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Analysis of the genetic diversity of beach plums by simple sequence repeat markers

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ABSTRACT. The purpose of this study was to measure the genetic diversity of wild beach plum and cultivated species, and to determine the species relationships using SSRs markers. An analysis of genetic diversity from ten beach plum germplasms was carried out using 11 simple sequence repeat (SSR) primers selected from 35 primers to generate distinct PCR products. From this plant material, 44 allele variations were detected, with 3-5 alleles identified from each primer. The analysis showed that the genetic similarity coefficient varied from 0.721 ± 0.155 to 0.848 ± 0.136 within each of the ten beach plum germplasms and changed within the range of 0.551 ± 0.084 to 0.695 ± 0.073 between any two pairs of germplasms. According to the genetic dissimilarity coefficient matrix, a cluster analysis of SSRs using the unweighted pair group mean average method in the NTSYSpc 2.10 software revealed that the ten germplasms could be divided into two groups at the dissimilarity coefficient of 0.606. Class I included 77.8, 12.5, 30, and 33.3% of MM, MI, NY, and CM, respectively. Class II contains the remaining 9 beach plum germplasms. The markers generated by 11 SSR primers proved very effective in distinguishing the beach plum germplasm resources. It was clear that the geographical distribution did not correspond

with the genetic relationships among the different beach plum strains. This result will be of value to beach plum breeding programs.

Key words: Beach plum; *Prunus maritima* Marshall; SSR markers; Genetic diversity

INTRODUCTION

The beach plum (*Prunus maritima* Marshall) is native to the sandy North Atlantic coast, from Newfoundland to North Carolina. Most beach plum populations are found geographically from northern Massachusetts to southern New Jersey (Clark et al., 2000). The beach plum is a stress-tolerant shrub that can grow well without much irrigation, even in low-nutrient sandy soils, saline land, old fields, and coastal beaches where many other plants cannot survive (Uva, 2003). The beach plum has a well-developed root system, and for this reason, it can serve as a rootstock to improve the stress tolerance of some tree scions. Aesthetically, it is appreciated for its profuse white blooms in spring and it can maintain their greenery until late autumn. In addition to its ornamental value, the fruit of the beach plum is purple to reddish (or even yellow), up to 1 inch in size, and is used for making jams, jellies, and wine. The beach plum, therefore, is a multipurpose crop. Although the study and utilization of beach plums have a long history and have been the basis for numerous cottage industries, research institutions have shown an increased interest in the last ten years. The leading researchers in this field are from Rutgers University, Cornell University, Cape May (New Jersey) Plant Materials Center of the US Natural Resources Conservation Service, Cooperative Extension of University of Massachusetts, and some private nurseries and farms (Clark et al., 2000).

Breeding of *Prunus* has been restricted due to the restricted genetic background of commercial cultivars, which also limits commercial production to specific areas and climatic zones. Introgression of genes from related species through inter-specific hybridization has been used in various breeding programs throughout the world to develop better-adapted cultivars and rootstocks (Martinez-Gomez et al., 2003a). Rootstock breeding programs using inter-specific hybridization have introduced useful traits, including size control, self-compatibility, adaptability to new environments, and pest resistance (Martinez-Gomez et al., 2003b). A more thorough evaluation of *Prunus maritima* could help in planning future hybridization strategies.

Among the different types of molecular markers, microsatellite or simple sequence repeat (SSR) markers are highly prized as molecular markers due to their co-dominance and high levels of polymorphism (Varshney et al., 2006). SSR markers were introduced in the early 1990s and are now being widely used for genetic characterization and diversity analyses of agricultural and horticultural crops. Understanding genetic variation between and within species is relevant to understanding the structures and dynamics of the species. The objective of this study was to analyze the level of polymorphism and genetic relationships between and within ten beach plum germplasms.

MATERIAL AND METHODS

Plant material

A total of 84 seedling offspring of ten beach plum genotypes from different states of the USA were used in this research (Table 1). Young leaves were collected from one-year-old branches and stored at -70°C until use.

