mean (UPGMA), based on total character difference, and bootstrap analysis was conducted using UPGMA search methods with 10,000 replicates, to obtain confidence values for the branches of the tree.

Results and Discussion

SSR MARKER CHARACTERIZATION. Bands or banding patterns that are unique to specific groups can be used for species identification or subgenus classification (Zhang et al., 2008). To identify the more common alleles shared by different species, 24 SSR primer sets were selected from over 170 pairs of primers that were previously developed for peach, cherry, and apricot (Zhang et al., 2008). In the present study, a total of 251 alleles were identified, with a mean of 10.5 alleles per SSR locus. Among the 24 loci, the CPPCT27 locus was the most polymorphic, with 17 alleles, whereas the UCD-CH12 locus was the least polymorphic, with only six alleles (Table 2).

Table 2. List of simple sequence repeat primers used in genetic relationship analysis and their amplified products in 40 *Prunus* accessions.

Primer	Primer sequence (5' → 3')	Motif	Source	Size range (bp)	Alleles (no.)
UDP96-001	AGTTTGATTTTCTGATGCATCC TGCCATAAGGACCGGTATGT	(CA)17	Cipriani et al. (1999)	100–150	8
UDP96-003	TTGCTCAAAAGTGTCTGTTGC ACACGTAGTGCAACACTGGC	(CT)11(CA)28	Cipriani et al. (1999)	85–150	16
UDP96-008	TTGTACACACCCTCAGCCTG TGCTGAGGTTTCAGGTGAGTG	(CA)23	Cipriani et al. (1999)	130–190	10
UDP97-401	TAAGAGGATCATTTTTGCCTTG CCCTGGAGGACTGAGGGT	(GA)19	Cipriani et al. (1999)	110–150	9
UDP97-403	CTGGCTTACAACCTCGCAAGC CGTCGACCAACTGAGACTCA	(AG)22	Cipriani et al. (1999)	95–170	15
UDP98-021	AAGCAGCAATTGGCAGAATC GAATATGAGACGGTCCAGAAGC	(GA)22(CA)11	Testolin et al. (2000)	100–150	12
UDP98-022	CTAGTTGTGCACACTCACGC GTCGAGGAACAGTAAGCCT	(TG)12(AG)24	Testolin et al. (2000)	85–165	13
UDP98-024	CCTTGATGCATAATCAAACAGC GGACACACTGGCATGTGAAG	(GT)19TC(TG)7	Testolin et al. (2000)	75–125	12
UDP98-406	TCGGAAACTGGTAGTATGAACAGA ATGGGTCGTATGCACAGTCA	(AG)15	Testolin et al. (2000)	70–105	8
UDP98-416	TTTTCTCAGCAGCCAAACAA ATGTTTCGTGCTTCTGCTCC	(AG)18	Testolin et al. (2000)	80–125	12
Pchgms2	GTCAATGAGTTTCAGTGTCTACACTC AATCATAACATCATTACGCCACTGC	(CT)24	Sosinski et al. (2000)	135–190	8
Pchgms4	ATCTTCACAAACCCTAATGTC GTTGAGGCAAAAGACTTCAAT	(CT)21	Sosinski et al. (2000)	160–200	9
PceGA59	AGAACCAAAAGAACGCTAAAATC CCTAAAATGAACCCCTCTACAAAT		Cantini et al. (2001)	140–300	12
PMS30	CTGTCGAAAGTTTGCCTATGC ATGAATGCTGTGTACATGAGGC		Cantini et al. (2001)	135–175	11
PMS40	TCACTTTCGTCCATTTTCCC TCATTTTGGTCTTTGAGCTCG		Cantini et al. (2001)	90–140	9
CPPCT27	GAGCAGTTCATAAGTTGGAACAA CGATAAAGATTTTGACTGCATGA	(CT)30	Aranzana et al. (2002)	65–110	17
UCD-CH11	TGCTATTAGCTTAATGCCTCCC ATGCTGATGTCATAAGGTGTGC	(CT)15	Struss et al. (2003)	95–170	10
UCD-CH12	AGACAAAGGGATTGTGGGC TTTCTGCCACAAACCTAATGG	(CA)14	Struss et al. (2003)	170–220	6
UCD-CH13	ACCCGCTTACTCAGCTGAAC TTAGCACTAAGCCTTTGCTGC	(CA)10	Struss et al. (2003)	135–270	8
UCD-CH19	GTACAACCGTGTTAACAGCCTG ACCTGCACTACATAAGCATTGG	(CA)12	Struss et al. (2003)	105–150	10
UCD-CH31	TCCGCTTCTCTGTGAGTGTG CGATAGTTTCTTCCAGACC	(CT)26	Struss et al. (2003)	90–200	10
UCD-CH39	CACTGTCTCCCAGGTTAAACTC CCTGAGCTTTTGACACATGC	(CT)28	Struss et al. (2003)	95–175	9
PacB35	ATTGCGATTTTCGGTCTGTT CCATCCCAAATTGCTTACTT	(CA)14(GA)11	Decroocq et al. (2003)	210–295	8
PacA10	TGAGCATAATTGGGGCAG GCCAGAGAAGCCATTTCAGT	(GA)14	Decroocq et al. (2003)	65–150	9

