Genetic Diversity and Phylogenetic Relationships among Plum Germplasm Resources in China Assessed with Inter-simple Sequence Repeat Markers

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ABSTRACT. Inter-simple sequence repeat (ISSR) markers were used to evaluate genetic similarity and interrelationship among 104 plum (*Prunus* L. spp.) and related accessions from the Chinese National Germplasm Repository for Plums and Apricots and the Tianshan Germplasm Repository for Wild Fruit Resources, including six plum species (*Prunus salicina* Lindl., *Prunus simonii* Carr., *Prunus ussuriensis* Kov. et Kost., *Prunus domestica* L., *Prunus cerasifera* Ehrh., and *Prunus spinosa* L.), two related species [apricot (*Prunus armeniaca* L.) and nanking cherry (*Prunus tomentosa* Thunb.)], eight putative hybrids between plum and apricot (plumcot), and six accessions of wild European plum (*P. domestica*). Out of the 42 ISSR primers, 12 were selected, which generated 103 markers in total, 99 of which were polymorphic. Possible accession-specific ISSR bands or patterns were also found. Some possible synonyms or homonyms were clarified or discussed, and closely related accessions such as bud mutants were discriminated. Based on the unweighted pair group method with arithmetic mean (UPGMA) analysis and principal coordinate analysis (PCoA) using the Jaccard coefficient, two different dendrograms were constructed—one including accessions grouped by species and one with all 104 accessions—and a two-dimensional plot was obtained. Three groups were formed in both dendrograms and PCoA plot: Group I including apricot ('Yinxiangbai') and plumcot types; Group II containing Asia-originated diploid species [e.g., *P. cerasifera*, *P. ussuriensis*, *P. tomentosa*, and Chinese plum-types (i.e., *P. salicina* and its hybrids)]; and Group III involving European-origin polyploid species (e.g., *P. spinosa* and *P. domestica*) and recently found wild European plum accessions in China. The dendrogram with accessions grouped by species implied that 1) plumcot types had closer relatedness with apricot than with plum; 2) *P. simonii* should be a variant of *P. salicina* while *P. ussuriensis* an independent species; 3) *P. domestica* was more closely related to *P. spinosa* than to *P. cerasifera*. Two accessions of European plum ('89-7-3' and 'Wanhei') were clustered into outgroups in the dendrogram with all 104 accessions, which could be grouped within Group III in the PCoA plot. The distribution of both European plum and Chinese plum-types across respective groups did not reflect the geographic origins. The present study also further confirmed that the wild plants found in Xinjiang of China were *P. domestica*.

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Plums are among the most important stone fruit crop in the world (Bhutani and Joshi, 1995). The largest producer is China, with an annual production of 4,635,600 metric tons in 2005 [Food and Agriculture Organization of the United Nations (FAO), 2006], accounting for 47% of the world production.

Plums belong to the genus *Prunus*. There are two important plum types: European plum (*Prunus domestica*, $2n = 6x = 48$) and Chinese plum-types, including both pure Chinese plum

(*Prunus salicina*, $2n = 2x = 16$) and its hybrids with other diploid plum species, such as *Prunus simonii*, *Prunus cerasifera*, *Prunus americana*, and others (Bhutani and Joshi, 1995). European plum is used for both drying and fresh markets, while Chinese plum-types are used mainly for fresh market.

European plum and Chinese plum-type cultivars, particularly the improved cultivars grown in California, have a narrow base of genetic diversity, making them not only limited in adaptability but also more susceptible to disease and insect attack (Ramming and Cociu, 1990). For example, eight of the top 10 plum cultivars in commercial production in California trace back to just five parents, all released by Luther Burbank (Okie and Weinberger, 1996).

Although plum production in China has increased rapidly in the past decade, much of the production is still based on selected old or local cultivars, such as 'Qiuli' and 'Wanhong' in northern China and 'Furongli' and 'Naili' in southern China, which have good flavor and adaptability but lack firm flesh and attractive appearance (Liu, 2004). Some improved plum cultivars, such as 'Friar', 'Blackamber', and 'Casselman', have been introduced from the United States into China recently, but most of them have insipid flavor, weak coldhardiness, and are susceptible to bacterial spot [*Xanthomonas arboricola* pv. *pruni* (Smith) Dye], the main plum disease in China (Liu, 1996).

Genetic variability is the prerequisite for any plant breeding program (Khush, 2002). Using diverse plum resources to broaden the genetic base of worldwide plum cultivars and improving local plum cultivars for development of plum industry in China are important objectives for plum breeders.

As an origin center of Chinese plum, China has rich plum germplasm resources. According to Zhang (1990), there are eight species, five botanical varieties, and ≈ 800 local cultivars distributed from Yi'an in Heilongjiang Province (lat. $47^{\circ}50'N$) to the middle parts of the Leizhou Peninsula (around lat. $21^{\circ}N$) in the southernmost parts of Guangdong Province. The Chinese National Germplasm Repository for Plums and Apricots was established at Xiongyue in Liaoning Province in 1986. It presently contains 600 accessions of local and improved cultivars and has been under an active collection program. The diversity of plum germplasm in China is attributed to natural and artificial selection for adaptation and other characteristics over a long period of time and in a wide array of ecological conditions.