Table 1. Origin of the materials used in the study.

Sample code	Origin	Sample	Sample number
UK	Unknown	Individuals from some wild plant	UK1, UK2, UK3, UK4, UK5, UK6, UK7, UK8, UK9
SM	Sandwich Massachusetts	Individuals from some wild plants	SM1, SM2, SM3, SM5, SM4, SM6, SM7, SM8, SM9
MI	Michigan	Individuals from some wild plants	MI1, MI2, MI3, MI4, MI5, MI6, MI7, MI8
NY	State of New York	Individuals from some wild plants	NY1, NY2, NY3, NY4, NY5, NY6, NY7, NY8, NY9, NY10
CM	Cape Code Massachusetts	Individuals from some wild plants	CM1, CM2, CM3, CM4, CM5, CM6, CM7, CM8, CM9
MD	Michigan	Seedling offspring of Dunbars	MD1, MD2, MD3, MD4, MD5, MD6, MD7, MD8, MD9
ME	Michigan	Seedling offspring of Ecos	ME1, ME2, ME3, ME4, ME5, ME6, ME7, ME8
MM	Michigan	Seedling offspring of Mini	MM1, MM2, MM3, MM4, MM5, MM6, MM7, MM8, MM9
MN	Michigan	Seedling offspring of Nana	MN1, MN2, MN3, MN4, MN5, MN6, MN7, MN8, MN9
MW	Michigan	Seedling offspring of Wild Goose	MW1, MW2, MW3, MW4

Primer design

After pre-screening, 35 primer (Genscript, Nanjing, Jiangsu, China) pairs were chosen that gave distinct, reproducible, and polymorphic amplification products at one or more loci in beach plums. A set of 11 SSR primer pairs were selected on the basis of previous reports of different *Prunus* species (Rahemi 2012), including BPPCT004, BPPCT007, BPPCT028, BPPCT032, BPPCT039, CPPCT026, CPPCT039, and UDP98-409 as representatives of peaches (Aranza and Dirlewanger 2002, Cipriani 1999), and CPDCT025, CPDCT027, and CPDCT042 for almonds (Table 2).

Table 2. Simple sequence repeat primers for *Prunus* species.