Both common and unique alleles were identified using 10 group-specific markers on a certain set of species (Table 3).

For example, one allele at the UDP96-003 locus was shared by accessions of *P. tomentosa*, *P. humilis*, *P. japonica*, and *P. glandulosa* and, therefore, was able to distinguish species in subgenus *Lithocerasus* from those in the other subgenera, whereas another allele from the same locus was shared by *P. triloba* and *P. salicina* (Fig. 1A). Similarly, the PacA10 locus was able to distinguish *P. salicina* (subgenus *Prunophora*) from *P. tomentosa*, *P. triloba*, *P. humilis*, *P. japonica*, *P. glandulosa*

(subgenus *Lithocerasus*), *P. davidiana*, and *P. persica* [subgenus *Amygdalus* (Fig. 1B)]; and the UCD-CH31 and UCD-CH39 loci were able to distinguish *P. tomentosa* from all other species. In addition, both the UDP96-008 and UDP97-401 loci yielded species-specific alleles for each of *P. japonica*, *P. humilis*, and *P. glandulosa*; and the UDP96-008, UDP98-024, and CPPCT27 loci yielded a common allele for *P. davidiana* and *P. persica* (subgenus *Amygdalus*) (Table 3).

In addition, we also found that subgenus *Cerasus* (*P. avium*, *Prunus fruticosa*, *Prunus cerasus*, *P. pseudocerasus*, *P. serrulata*,

Table 3. Ten group-specific simple sequence repeat markers applied to genetic relationship analysis among 40 *Prunus* accessions distinguish a set of species by generating common and unique bands.

SSR marker	Species	Band size (bp)
UDP96-003	<i>Prunus triloba</i> , <i>Prunus salicina</i>	80
	<i>Prunus tomentosa</i> , <i>Prunus humilis</i> , <i>Prunus japonica</i> , <i>Prunus glandulosa</i>	90
UDP96-008	<i>P. humilis</i> , <i>P. japonica</i> , <i>P. glandulosa</i>	140
	<i>Prunus davidiana</i> , <i>Prunus persica</i>	142
UDP97-401	<i>P. humilis</i> , <i>P. japonica</i> , <i>P. glandulosa</i>	120
UDP98-024	<i>P. davidiana</i> , <i>P. persica</i>	105
UDP98-416	<i>P. salicina</i> , <i>P. humilis</i> , <i>P. japonica</i> , <i>P. glandulosa</i>	90
Pchgms2	<i>Prunus avium</i> , <i>Prunus fruticosa</i> , <i>Prunus cerasus</i> , <i>Prunus pseudocerasus</i> , <i>Prunus serrulata</i> , <i>Prunus mahaleb</i> , <i>Prunus yedoensis</i> , <i>P. cerasus</i> × <i>Prunus canescens</i> , <i>P. avium</i> × <i>P. pseudocerasus</i>	135
CPPCT27	<i>P. davidiana</i> , <i>P. persica</i>	110
UCD-CH31	<i>P. triloba</i> , <i>P. salicina</i> , <i>P. humilis</i> , <i>P. japonica</i> , <i>P. glandulosa</i> , <i>P. davidiana</i> , <i>P. persica</i> , <i>Prunus armeniaca</i> , <i>Prunus sibirica</i>	155
	<i>P. tomentosa</i>	158
UCD-CH39	<i>P. tomentosa</i>	95
PacA10	<i>P. tomentosa</i> , <i>P. triloba</i> , <i>P. humilis</i> , <i>P. japonica</i> , <i>P. glandulosa</i> , <i>P. davidiana</i> , <i>P. persica</i>	95
	<i>P. armeniaca</i> , <i>P. sibirica</i>	98