Prunus domestica has been cultivated in Europe for at least 2000 years, but no distinctly wild form is known (Westwood, 1993). The genetic origin of European plum remains a controversial issue. Crane and Lawrence (1952) suggested that *P. domestica*, a hexaploid, originated as a hybrid between *Prunus cerasifera*, a diploid, and *Prunus spinosa*, a tetraploid, via either chromosome doubling of the hybrid triploid or as a product of unreduced gametes from both parents. Zohary (1992) proposed that *P. domestica* was an autopolyploid derived from *P. cerasifera* rather than an allopolyploid. Eryomine (1991) even suggested that many other species, including *Prunus microcarpa* C.A. Mey., *Prunus salicina*, *Prunus armeniaca*, and *Prunus persica* (L.) Batsch, could be included.

Recent surveys from Xinjiang in northwestern China found apparently native stands of *P. domestica* in wild forests along the Ili River. Neither of the presumed parental species was found nearby (Lin and Shi, 1990). This finding may shed light on the genetic origin of European plum after in-depth investigation on wild populations. The wild European plum may also be helpful to broaden the genetic base of cultivated plum cultivars.

Before these cultivated or wild resources can be exploited efficiently, they must be systematically evaluated for genetic diversity assessment. We had evaluated the genetic diversity of Chinese plum resources on the basis of morphological traits and isozyme polymorphisms (Zhang and Zhou, 1998); however, morphological traits were highly influenced by the environment, so estimates of genetic diversity were not precise and isozymes were few in number and hence were not very efficient for characterization of the genetic diversity in the germplasm (Khush, 2002).

The advent of DNA molecular markers has ushered in a new epoch in the efficient characterization and identification of germplasm resources. Restriction fragment length polymorphism (RFLP) analysis was the first molecular technique to be used for this purpose. However, RFLPs have the disadvantages of being time-consuming and labor-intensive, and they often involve the use of radioactive materials (Fang et al., 1997). Moreover, this procedure also requires relatively large quantities of sample DNA. Randomly amplified polymorphic DNA (RAPD) analysis is a simple, quick, and convenient procedure requiring much smaller quantities of template DNA, but the reproducibility of this approach has often been questioned (Jones et al., 1997; Virk et al., 2000). Microsatellites or simple sequence repeat (SSR) markers with tandem repeats of a basic motif of <6 bp are the most polymorphic and hence are highly useful markers; however, their development is laborious and expensive (Ammiraju et al., 2001).

Recently, inter-simple sequence repeat (ISSR) markers (Gupta et al., 1994; Zietkiewicz et al., 1994) have emerged as an alternative system with the reliability and advantages of microsatellites (SSR). This approach had been used in several fruit crops, such as blueberry [*Vaccinium* L. (Levi and Rowland, 1997)], strawberry [*Fragaria* L. (Cekic et al., 2001)], citrus [*Citrus* L. (Sankar and Moore, 2001)], grape [*Vitis vinifera* L. (Moreno et al., 1998)], trifoliate orange [*Poncirus trifoliata* (L.) Raf. (Fang et al., 1997)], and cashew [*Anacardium occidentale* L. (Archak et al., 2003)] for the purposes of cultivar identification, germplasm collection or natural population diversity evaluation, phylogenetic relationship analysis, genetic linkage mapping, and marker assisted selection. For plums, phenetic characterization of 24 Chinese plum-types and four European plum cultivars has been reported (Goulao et al., 2001), but no studies on the diversity of the Chinese plum collection have been conducted.

To draw information on genetic variation of plum germplasm resources in China and advance their collection, management, and utilization, a subset of plum collection from two germplasm repositories in China was characterized with ISSR markers in the present study, specifically aiming to 1) identify duplicated or mislabeled accessions, 2) evaluate diversity to facilitate its use in breeding and in developing a collection strategy, and 3) analyze the genetic relationship of plum species, focusing on the origin of European plum.

Materials and Methods

PLANT MATERIAL. One hundred four (104) accessions were tested, which represented six plum species (*P. salicina*, *P. simonii*, *P. ussuriensis*, *P. domestica*, *P. cerasifera*, and *P. spinosa*), two related species (*P. armeniaca* and *P. tomentosa*), eight plumcots, and six accessions of wild European plum (Table 1); and all were collected from the Chinese National

Table 1. Accessions of *Prunus* assayed with ISSR markers in present study.