No	Primer	Range size (bp)	Ref.	Species	Forward primer (5'-3')
1	*BPPCT004*	*200*	Dirlewanger et al. (2002)	*Peach*	*CTGAGTGATCCATTTGCAGG*
2	*BPPCT007*	*143-15*	Dirlewanger et al. (2002)	*Peach*	*TCATTGCTCGTCATCAGC*
3	BPPCT010	131	Dirlewanger et al. (2004)	Peach	AAAGCACAGCCCATAATGC
4	BPPCT014	215	Dirlewanger et al. (2004)	Peach	TTGTCTGCCTCTCATCTTAACC
5	BPPCT016	89-103	Dirlewanger et al. (2002)	Peach	GATTGAGAGATTGGGCTGC
6	BPPCT017	151-18	Dirlewanger et al. (2002)	Peach	TTAAGAGTTTGTGATGGGAACC
7	BPPCT023	183-23	Dirlewanger et al. (2002)	Peach	TGCAGCTCATTACCTTTTGC
8	BPPCT025	178-20	Dirlewanger et al. (2002)	Peach	TCCTGCGTAGAAGAAGGTAGC
9	BPPCT026	134-14	Dirlewanger et al. (2002)	Peach	ATACCTTTGCCACTTGCG
10	*BPPCT028*	*155-16*	Dirlewanger et al. (2002)	*Peach*	*TCAAGTTAGCTGAGGATCGC*
11	*BPPCT032*	*202-20*	Dirlewanger et al. (2002)	*Peach*	*TTAAGCCACAACATCCATGAT*
12	*BPPCT039*	*148-15*	Dirlewanger et al. (2002)	*Peach*	*ATTACGTACCCTAAAGCTTCTGC*
13	CPDCT004	131-15	Mnejja et al. (2005)	Almond	TCTCAGGTTCGTATCCCCTCT
14	*CPDCT025*	*172-19*	Mnejja et al. (2004)	*Almond*	*GACCTCATCAGCATCACCAA*
15	*CPDCT027*	*156-17*	Mnejja et al. (2005)	*Almond*	*TGAGGAGAGCACTGGAGGAG*
16	CPDCT034	157-17	Mnejja et al. (2005)	Almond	GAGAACCTTTTGTTTGGCCTTA
17	*CPDCT042*	*164-18*	Mnejja et al. (2005)	*Almond*	*ACGCGTTACAAGTGAGATGC*
18	CPPCT002	100	Aranzana et al. (2002)	Almond	GGAGCTGCAATATTGCTG
19	CPPCT005	122-16	Aranzana et al. (2002)	Peach	CATGAACTCTACTCTCCA
20	CPPCT006	190-21	Aranzana et al. (2002)	Peach	AATTAACTCCAACAGCTCCA
21	CPPCT008	153-19	Aranzana et al. (2002)	Peach	GAGCTCTCACGCATTAGTTT
22	CPPCT022	217-28	Aranzana et al. (2002)	Peach	CAATTAGCTAGAGAGAATTATTG
23	*CPPCT026*	*180*	Aranzana et al. (2002)	*Peach*	*AGACGCAGCACCCAAACTAC*
24	CPPCT033	127-20	Aranzana et al. (2002)	Peach	TCAGCAAACTAGAAACAAACC
25	*CPPCT039*	-	Dirlewanger et al. (2004)	*Peach*	*GCACCAGTTCTTCGTCATCTC*
26	CPSCT012	126-150	Mnejja et al. (2004)	Plum	ACGGGAGACTTTCCCAGAAG
27	CPSCT034	230	Mnejja et al. (2004)	Plum	AGGTGGACAATAGCCGTGAT
28	pchgms5	160	Sosinski et al. (2000)	Peach	CCAGTAGATTTCAACGTCATCTACA
29	PMS2	132-152	Cantini et al. (2001)	Sweet cherry	CACTGTCTCCCAGGTTAAACT
30	PS01H03	-	Sosinski et al. (2000)	Sour cherry	TGAGGAGCATAATGACAGT
31	UDP96-001	122-140	Cipriani et al. (1999)	Peach	AGTTTGATTTTCTGATGCATCC
32	UDP96-019	211-222	Cipriani et al. (1999)	Peach	TTGGTCATGAGCTAAGAAAACA
33	UDP98-412	101-141	Testolin et al. (2000)	Peach	GGGAGGTTACTATGCCATGAAG
34	*UDP98-409*	*126-143*	Cipriani et al. (1999)	*Peach*	*GCTGATGGGTTTTATGGTTTTC*
35	UDP98-412	124-132	Testolin et al. (2000)	Peach	AGGGAAAGTTTCTGCTGCAC

Eleven pairs of primers marked in italics were the effective primers used in this study.

Genomic DNA extraction

Genomic DNA was extracted from the fresh, young leaves using a modified CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson, 1980; Bousquet et al., 1990) by the Plant DNA Extraction Kit (Bio Take Corporation, Beijing, China). The extracted DNA was purified, and, after checking for quality by electrophoresis on a 1% agarose gel (Genmed, Shanghai, China), was diluted to a final concentration of 20 ng/μL with 1X TE buffer and stored at -20°C.

PCR amplification and verification of genomic DNA

In total, 11 primer pairs of beach plum SSRs were used for PCR amplification. The PCR amplification was carried out in a 20-μL reaction system containing 2 μL genomic DNA (20 ng/μL), 0.8 μL 10 pmol of each primer, 0.1 μL *Taq* DNA polymerase (5 U/μL, Promega, Madison, WI, USA), 2 μL 10X buffer, 1.6 μL 25 mM $MgCl_2$, and 1.2 μL 2.5 mM dNTPs. The amplification reaction was performed in an Eppendorf Authorized Thermal Cycler using the following temperature cycling parameters: an initial denaturation for 5 min at 94°C; 35 cycles of denaturation at 94°C for 0.5 min, a primer set-specific annealing temperature between 45° and 68°C for 0.5 min, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR products were resolved by 8% non-denaturing polyacrylamide gel electrophoresis to check the DNA banding patterns.