Fig. 1. Simple sequence repeat band patterns of 21 *Prunus* accessions generated by UDP96-003 and PacA10 markers. (A) Marker UDP96-003: allele “a” was shared by *Prunus tomentosa*, *Prunus humilis*, *Prunus japonica*, and *Prunus glandulosa*; allele “b” was shared by *Prunus triloba* and *Prunus salicina*. (B) Marker PacA10: allele “c” was shared by *Prunus armeniaca* and *Prunus sibirica*; allele “d” was shared by *P. tomentosa*, *P. triloba*, *P. humilis*, *P. japonica*, *P. glandulosa*, *Prunus davidiana*, and *Prunus persica*. *Prunus* accession numbers are detailed in Table 1; M = DNA size markers.

Prunus mahaleb, *Prunus yedoensis*, *P. cerasus* × *Prunus canescens*, and *P. avium* × *P. pseudocerasus*) could be distinguished by a unique allele at the Pchgms2 locus. Although subgenus *Cerasus* exhibits a wide geographic distribution, all the specimens included in the present study possessed the same allele, whereas the other species exhibited polymorphism. During the optimization of Pchgms2 amplification, only a single allele was amplified under different annealing temperatures (52, 55, 58, and 60 °C) in the 22 accessions representing subgenus *Cerasus*. Thus, the locus Pchgms2 was very useful for identifying subgenus *Cerasus*.

Among the 24 loci, the UCD-CH12 locus was determined to be the most suitable for homology analysis, with four common alleles shared by the 17 species and 2 hybrids analyzed, and

could be useful for elucidating relationships within the genus. Thus, it might eventually be possible to classify *Prunus* species using only one or two SSR markers. Cipriani et al. (1999) reported that 59% of peach SSR primer pairs amplified products of expected size in other *Prunus* species. Our results also show that the examined *Prunus* species were closely related and that SSR primer pairs exhibit high transferability within the genus. Although Dirlewanger et al. (2002) reported that the transferability of primer pairs across species is not related to genetic distance, our results indicated that more closely related species were more likely to share similar alleles. For example, *P. humilis*, *P. japonica*, and *P. glandulosa* shared a distinguishing allele with *P. salicina* at locus UDP98-416; and *P. humilis*, *P. japonica*, *P. glandulosa*, *P. triloba*, and *P. tomentosa* shared a distinguishing allele with *P. davidiana* and *P. persica* at locus PacA10 (Table 3), which indicated that subgenus *Lithocerasus* was more closely related to subgenus *Prunophora* than subgenus *Cerasus*.

PHYLOGENETIC ANALYSIS. All the accessions from subgenus *Cerasus* clustered together (Fig. 2), thus providing support for their common genetic origin. *P. cerasus* and *P. fruticosa* were more closely related to each other than to *P. avium*, which supports the hypothesis that *P. cerasus* arose as an allotetraploid from the hybridization of *P. avium* ($2n = 2x = 16$) and *P. fruticosa* ($2n = 4x = 32$; Olden and Nybom, 1968). Meanwhile, the hybrid ‘Gisela 5’ (*P. cerasus* × *P. canescens*) connected the *P. avium* group to the group that contained *P. cerasus* and *P. fruticosa*, and the hybrid ‘Colt’ (*P. avium* × *P. pseudocerasus*) was grouped between the cherries from Asia and Europe.

Fig. 2. Dendrogram of 40 *Prunus* accessions based on unweighted pair group method with arithmetic mean analysis of simple sequence repeat data. Numbers below the lines indicate bootstrap values (percentage of 1000 replicates). Bootstrap values greater than 50% are shown.

Although *P. mahaleb* originated in Europe, it was closely related to cherries from Asia. Generally, subgenus *Cerasus* is more divergent from the other groups. Subgenus *Cerasus* was also more closely related to subgenus *Prunophora* than to subgenus *Amygdalus*, which confirms the hypothesis that cherry is genetically more distant from peach than from apricot (Badenes and Parfitt, 1995; Chin et al., 2014; Watkins, 1976). Successful hybridization among *Prunus* species has been reported [e.g., *Prunus amygdalus* × *P. persica* (Felipe, 2009), *Prunus domestica* × *P. armeniaca* (Jones, 1928), *P. davidiana* × *Prunus mira* (Shoferistov, 1989)], which indicates that hybridization and crossing could occur among plum, apricot, and peach, but not with cherry.