Accession no.	Planting no. ^z	Accession name	Species ^y	Origin
1	A1-1	Xingmeili	Plumcot	China
2	A1-2	Retsudo Burubetsuto	Plumcot	Japan
3	A1-3	Zhuanzihong	Plumcot	China
4	A1-4	Tanchengxingmei	Plumcot	China
5	A2-6	Yingtaoli	<i>P. cerasifera</i>	China
6	A2-7	Hongyeli	<i>P. cerasifera</i>	China
7	A2-11	Huangniuxinli	<i>P. simonii</i>	China
8	A2-12	Xiangjiaoli	<i>P. simonii</i>	China
9	A2-13	Bianyan	<i>P. simonii</i>	China
10	A2-14	Xiangbianli	<i>P. simonii</i>	China
11	A2-15	Xiangbianli	<i>P. simonii</i>	China
12	A3-15	Bingtangli	<i>P. domestica</i>	Unknown
13	A3-14	Bingtangli	<i>P. domestica</i>	Unknown
14	A4-12	Angeleno	<i>P. salicina</i> hybrid	United States
15	A4-11	89-7-3	<i>P. domestica</i>	United States
16	A4-10	89-7-4	<i>P. domestica</i>	United States
17	TGRWF	Xinjiang #1	Wild <i>P. domestica</i>	China
18	A4-9	483	<i>P. domestica</i>	United States
19	A4-8	89-7-6	<i>P. domestica</i>	United States
20	A4-7	Dameigui	<i>P. domestica</i>	Unknown
21	A3-6	Li #8	<i>P. domestica</i>	Italy
22	TGRWF	Xinjiang #2	Wild <i>P. domestica</i>	China
23	A4-5	Wanhei	<i>P. domestica</i>	Unknown
24	A3-5	Tianli	<i>P. domestica</i>	Unknown
25	A4-4	Ganli	<i>P. domestica</i>	Unknown
26	A4-3	Richard Early	<i>P. domestica</i>	Italy
27	A3-3	Ainula	<i>P. domestica</i>	China
28	A4-2	Mt. Royal	<i>P. domestica</i>	United States
29	TGRWF	Xinjiang #3	Wild <i>P. domestica</i>	China
30	A3-1	Ruth Grestetter	<i>P. domestica</i>	Germany
31	A5-1	Mulili	<i>P. salicina</i>	China
32	A6-1	Methley	<i>P. salicina</i> hybrid	United States
33	A5-2	Longyuanqiuli	<i>P. salicina</i>	China
34	A6-2	President	<i>P. domestica</i>	England
35	A5-3	Freedom	<i>P. salicina</i> hybrid	United States
36	A6-3	Homeside	<i>P. salicina</i> hybrid	United States
37	A5-4	Friar	<i>P. salicina</i> hybrid	United States
38	A6-4	Suili #3	<i>P. salicina</i>	China
39	A5-5	Akihime	<i>P. salicina</i>	Japan
40	A6-5	Dahainiuxinli	<i>P. salicina</i>	China
41	A5-6	Product	<i>P. salicina</i>	United States
42	A6-6	Cuoli	<i>P. salicina</i>	China
43	A5-7	Pingguoli	<i>P. salicina</i>	China
44	A6-7	Lushanli	<i>P. salicina</i>	China
45	A5-8	Kuandiandali	<i>P. salicina</i>	China
46	A5-9	Qiuli	<i>P. salicina</i>	China
47	A6-9	Tongpannai	<i>P. salicina</i>	China
48	A5-10	Lihe #1	<i>P. salicina</i>	China
49	A6-10	Wanhong	<i>P. salicina</i>	China
50	A5-11	Lihe #2	<i>P. salicina</i>	China
51	A6-11	Huanai	<i>P. salicina</i>	China
52	A5-12	Lihe #4	<i>P. salicina</i>	China
53	A6-12	Ruth Grestetter	<i>P. domestica</i>	Germany
54	A5-13	Ozark Premier	<i>P. salicina</i> hybrid	United States
55	A6-13	Xiangyanghong	<i>P. salicina</i>	China
56	A6-14	Kaiyuanli	<i>P. salicina</i>	China
57	A6-15	Yanjili	<i>P. salicina</i>	China
58	A5-15	Au-Producer	<i>P. salicina</i> hybrid	United States

continued next page

Table 1. Continued.

Accession no.	Planting no. ^z	Accession name	Species ^y	Origin
59	A8-15	Tiankanmali	<i>P. salicina</i>	China
60	A7-15	2-214	<i>P. domestica</i>	United States
61	A7-14	Batangli	<i>P. salicina</i>	China
62	A8-14	Huangganhe	<i>P. salicina</i>	China
63	A7-13	2-213	<i>P. salicina</i>	United States
64	A7-12	Sanhuali	<i>P. salicina</i>	China
65	A8-11	Huangguli	<i>P. salicina</i>	China
66	A7-9	Qingnai	<i>P. salicina</i>	China
67	A8-9	Zhushali	<i>P. salicina</i>	China
68	A7-8	Suilinglimei	<i>P. salicina</i>	China
69	A8-8	Qingpihongxin	<i>P. salicina</i>	China
70	A8-7	Santa Rosa	<i>P. salicina</i> hybrid	United States
71	A8-6	Ozark Premier	<i>P. salicina</i> hybrid	United States
72	A8-5	Au-cherry	<i>P. salicina</i> hybrid	United States
73	A8-4	Furenli	<i>P. salicina</i>	China
74	A8-3	Taiyo	<i>P. salicina</i>	Japan
75	A8-2	Robusto	<i>P. salicina</i> hybrid	United States
76	A7-2	Guali	<i>P. salicina</i>	China
77	A9-1	Gecko	<i>P. salicina</i>	Japan
78	A9-2	Hongrouli	<i>P. salicina</i>	China
79	A9-3	Jiaqingzi	<i>P. salicina</i>	China
80	A9-4	Ziyu	<i>P. salicina</i>	China
81	A9-5	Xiangjiaoli	<i>P. salicina</i>	China
82	A10-6	Yuhuangli	<i>P. salicina</i>	China
83	B7-2	Jinshuai	<i>P. salicina</i>	China
84	B7-1	Changli #15	<i>P. salicina</i>	China
85	A14-7	Dongbeimeili	<i>P. salicina</i>	China
86	B5-4	Fortune	<i>P. salicina</i> hybrid	United States
87	A15-14	Beauty	<i>P. salicina</i>	United States
88	B9-1	Oishi Wase	<i>P. salicina</i>	Japan
89	C5-3	Yinxiangbai	<i>P. armeniaca</i>	China
90	C1-3	Taianxingmei	Plumcot	China
91	C1-4	Limixing	Plumcot	China
92	TGRWF	Xinjiang #4	Wild <i>P. domestica</i>	China
93	TGRWF	Xinjiang #5	Wild <i>P. domestica</i>	China
94	TGRWF	Xinjiang #6	Wild <i>P. domestica</i>	China
95	A16-8	Hollywood	<i>P. salicina</i> hybrid	United States
96	E14-2	Maoyingtao	<i>P. tomentosa</i>	China
97	A1-5	Xingmei	Plumcot	China
98	A1-6	Xingli	Plumcot	United States
99	A2-2	Heicili	<i>P. spinosa</i>	China
100	A2-3	Xiangjiaoli	<i>P. ussuriensis</i>	China
101	A3-13	Stanley	<i>P. domestica</i>	United States
102	A3-11	Danding	<i>P. domestica</i>	Unknown
103	A5-14	Naili	<i>P. salicina</i>	China
104	A12-2	Oishi Nakade	<i>P. salicina</i>	Japan