Data analysis

Amplification products were scored as “present” or “absent” and transferred to a binary code with 1 or 0, respectively. Only distinct, reproducible, and well-resolved SSR fragments could be taken into account and scored as polymorphic markers. Genetic similarities (GS) were calculated using NTSYSpc version 2.10. Next, phylogenetic trees (dendrograms) were constructed using the unweighted pair group mean average method (UPGMA), also in NTSYSpc. Genetic similarity data are reported as the average and standard deviation (SD) that were tabulated in Excel 2003. The coefficient of variation (CV) was calculated as SD/average x 100%. Average genetic diversity (AGD), the average number of polymorphic loci in all pairwise comparisons within each species, which was also obtained from NTSYSpc.

RESULTS

SSR banding patterns

A total of 35 pairs of SSR primers were used in this study, including 25 pairs specific for the peach, 6 pairs for the apricot, 2 pairs for the plum, 1 pair for the sweet and 1 pair for the sour cherry. Eight DNA samples of the 84 beach plum samples were randomly selected for PCR screening (Figure 1) using the 35 pairs of SSR primers. Four of the 35 pairs of SSR primers could not amplify the beach plum samples, including 2 peach SSR primers (BPPCT023 and PCHGMS55), 1 apricot SSR primer (CPDCT004), and 1 sour cherry SSR primer (PS01H03), with which about 92.0, 83.3, 100, and 100% amplified products successfully with peach, apricot, plum, and sweet cherry samples, respectively. However, only the primers for the peach and apricot resulted in polymorphic bands after amplification, and the effective amplification rate for each was 34.8 and 60%, respectively.

Only 11 of the 35 primers produced clear PCR products, and the band patterns from the different samples showed clear differences (Table 2). Further, these 11 pairs of SSR primers were used to study the genetic polymorphisms in 84 seedlings from 10 beach plum germplasms. The band patterns generated by BPPCT039 are shown in Figure 2. The results showed that a total of 63 gene loci could be detected from the 84 plant materials with these 11 primer pairs, and 44 polymorphic alleles were detected, and with an average of 4 allelic variations per SSR primer (Table 3). These sites varied by 150-300 bp (Figure 2). The highest rate of polymorphism (100%) was obtained by primers BPPCT039, CPPCT026, and CPPCT039, and the lowest rate of polymorphism, obtained by primers BPPCT004 and CPDCT042, was less than 50%.

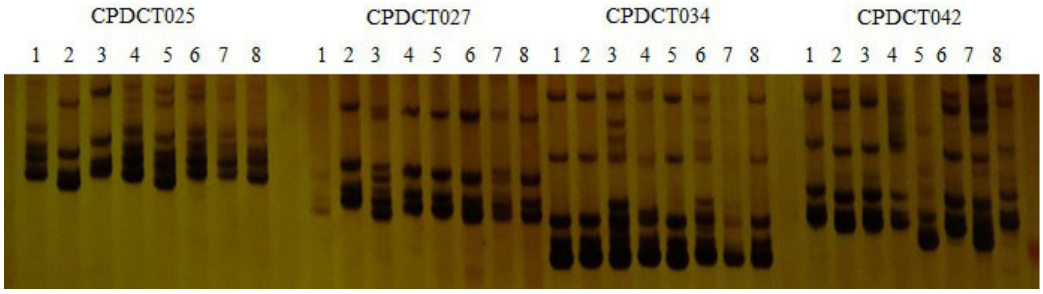

Figure 1. Amplification results of eight DNA samples. *Lanes 1-8* correspond to UN8, SM4, MI3, CM4, ME6, MM8, MN9, and UN2, respectively.