The *P. salicina* accessions formed a group with high bootstrap values, along with a group that included *P. humilis*, *P. japonica*, and *P. glandulosa* (Fig. 2), which together formed a group with *P. tomentosa* and *P. triloba*. The apricot group (*P. armeniaca*, *P. sibirica*) was more similar to the plum group (*P. salicina*) than to the peach group (*P. davidiana*, *P. persica*). A previous morphological study suggested that the evolution

of these species progressed from peach > apricot > plum > cherry (Yü and Li, 1986), which is corroborated by the molecular analysis of the present study.

GENETIC DIVERSITY OF FOUR SPECIES FROM SUBGENUS LITHOCERASUS. The four species from subgenus *Lithocerasus* that were included in the present study failed to cluster into a single group and, instead, were grouped with members of the subgenus *Prunophora*. This grouping suggests that subgenus *Lithocerasus* is closely related to *P. salicina*. A close relationship was previously reported between *P. glandulosa* and *P. japonica*, which belong to subgenus *Prunophora* and subgenus *Amygdalus*, respectively, based on RAPD analysis (Shimada et al., 2001). The present study grouped *P. japonica*, *P. glandulosa*, and *P. humilis*, and within this group, *P. glandulosa* was more closely related to *P. japonica* than to *P. humilis*. In addition, a previous isozyme study (Mowrey and Werner, 1990) indicated that *P. japonica* only differed from *P. glandulosa* by one of 110 alleles and, moreover, that *P. japonica* and *P. glandulosa* grouped together in subgenus *Cerasus*. However, although the present study confirms the genetic similarity of *P. humilis*, *P. japonica*, and *P. glandulosa*, the species were placed in subgenus *Prunophora* (Fig. 2), in agreement with the ITS sequence analysis of Bortiri et al. (2006), which indicated that *P. tomentosa* and *P. glandulosa*

were more closely related to subgenus *Prunophora* than to subgenus *Amygdalus*.

Evidence from interspecific crosses also supports the classification of subgenus *Lithocerasus* as distinct from subgenus *Cerasus* and suggests that subgenus *Lithocerasus* is more closely related to subgenus *Prunophora*. For example, members of subgenus *Lithocerasus* section *Microcerasus* have been reported to hybridize readily with members of subgenus *Prunophora* and, to some extent, with members of subgenus *Amygdalus*, but generally fail to hybridize with members of subgenus *Cerasus* (Garley, 1980; Kataoka et al., 1988). Furthermore, *P. japonica* is also capable of growing on rootstocks of both *P. glandulosa* and *P. armeniaca* (Fan, 1999), and the grafting compatibilities between some members of subgenus *Lithocerasus* and subgenera *Prunophora* or *Amygdalus* is greater than that of subgenus *Cerasus* (Ding, 1997; Mizutani et al., 1985). Thus, our results support a classification system that separates bush cherry and rock cherry into either subgenus *Lithocerasus* or subgenus *Prunophora*.

THE STATUS AND RELATIONSHIP OF *P. TOMENTOSA* AND *P. TRILOBA*. The species *P. tomentosa* and *P. triloba* are morphologically similar and are mainly distinguished by fruit morphology. The fruit of *P. triloba* is larger and dry at maturity, whereas the fruit of *P. tomentosa* is smaller and remains fleshy. However, it is difficult to distinguish the two species without flowers or fruit, and few studies have investigated their phylogenetic relationship. The topology of the present study agrees with the species' morphological similarity, since *P. tomentosa* and *P. triloba* were grouped together and formed a sister group to subgenus *Prunophora*. Previously, RAPD analysis (Shimada et al., 2001) placed the two species in subgenus *Amygdalus*; however, isozyme analysis grouped the species together as a separate clade that was closer to members of subgenus *Prunophora* than to those of subgenus *Cerasus* (Mowrey and Werner, 1990). Further, according to the analysis of nrITS sequences and plastid markers (Chin et al., 2014), *P. tomentosa* and *P. triloba* are grouped together and are more closely related to *P. glandulosa* (subgenus *Lithocerasus*) and subgenus *Prunophora* than to subgenus *Amygdalus* or subgenus *Cerasus*. Our findings support the conclusions drawn from the analysis of isozymes, nrITS sequences, and plastid markers.