^zTGRWF refers to samples collected from the Tiansan Germplasm Repository for Wild Fruit Resources, located in Xinyuan County, Xinjiang Province, China.

^yPlumcot = natural or artificial hybrid between plum and apricot (*P. armeniaca*); *P. salicina* hybrid = hybrid between *P. salicina* and other diploid plum species.

Germplasm Repository for Plums and Apricots located in Xiongyue, Liaoning Province, China, except for six accessions of wild European plum, which were sampled from the Tianshan Germplasm Repository for Wild Fruit Resources located in Xinyuan, Xinjiang Province, China.

DNA EXTRACTION. Samples were taken from three plants of each accession. DNA extraction was performed using the CTAB method (Doyle and Doyle, 1990) with modification.

Briefly, ≈ 0.1 g of frozen leaves was put into a 1.5-mL Eppendorf tube and ground to a fine powder in liquid nitrogen with a specially designed grinder. The powder was added to 650 μ L of extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, 2% BME, 1% PVP) and incubated at 65 °C for 30–60 min. After this mixture had been placed in an ice-bath for 5 min, DNA was extracted once with one volume of chloroform/isoamyl alcohol (24:1, v/v). The DNA suspension

was mixed gently and centrifuged at 15,300g_n (4 °C) for 15 min. The top aqueous phase was collected into a new tube, and 2 µL of RNase (10 mg·mL⁻¹) was added, mixed, and then incubated at 37 °C for 30–60 min. DNA was extracted once more with one volume of the chloroform/isoamyl mixture, the top aqueous phase was collected again, and the DNA was precipitated with two volumes of cold ethanol. The DNA pellet was washed three times with 70% ethanol and dissolved in 100 µL of sterile water after the ethanol evaporated completely. DNA quality was checked on a 1% agarose gel, and the concentration was determined by spectrophotometry at 260 nm. A portion of the DNA was diluted to 20 ng·µL⁻¹ for use, and the stock and diluted portions were stored at -20 and 4 °C, respectively.

PCR PROCEDURE. PCR reaction mixtures had a total volume of 20 µL containing 60 ng of DNA template, 0.25 µM primer, 0.2 mM dNTPs, 0.25 unit of *Taq* DNA polymerase, 3.0 mM MgCl₂, 2 µL 10× buffer [20 mM MgSO₄, 100 mM KCl, 80 mM (NH₄)₂SO₄, 100 mM Tris-HCl, 0.5% NP-40, pH 9.0]. Reaction mixtures were overlaid with 50 µL of mineral oil.

Amplifications were performed on a DNA Thermal Cycler 9700 (PerkinElmer, Wellesley, MA) with the following temperature profile: a pre-denaturation step of 5 min at 94 °C followed by 40 cycles of denaturation, 45 s at 94 °C; annealing, 1 min at 52 °C; extension, 90 s at 72 °C. A final extension was allowed for 5 min at 72 °C.

Amplified products were separated by electrophoresis on 2% agarose gel in 1× TAE buffer under a voltage of 40 V·cm⁻¹ for ≈1.5 h and visualized with staining by ethidium bromide, and then ultraviolet-transilluminated gels were photographed.

DATA ANALYSIS. Amplification fragments were scored as present (1) or absent (0). Faintly stained bands were not included in the analysis. Each reaction was repeated twice, hence only reproducible products were included in the study. Based on the number of shared amplification products, data for all 12 primers were assembled into a data matrix. The UPGMA cluster analysis and PCoA were performed using the NTSYS-pc version 2.1 software (Rohlf, 2000) based on the Jaccard coefficient of similarity, and two dendrograms, one with accessions grouped by species and one including all 104 accessions, and a scatter plot were obtained. Mantel tests (Mantel, 1967) were carried out to calculate goodness-of-fit between the cluster analyses and original matrices.