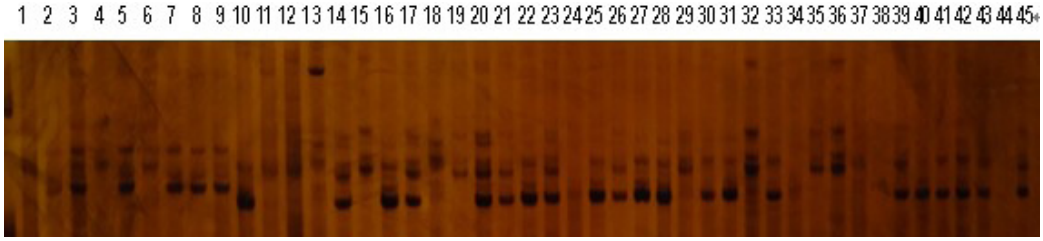

Figure 2. Simple sequence repeat patterns generated by the BPPCT039 primer. *Lanes 1-45* correspond sequentially toUK1, UK2, UK3, UK4, UK5, UK6, UK7, UK8, UK9, SM1, SM2, SM3, SM5, SM4, SM6, SM7, SM8, SM9, MI1, MI2, MI3, MI4, MI5, MI6, MI7, MI8, NY1, NY2, NY3, NY4, NY5, NY6, NY7, NY8, NY9, NY10, CM1, CM2, CM3, CM4, CM5, CM6, CM7, CM8, and CM9.

Table 3. Amplification results of 11 SSR primers.

Primer Name	Species	Total No. of loci	No. of polymorphic alleles	Polymorphic proportion
BPPCT004	Peach	7	3	42.86
BPPCT007	Peach	7	4	57.14
BPPCT028	Peach	6	4	66.67
BPPCT032	Peach	7	5	71.43
BPPCT039	Peach	4	4	100.00
CPDCT025	Almond	5	4	80.00
CPDCT027	Almond	6	4	66.67
CPDCT042	Almond	6	3	50.00
CPPCT026	Peach	5	5	100.00
CPPCT039	Peach	4	4	100.00
UDP98-409	Peach	6	4	66.67
Total	-	63	44	69.84

Genetic analysis

According to the polymorphic bands resulting from amplification with the SSR primers, the genetic deviations between different germplasm resources were analyzed by NTSYSpc (Figure 3). The genetic similarity coefficients of the ten beach plum germplasms were in the range of 0.551 ± 0.084-0.695 ± 0.073, and the mean genetic similarity coefficient was 0.626 ± 0.032 (Table 4). This result showed that the genetic similarity between MD (Dunbars) and MM (Mini) was the lowest (0.551 ± 0.084), while the genetic similarity between SM (Sandwich Massachusetts) and MI (Michigan) was the highest (0.695 ± 0.073). The genetic distance within the ten germplasms varied from 15.25 to 27.91%, and the greatest distance was MM (27.91%), the least was MW (Wild Goose) (15.25%) (Table 5). The variation coefficients of genetic similarity differed among the samples. The mean variation coefficient between the different beach plum germplasms was 5.1%. However, the mean variation coefficient within one germplasm resource was large (21.5%), over 4 times that of the other resources (Table 5). These results suggested that regardless of whether the seedlings were progeny of wild beach plums or beach plum cultivars, the genetic distance between the individuals was large.