Traditionally, *P. triloba* has been placed in subgenus *Amygdalus*; however, this classification has been questioned. For example, Zhao (1996) suggested that *P. triloba* be placed in subgenus *Cerasus*, along with *P. tomentosa*, based on morphology. In addition, although *P. triloba* can be used as a rootstock for plum, apricot, and peach, it causes dwarfing in peach (Ding, 1997), a phenomenon that might be explained by genetic distance, since *P. triloba* is more closely related to subgenus *Prunophora* (plum and apricot) than to subgenus *Amygdalus* (peach, nectarine, and almond). Our results also support this point of view.

Meanwhile, *P. tomentosa* cannot be used directly as a rootstock for sweet cherry, owing to the incompatibility of the two species, although using *P. tomentosa* as a rootstock and Fuli (*P. salicina*) as an interstock produces sweet cherries that grow well and fruit earlier. Grafting compatibility also suggests that *P. tomentosa* is more closely related to subgenus *Prunophora* than to subgenus *Cerasus*, since a hybrid rootstock for peach was derived from a cross between *P. tomentosa* and *Prunus cerasifera* (Warner, 1998). The interspecific hybridization of *P. tomentosa* and *P. salicina* (Zhang and Gu, 2015) also supports the conclusion that *P. tomentosa* is more closely related to subgenus *Prunophora* than to subgenus *Cerasus*. Therefore, the results of the present study suggest that *P. triloba* and *P. tomentosa* should be assigned to the same group, either in subgenus *Lithocerasus* or subgenus *Prunophora*, rather than in subgenus *Amygdalus*.

IMPLICATIONS FOR *PRUNUS* ROOTSTOCK BREEDING. The genetic resources of Chinese *Prunus* species have made important contributions to global fruit production, either by direct planting or as breeding material. In addition, Chinese *Prunus* species may also be useful as a source of genetic variation for breeding *Prunus* rootstocks, especially since interspecific crosses in the genus are usually feasible and efficient (in terms of selection pressure). Such interspecific crosses permit the introgression of beneficial genes for tolerance to drought and cold and resistance to pests and disease, from wild species to cultivated species, which can result in progeny that can be cultivated over a wide range of climates or have a wide range of compatibility with other cultivars.

To identify suitable rootstocks for stone fruits, grafting experiments have typically focused on more primitive species from China, such as *P. pseudocerasus*, *P. canescens*, *P. tomentosa*, *P. humilis*, *P. japonica*, and *P. triloba*, which are characterized by their cold resistance, dwarfing, and early fruiting. Further, distant hybridization within *Prunus* species has been widely applied as the preferred strategy for fruit production and has yielded a number of cultivars. For example, 'Colt' (*P. avium* × *P. pseudocerasus*) and 'Gisela 5' and 'Gisela 6' (*P. cerasus* × *P. canescens*) have been important rootstocks for sweet cherry (Bielicki and Rozpara, 2010), whereas a hybrid rootstock from a cross between *P. tomentosa* and *P. cerasifera* is used for peach (Warner, 1998), and an interspecific hybrid of *P. tomentosa* and *P. salicina* exhibited dwarfing, large fruit, and cold resistance (Zhang and Gu, 2015). However, an improved understanding of the genetics of *Prunus* species is fundamental to the development of new breeding programs, and the close relationships identified in the present study will improve *Prunus* breeding programs by helping breeders choose parental combinations and allow the combination of some major characteristics from wild species.

Conclusions

The findings of the present study clarify the relationships and genetic diversity of 10 *Prunus* species from China. Although members of subgenus *Lithocerasus* are morphologically similar to members of subgenus *Cerasus*, the two groups are genetically distinct, and our analysis also indicated that the four examined species of subgenus *Lithocerasus* (i.e., *P. humilis*, *P. japonica*, *P. glandulosa*, and *P. tomentosa*) were more closely related to members of subgenus *Prunophora* than to those of subgenus *Amygdalus* or subgenus *Cerasus*. Furthermore, *P. triloba* was genetically distant from members of subgenus *Amygdalus* but was closely related to *P. tomentosa*. Therefore, we suggest that *P. triloba* be grouped with *P. tomentosa* and placed in either subgenus *Lithocerasus* or subgenus *Prunophora*. The results of our molecular analysis can also be used to direct breeding programs for *Prunus* rootstocks.

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