Results

In a pilot experiment, the effects of various levels of magnesium concentrations (1.0, 1.5, 2.0, 2.5, and 3.0 mM), template DNA concentrations (10, 20, 40, 60, and 80 ng), dNTP concentrations (0.1, 0.15, 0.2, 0.25, and 0.3 mM), *Taq* polymerase contents (0.25, 0.5, 1.0, 1.5, and 2.0 U), primer concentrations (0.1, 0.15, 2.0, 2.5, and 3.0 mM), and annealing temperatures (49, 49.3, 50, 50.8, 52.1, 53.7, 55.6, 57.1, 58.3, 59.2, 59.8, and 60 °C) on amplification results were examined using the DNA samples of ‘Xingmeili’ (accession no. 1) and ‘Stanley’ (101) and optimized conditions, as described in Materials and Methods, were obtained. Under the optimized reaction system, 42 ISSR primers from University of British Columbia, Canada, were tested, among which 12 primers (Table 2) were selected for their reproducible and clear band patterns.

A total of 103 bands were scored with an average band number of 8.6 per primer across 104 accessions assayed. Among the 103 bands, 99 bands (96.1%) were polymorphic,

Table 2. ISSR primers used for the genetic diversity evaluation of 104 plum and related accessions and their amplification performance.

Primer code	Sequence ^a	Total no. of bands	No. of polymorphic bands	Percent of polymorphic bands (%)
810	(GA) ₈ T	8	8	100
818	(CA) ₈ G	9	9	100
825	(AG) ₈ T	7	5	71.4
827	(AC) ₈ G	4	4	100
841	(GA) ₈ YC	9	9	100
845	(CT) ₈ RG	10	10	100
848	(CA) ₈ RG	6	5	83.3
853	(TC) ₈ RT	10	10	100
855	(AC) ₈ YT	6	5	83.3
857	(AC) ₈ YG	12	12	100
868	(GAA) ₅	9	9	100
880	(GGAGA) ₃	13	13	100
Total		103	99	96.1

^aY = C or G; R = A or T.

and only four bands were monomorphic. These results indicated a high level of diversity in the 104 accessions under investigation.

Twenty-six accession-specific band patterns were identified (Table 3). In total, seven accession-specific band patterns were obtained for accession 23 (‘Wanhei’, a European cultivar with unknown name), three for accession 89 (‘Yingxiangbai’, a white-flesh local apricot cultivar), three for accession 96 (*P. tomentosa*, a common rootstock for plum in northern China), two for accessions 6 (‘Hongyeli’, red-leaf plum, a variant of *P. cerasifera*) and 101 (‘Stanley’, a U.S. hybrid-derived European plum cultivar), and one for accessions 2, 13, 16, 60, 99, 100, and 102, respectively.

There were two possible accession-specific bands among the total 103 bands obtained. One was Primer 853-450 bp for all cultivated European plum and another Primer 827-400 bp for ‘2-214’ (accession no. 60).

Genetic similarities between accessions were estimated using the Jaccard coefficient, and the similarity coefficient matrix was established (data not shown). The similarity values varied from 0.19 for ‘89-7-3’ (15) versus ‘Limixing’ (91) to

Table 3. ISSR primers generating unique band patterns to some *Prunus* accessions tested.

Accession name (no.)	Primers generating unique band pattern
Hongyeli (6)	Primers 818, 857
Wanhei (23)	Primers 818, 845, 853, 855, 857, 868, 880
Maoyingtao (96)	Primers 818, 853, 855
Bingtangli (13)	Primer 845
89-7-3 (15)	Primers 845, 880
Heicili (99)	Primer 848
Stanley (101)	Primers 848, 857
Retsudo burubetsuto (2)	Primer 853
Yinxiangbai (89)	Primers 853, 855, 868
Xiangjiaoli (100)	Primer 855
2-214 (60)	Primer 857
Danding (102)	Primer 857
89-7-4 (16)	Primer 880

Table 4. Average Jaccard coefficients of 10 *Prunus* species or groups within and between them based on ISSR data

Species ^z	●	○	☆	◇	◆	■	□	▲	△	※
● <i>P. salicina</i> and its hybrids (59)	0.74 ^y									
○ <i>P. simonii</i> (5)	0.68	0.91								
☆ <i>P. ussuriensis</i> (1)	0.60	0.54	1							
◇ <i>P. cerasifera</i> (2)	0.52	0.49	0.57	0.88						
◆ <i>P. spinosa</i> (1)	0.47	0.43	0.56	0.42	1					
■ <i>P. domestica</i> (20)	0.39	0.41	0.41	0.46	0.53	0.67				
□ Wild <i>P. domestica</i> (6)	0.41	0.45	0.48	0.47	0.55	0.56	0.91			
▲ <i>P. armeniaca</i> (1)	0.42	0.41	0.37	0.37	0.36	0.37	0.40	1		
△ Plumcot (8)	0.51	0.48	0.44	0.43	0.41	0.41	0.42	0.55	0.72	
※ <i>P. tomentosa</i> (1)	0.50	0.49	0.44	0.43	0.44	0.39	0.41	0.37	0.42	1

^zNumbers in parentheses indicate the numbers of accessions included; *P. salicina* include both pure *P. salicina* and hybrids between *P. salicina* and other diploid plum species.