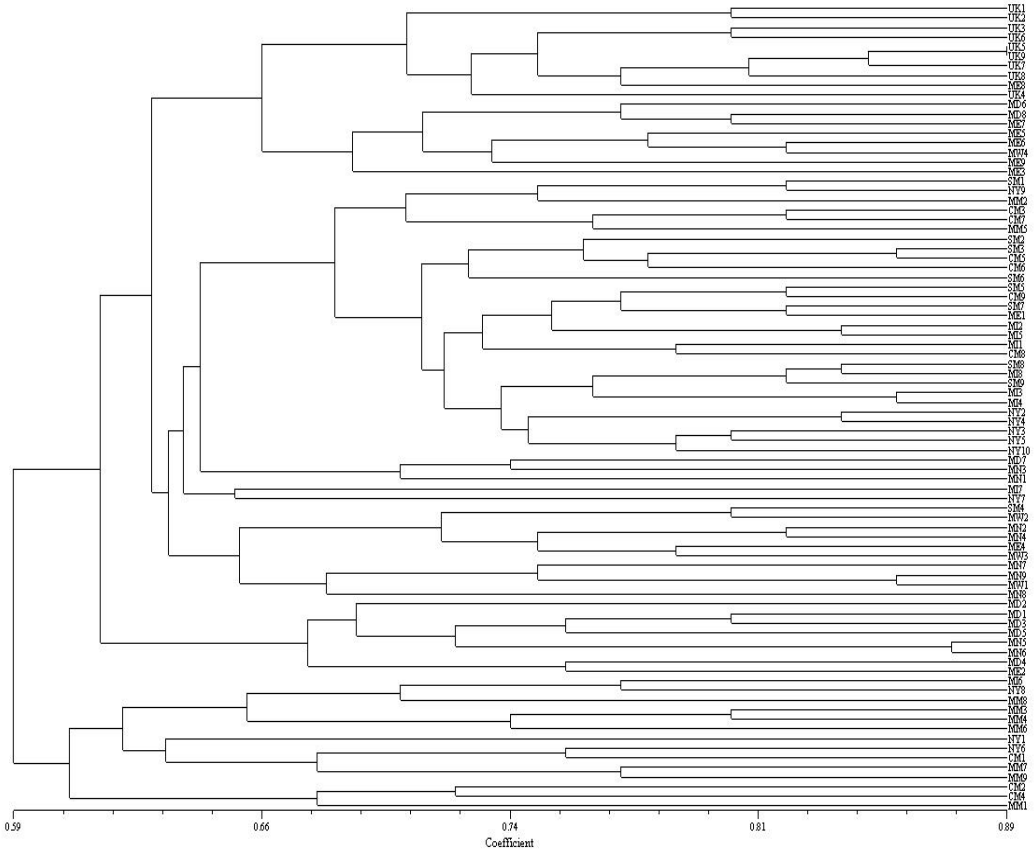

Figure 3. Cluster analysis of 84 beach plum materials based on SSR markers. The numbers in the figure are accession numbers as seen in Table 1.

Table 4. Genetic similarity between any two pairs of the ten provenances of beach plums based on SSR data.

Beach plum	UK	SM	MI	NY	CM	MD	ME	MM	MN
SM	0.656								
MI	0.612	0.695							
NY	0.629	0.673	0.667						
CM	0.641	0.681	0.649	0.661					
MD	0.627	0.609	0.605	0.579	0.563				
ME	0.658	0.643	0.622	0.623	0.635	0.633			
MM	0.593	0.604	0.602	0.614	0.631	0.551	0.588		
MN	0.598	0.656	0.631	0.624	0.624	0.617	0.635	0.584	
MW	0.627	0.673	0.602	0.621	0.629	0.608	0.659	0.582	0.675

Table 5. Average genetic diversity (AGD) within the ten provenances of beach plums.

Beach plum	AGD (%)
UK	19.49
SM	21.53
MI	25.20
NY	27.09
CM	24.04
MD	23.50
ME	24.85
MM	27.91
MN	27.03
MW	15.25

Genetic relationships among the 84 strains of ten beach plum germplasms

According to the genetic dissimilarity coefficient matrix, the 84 strains were divided into 2 large classes with a dissimilarity coefficient of 0.606 in the UPGMA tree. Class I included 77.8, 12.5, 30, and 33.3% of MM, MI, NY, and CM, respectively. Class II contains the remaining 9 beach plum germplasms. Class II could be subdivided into 3 groups at a dissimilarity coefficient of 0.632 in the UPGMA tree. One group contained all the members of UK and 66.7% of ME, and also contained 10, 25, and 25% of NY, MD, and MW, respectively. Another group mainly contained 6 beach plum germplasms, including 100% of SM, 87.5% of MI, 60% of NY, 66.7% of CM, 77.8% of MN, 75% of MW, and 25%, 22.2%, 22.2% of MD, ME, and MM, respectively. The remaining group contained 62.5% of MD, 22.2% of MN, and 11.1% of ME.