^yValues on the diagonal line are the similarities within species or groups.

1.00 for 'Xiangjiaoli' (8) versus 'Bianyan' (9) and 'Xinjiang #4' (92) versus 'Xinjiang #5' (93). Apparently, 'Xiangjiaoli' (8) and 'Bianyan' (9) are duplicated accessions, as are 'Xinjiang #4' (92) and 'Xinjiang #5' (93).

'Tianli' (24) versus 'Ganli' (25); 'Jinshuai' (83) versus 'Changli #5' (84); and 'Pingguoli' (43) versus 'Kuandiandali' (45), which were all difficult to discriminate due to their morphological similarity, showed similarity coefficients of 0.97, 0.91, and 0.90, respectively. 'Tianli' (24) versus 'Ganli' (25) were both introduced European plum cultivars with unknown original names, and their Chinese translated names, "tianli" and "ganli," mean "sweet plum" in Chinese. The similarity based on ISSR data suggested that the two accessions were possible synonyms.

Three accessions (8, 81, and 100) have the same name, 'Xiangjiaoli', and they belong to three different species, *P. simonii*, *P. salicina*, and *P. ussuriensis*, respectively. Similarity coefficients between them range from 0.56 for accession 8 versus accession 100 to 0.72 for accession 8 versus accession 81, correlating with the growth habit differences between the three mentioned accessions: *P. simonii* is upright, *P. salicina* is intermediate, and *P. ussuriensis* is spreading.

The similarity coefficients of four accession pairs with same names, 'Xiangbianli' (10) versus 'Xiangbianli' (11); 'Bingtangli' (12) versus 'Bingtangli' (13); 'Ruth Grestetter' (30) versus 'Ruth Grestetter' (53); and 'Ozark Premier' (54) versus 'Ozark Premier' (71) were 0.75, 0.76, 0.55, and 0.80, respectively, indicating that four pairs were all homonymous pairs incited by mislabeling.

Jaccard coefficients of similarity of 'Lihe #1' (48), 'Lihe #2' (50), and 'Lihe #4' (52) versus 'Qiuli' (46) were 0.96, 0.93, and 0.98, respectively, and these results supported the suggestion that the first three were probable bud mutants or seedlings of 'Qiuli' (Zhang and Zhou, 1998). The two pairs of improved plum cultivars, 'Oishi wase' (88) versus 'Oishi nakade' (104) and 'Freedom' (35) versus 'Fortune' (86) were sibling lines, but the similarity coefficients between them were only 0.66 and 0.64, respectively, showing that the similarity coefficients based on ISSR data did not coincide completely with their pedigree information.

The average Jaccard coefficients within and between species or groups (Table 4) indicated that similarities within species were higher than those between species. Genetic variability within *P. salicina* was lower than within *P. domestica*, and either five accessions of *P. simonii* or six accessions of wild

European plum presented low genetic variability. The dendrogram using accessions grouped by species (Fig. 1) was constructed with a cophenetic correlation coefficient of 0.85 by Mantel test. The cophenetic correlation coefficient is considered to be a good representation of the data matrix in the dendrogram if it is ≥ 0.8 (Romesburg, 1990). This dendrogram showed that *P. simonii* was closer to *P. salicina* than was *P. ussuriensis* to *P. salicina*, although both were very similar to *P. salicina* in morphological traits. *P. domestica* was more closely related to *P. spinosa* than to *P. cerasifera*. Hybrids of plum and apricot were more similar to apricot than to plum.

The clustering results including all 104 accessions tested (Fig. 2),

Fig. 1. Dendrogram of 10 *Prunus* species or groups generated by ISSR data using UPGMA cluster analysis based on the Jaccard coefficient.

Fig. 2. Dendrogram of 104 plum and related accessions generated by ISSR data using UPGMA cluster analysis based on Jaccard similarity coefficients; ● = *P. salicina* and its hybrids, ○ = *P. simonii*, ☆ = *P. ussuriensis*, ◇ = *P. cerasifera*, ✱ = *P. tomentosa*, ■ = *P. domestica*, □ = wild *P. domestica*, ◆ = *P. spinosa*, ▲ = *P. armeniaca*, and △ = hybrids of plum and apricot (*P. armeniaca*). The numbers following the symbols are accessions numbers as seen in Table 1. Scale on bottom is the Jaccard coefficient of similarity.

with a cophenetic correlation coefficient of 0.96 by Mantel test, showed that they were divided into three main groups and outgroups. Group I was composed of all eight accessions of plumcot types and one apricot cultivar, Yinxiangbai (89). Group II consisted of all diploid plum accessions and three European cultivar types [i.e., ‘President’ (34), ‘Ruth Grestetter’ (53), and ‘2-214’ (60)]. This group has three subgroups, such as cherry plum (containing two accessions of *P. cerasifera*, 5 and

6), Chinese plum-types, and *P. tomentosa* (96). Group III included the tetraploid species [‘Heicili’ (99)] and all hexaploid European plum, including 6 accessions of wild European plum (17, 22, 29, 92, 93, and 94), except for three accessions of European plum including accessions 34, 53, and 60 which were mixed into Group II, ‘89-7-3’ (15) and ‘Wanhei’ (23) which were in the outgroups.

Associations among 104 accessions were also revealed by PCoA (Fig. 3). In the PCoA plot, similar grouped results were obtained, but two European plum accessions, ‘89-7-3’ (15) and ‘Wanhei’ (23), which were divided into outgroups in the clustering result (Fig. 2), could be grouped within Group III, and additional information was revealed (e.g., the plumcots were displaced between apricot and Chinese plum-types that reflected their phylogenetic relationships), while Eurasian species (*P. spinosa* and *P. cerasifera*) between European plum and Chinese plum-types that reflected their geographic origins.

Discussion

ISSR PCR using primers based on dinucleotide, tetranucleotide, or pentanucleotide repeats has now become fashionable among researchers (Zietkiewicz et al., 1994). ISSRs have been used in fruit trees (Archak et al., 2003; Cekic et al., 2001; Fang et al., 1997; Goulao et al., 2001; Levi and Rowland, 1997; Moreno et al., 1998; Sankar and Moore, 2001), but little information is available for evaluation and identification of plum resources using ISSR techniques. Our study further supports ISSR as a reliable, quick, uncomplicated, and cheap method of identification for the large number of accessions held in most collections (Gilbert et al., 1999). This method could be used to eliminate duplicated collections, to

discriminate among homonyms or synonyms, to identify closely related accessions, to measure genetic diversity, and to reveal genetic relationships.

Zietkiewicz et al. (1994) noted that primers anchored at the 5′ end displayed more specificity than those anchored at the 3′ end. Fang et al. (1997) also found that in citrus, 5′-anchored primers generally had more specificity and thus generated fewer but larger fragments than 3′-anchored ones. However, 10 of 12

Fig. 3. Two-dimensional plot of 104 plum and related accessions generated by ISSR data using principal coordinate analysis (PCoA) based on the Jaccard coefficients of similarity; ● = *P. salicina* and its hybrids, ○ = *P. simonii*, ☆ = *P. ussuriensis*, ◇ = *P. cerasifera*, ※ = *P. tomentosa*, ■ = *P. domestica*, □ = wild *P. domestica*, ◆ = *P. spinosa*, ▲ = *P. armeniaca*, and △ = hybrids of plum and apricot (*P. armeniaca*).

primers used in this study were anchored at the 3' end, and they produced clear patterns too, which is in agreement with the results obtained by Huang and Sun (2000). Wang et al. (1994) reported that (AT)_n was the most abundant microsatellite in plant nuclear genomes, but in the present study the primer UBC 880 with the motif of (GGAGA)₃ generated maximum fragments, suggesting that this pentanucleotide repeat was most frequent among eight motifs tested in plum genomes.

Regarding the effects of primers with same motifs but differently anchored nucleotides, primers UBC 827, 855, and 857 amplified 4, 6, and 12 bands, respectively, and this result supported the observation by Ammiraju et al. (2001) that anchored nucleotides at the 5' and 3' ends of the primers play important roles in detecting polymorphism.

Somewhat unexpectedly, the European plum accessions—'President' (34), 'Ruth Grestetter' (53), and '2-214' (60)—were clustered into same group as diploid accessions, mainly Chinese plum-types. Similar overlaps were observed in the plum dendrogram generated with RAPD characters by Shimada et al. (1999), where three Chinese plum-types, namely 'King', 'Jinsantou', and 'Beauty', were classified into the "European plum group"; these results were considered plausible if these cultivars are interspecific hybrids between Japanese plum (*P. salicina*) and related species such as *P. cerasifera* or *P. simonii*.

In general, the phenogram showed clustering in good accordance with the classification previously established. One apricot accession (89) was clustered into the same group as all plumcot types, suggesting that seven accessions of hybrids between plum and apricot were possibly more closely related to apricot than to plum. Possible explanations for this were 1) repeated back-crossing plum–apricot hybrids with apricots or 2) favorable selection of the apricot-like descendant from segregating populations as the main objective for the hybridization between plum and apricot in apricot breeding programs as

necessary to select a new apricot cultivar with a late flowering time so as to elevate the resistance to spring frost injury for apricot, which has been a serious problem for apricot industry worldwide.

Group II, a diploid species group, included all accessions of *P. salicina*, *P. cerasifera*, *P. simonii*, *P. ussuriensis*, *P. tomentosa*, and *P. salicina* hybrids, among which the first five species were all of Asian origin and the last one was interspecific hybrid between *P. salicina* and other diploid species. Three subgroups were formed in this group, such as the cherry plum subgroup including 'Yingtaoli' (5) and 'Hongyeli' (6), the Chinese plum subgroup including *P. simonii*, *P. salicina*, *P. ussuriensis*, and three accessions of *P. domestica* (accessions 34, 53, and 60), and *P. tomentosa* subgroup (96).