Based on the results above, we realized that the geographical distribution is not consistent with the genetic relationships among the different beach plum germplasm resources. The genetic relationships among the wild genotypes were close, apart from 10 strains of UK, 4 other wild genotypes (SM, MI, NY, and CM) could be divided into one group. The genetic relationship between the cultivated species Nana (MN) and Wild Goose (MW) was close, so that in one group, others 3 species (MD, ME, and MM) distributed in 3 different groups alone. Therefore, the relationships between the cultivated species and the wild genotypes are inseparable, which is facilitated by the method of breeding cultivated beach plums that uses artificial selections from wild progeny. Therefore, although MM was the predominate strain in class I, this class also contained some MI, NY, and CM strains. In class II, MN, MW, and wild species were predominant, although there was also a small representation of MD, ME, and MM.

DISCUSSION

It can be difficult and time consuming to distinguish beach plum cultivars based on morphological characteristics alone, and for this reason, it is important to develop molecular markers to aid in cultivar identification. Several marker systems have been applied to perennial ryegrass, including amplified fragment-length polymorphism (AFLPs) (Fang et al., 2005), random amplified polymorphism DNA (RAPDs) (Huff, 1997; Ravi et al., 2003), restriction fragment length polymorphism (RFLPs) (Charmet et al., 1997), and SSRs (Flajoulot et al., 2005; Li et al., 2010). Due to the high level of polymorphism and co-dominance of SSRs markers, these markers are particularly useful for genetic mapping, for studies on genetic variation, and for determining genetic relationships. In this study, we used SSRs markers to fingerprint and evaluate genetic relationships among beach plum cultivars. The high polymorphism may be due to the reasons that most beach plum cultivars develop from seedlings, and the genetic difference among this beach plum cultivars is relatively obvious. This study demonstrated that SSRs primer can be applied efficiently to study beach plum germplasm resources.

Many studies indicate that microsatellite sequences are highly conserved in *Prunus* (Hormaza, 2002; Serrano et al., 2002), and many SSR primer pairs have been developed for *Prunus* species and used in their genetic analysis (Cipriani et al., 1999; Aranzana et al., 2002; Dirlewanger et al., 2002; Wang et al., 2011). However, there are no studies on the utilization of SSR primers to analyze beach plum germplasm resources. DNA markers have been shown transfer between plant species, so other *Prunus* SSR primer pairs were used to identify beach plum germplasms. In this study, SSR amplification products from beach plums were sequenced and compared to those obtained from peaches and almonds. The result showed that the sequences obtained contained the SSR loci and that the number of sequence repeats was different between species. This study also demonstrated that the SSR primer pairs developed for peaches and almonds could be used for the identification of beach plums.

Here, we report the amplification of 11 SSR loci and the use of the SSRs to estimate the genetic diversity in 10 beach plum germplasms. All 84 strains from these 10 germplasms were grouped into 2 main clusters in the dendrogram. From the results, it was clear that the geographical distribution did not correspond with the genetic relationships among the different beach plum strains. This result was different from those previously reported, such as Hurtado et al. (2001), that used the RFLP, RAPD, and AFLP markers to separate 16 apricot cultivars into clusters of genetic similarity that were very similar to clusters of their known geographic origins. This difference merits further study. Genetically, the cultivars “Nana” and “Wild goose” were the closest in the dendrogram, which indicated that these two cultivars shared a common ancestor may have the same origin. In conclusion, the markers generated by 11 SSR primers proved very effective in distinguishing the beach plum germplasm resources. These markers can be used for DNA fingerprinting and estimates of the genetic relatedness of beach plums. The results from this study may be valuable for breeding programs design and genetic resources management of beach plums.

Conflicts of interest

The authors declare no conflict of interest.

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