Chinese plum, *P. salicina*, was native to China, and introduced into Japan no earlier than 1500 AD. Many

cultivars were introduced from Japan into California around 1870 and subsequently to Europe. Luther Burbank introduced several Japanese plum cultivars and hybridized them with American native species, resulting in a series of improved plum cultivars, which laid a foundation for fresh market plum production worldwide (Ryugo, 1988). Five plum cultivars from Japan, including 'Akihime' (39), 'Taiyo' (74), 'Gecko' (77), 'Oishi wase' (88), and 'Oishi nakade' (104), were distributed across the Chinese plum subgroup in present dendrogram, indicating that diverse plum resources had been introduced into Japan from China. All the improved Chinese plum hybrids, although most of them derived from interspecific hybridization, were grouped together with pure Chinese plum cultivars possibly due to back-crossing or selection bias, and this result was in agreement with the *P. salicina*-predominating genetic components of improved Chinese plum hybrids (Byrne and Littleton, 1988).

The clustering results did not reflect the geographic origin for both Chinese plum-types in Group II and European plum in Group III (Fig. 2), but the plumcot types were plotted between apricot and Chinese plum and Eurasian species (*P. spinosa* and *P. cerasifera*) between *P. domestica* and Chinese plum (*P. salicina* and *P. simonii*) in the PCoA plot (Fig. 3), which was consistent with their genetic origin and geographic distribution, respectively.

Two European cultivars, '89-7-3' (15) and 'Wanhei' (23), were grouped into distinct outgroups, and another three cultivars [i.e., 'President' (34), 'Ruth Grestetter' (53), and '2-214' (60)] were intermixed with diploid accessions, suggesting that they may be useful materials to broaden the genetic background of European plum cultivars.

As to the taxonomic placement of *P. simonii*, three opinions exist: the first one is that *P. simonii* is derived from hybridization between apricot and plum (Faust, 1999; Luo et al., 1992; Ruan et al., 2002); the second is that it is thought as an independent species (Qu and Sun, 1990; Wu, 1984; Yu, 1984;

Zhang and Zhou, 1998); and the last opinion considers *P. simonii* to be a variant of *P. salicina* (Okie and Weinberger, 1996; Yoshida, 1987). Our results support *P. simonii* as a variant of *P. salicina* because all five accessions of *P. simonii* formed a subcluster underneath Group II and were inserted among Chinese plum-types, coinciding with the clustering results with RAPD markers (Boonprakob and Byrne, 2001; Liu et al., 2006).

Prunus salicina was native to Yangtze River basin of China, while *P. simonii* was native to northern China. The present study implied that one or a few individuals of *P. salicina* from Yangtze River basin colonized in a vacant habitat of northern China and a variant was formed as the gene pool of *P. simonii* had low levels of genetic variation (Table 4). In other words, the “founder effect” may have played an important role in the speciation of *P. simonii*.

Although most Chinese taxonomists thought *P. ussuriensis* to be an independent species, Faust (1999) suggested that it was only a minor variation of *P. salicina*. Compared with *P. simonii*, *P. ussuriensis* was distinct from *P. salicina* and its hybrids, so it seems reasonable to consider *P. ussuriensis* as an independent species.

The discovery of putative wild *P. domestica* in a canyon in the Yili region in Xinyuan County, Xinjiang, China (Lin and Shi, 1990), suggested the possibility that European plum may have originated in China. The plants found in Xinjiang, according to their morphological characteristics (Zhang et al., 1998) and chromosome numbers (Lin et al., 1991), belong to *P. domestica*. In present study, six accessions collected from the Tiansan Germplasm Repository for Wild Fruit Resources were clustered into a subcluster underneath Group III, the European plum group, further confirming that they belong to *P. domestica*. High average Jaccard coefficients were presented for six accessions of wild *P. domestica* from six populations from Xinjiang, China (Table 4). This result implied that the cultivated *P. domestica* was brought to Xinjiang and had naturalized there and that Xinjiang was not the origin center of *P. domestica*, as a high level of diversity should exist in the center of origin of a wild species (Vavilov, 1930). A similar result was obtained by us with RAPD markers (Liu et al., 2006).

Our results suggest that *P. domestica* was derived from *P. spinosa* because both were closely related. The limitation of our experiment is that only one accession of *P. spinosa* is used, so collecting more accessions of related species and further study on the origin of European plum based on DNA techniques is needed to resolve this question. The evaluation of genetic relationships between European plum-related species is presently under way using cpDNA and mtDNA PCR-RFLP methods in our laboratory.

Classification of stone fruit species—including plum, apricot, cherry, and peach—has long been complicated and problematic due to the diverse phenotypes and large number of species. Some Chinese pomologists still put plum, apricot, cherry, and peach into separate genera, as *Prunus* L., *Armeniaca* Mill., *Cerasus* Mill., and *Amygdalus* L., respectively (Yu, 1984), and refer to the plum genus as the “small *Prunus*,” but the most widely adopted classification of stone fruit species places all of them under one genus (i.e., *Prunus*), the so-called “large *Prunus*” (Lee and Wen, 2001). Our results support the classification of stone fruit species under “large *Prunus*” because apricot (89), plumcot types (1, 2, 3, 4, 90, 91, 97, and 98), and *P. tomentosa* [named *Cerasus tomentosa* (Thunb.) Wall. by “small

Prunus” taxonomists] (96) were grouped with the diploid plum species group earlier than European plum (Fig. 2).

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