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Original Article

Genetic Diversity and Population Structure Analysis of Sand Pear (*Pyrus pyrifolia*) 'Nakai' Varieties Using SSR and AFLP Markers

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Abstract

In this study, the technologies of simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) markers were used to analyze the genetic diversity of 30 sand pear (*Pyrus pyrifolia*) 'Nakai' varieties. Ten pairs of SSR polymorphic primers were selected to amplify *P. pyrifolia* 'Nakai' varieties. A total of 90 alleles were detected. The polymorphism information content index was between 0.5578 and 0.8423, with an average of 0.7585. The selected 10 pairs of AFLP primer combinations were used to amplify the analyzed pear varieties, and 1,046 polymorphic loci were detected. The average amplification results of each primer combination detected 105 bands with an average polymorphism percentage of 86.46%. The combined data of SSR and AFLP analysis showed that the analyzed *P. pyrifolia* 'Nakai' varieties were characterized by extremely rich genetic diversity and were highly representative. According to the results of SSR, AFLP, and SSR+AFLP cluster analysis, the analyzed *P. pyrifolia* 'Nakai' varieties can be categorized into three clusters. The results of genetic structure showed that the hybridization between these *P. pyrifolia* 'Nakai' varieties resulted in the heterozygosity of genotypes. In addition, we found that 'Nijisseik', 'Ejima', and 'Fuli' are good parent resources among the pear varieties through observing the genetic background of the analyzed pear varieties. This study reveals the genetic diversity levels of *P. pyrifolia* 'Nakai' varieties at the molecular level, which was important in molecular identification and protection of pear germplasm resources, as well as pear variety breeding and genetic improvement.

Keywords: cluster analysis; genetic polymorphism; genetic structure; molecular markers; pear varieties

Introduction

Pear belongs to the *Rosaceae* family and is mainly divided into white pear, sand pear, autumn pear, and other varieties. Most pear varieties originating in East Asia can be divided into five groups, namely, Ussurian pear, Chinese white pear, Chinese sand pear, Xinjiang pear, and Japanese pear, according to their geographical distribution (Teng and Tanabe, 2004). As the origin of the pear, China is the largest pear-producing country in the world and one of the main origin centers of pear plants. The pear variety resources are abundant in China with complex and changeable phenotypes (Zhang *et al.*, 2016). The breeding and popularization of pear varieties accelerate the exchange of germplasm resources in different places and also increase the genetic diversity of pear varieties. However, with the increase of pear varieties, it is more and more difficult to

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distinguish different varieties, which are only depending on phenotypic characterization and protein marker analysis. Therefore, identifying pear varieties and determining genetic correlation are necessary in cultivating new varieties and in collecting and preserving germplasm resources (Tana *et al*., 2015; Yue *et al*., 2018).

The traditional morphological feature identification method is difficult to apply and tends to change due to environmental factors. This method, which requires strong professional basic knowledge, is also time consuming and laborious (Jinbo *et al*., 2011). However, DNA molecular marker technology can be used without the influence of environmental conditions. This technology can quickly and accurately identify the genetic specificity of varieties at the molecular level, and overcome the deficiencies of traditional morphological markers, such as a long identical cycle, large amount of errors and requiring special skills acquired through extensive experience (Poczai *et al*., 2013). Therefore, this technology has been widely applied in the analysis of genetic diversity, variety identification, and establishment of fingerprints in various fruit trees such as strawberries (Sánchez-Sevilla *et al*., 2015), hawthorn (Güney *et al*., 2018), citrus (Rao *et al*., 2008), and peach (Cheng and Huang, 2009). At present, various types of molecular markers have been widely applied in the study of genetic diversity of pear varieties. In pear tree fingerprinting studies, random amplification of polymorphic DNA (RAPD) (Lisek and Rozpara, 2010), amplified fragment length polymorphism (AFLP) (Yamamoto *et al*., 2007), simple sequence repeat (SSR) (Yue *et al*., 2014; Puskás *et al*., 2016), inter-simple sequence repeat amplification (ISSR) (Monte-Corvo *et al*., 2001), and sequence-related amplification polymorphism (SRAP) (Zhang *et al*., 2012) were all used to evaluate genetic variability and identify germplasm resources. Therefore, in this study, SSR and AFLP markers were used to analyze the genetic diversity and population structure of *P. pyrifolia* 'Nakai' varieties to reveal their genetic diversity at the molecular level and provide a basis for molecular identification of pear variety resources and protection of variety rights, as well as pear variety breeding and genetic improvement.

Materials and Methods

Materials

A total of 30 *P. pyrifolia* 'Nakai' varieties were collected, which included 23 Chinese cultivars, 5 Japanese cultivars and 2 Korean cultivars (Table 1). These were provided by Yangtze University and Fruit and Tea Research Institute of Hubei Academy of Agricultural Sciences.

DNA extraction and molecular marker polymorphism screening

The leaves were frozen in liquid nitrogen, and the DNA was extracted using CATB method (Ye *et al*., 2017). The quality and concentration of DNA were determined using 1% agarose gels and a NanoPhotometer® spectrophotometer (Implen, CA, USA), respectively. The extracted DNAs were diluted to 40 ng μL^{-1} for PCR reaction.

To fully reflect the genetic diversity of *P. pyrifolia* 'Nakai' varieties, the SSR primers refer to the genetic linkage map of pears constructed by Terakami *et al*. (2009) and Celton *et al*. (2009). Seventeen pairs of SSR primers distributed in 17 linkage groups of pears were selected for screening, and the primer sequences were reported by Yamamoto *et al*. (2002) and Liebhard *et al*. (2002) (Table 2). Primer pairs were prescreened on four genotypes ('Eli 2', 'Housui', 'Jingli 1', and 'Wonhwang') in order to identify primers that generated clear bands for further amplification reactions for *P. pyrifolia* 'Nakai' varieties.

Finally, 10 pairs of SSR primers and 10 pairs of appropriately combined AFLP primers (Table 3) were determined for further amplification of 30 sand pear DNA samples. All primers were synthesized by Sangon Biological Engineering Technology and Service Co. Ltd, Shanghai.

SSR analysis

PCR reactions were carried out in a 20 μL mixture: 14.8 uL of ddH_2O, 0.4 uL of dNTP mixture, 2 uL of 10×PCR Buffer, 0.3 uL of forward primer (20 uM), 0.3 uL of reverse primer (20 uM), 2 μL of DNA template and 0.2 uL of Taq DNA polymerase (TaKaRa, Dalian, China). The SSR PCR reaction was carried out as follows: 94 °C for 5 min for the initial denaturing step, followed by 35 cycles at 94 °C for 30 s, 55 °C for 35 s, and 72 °C for 40 s for denaturing, annealing, and extension, respectively, followed by a final extension step at 72 °C for 3 min.

AFLP analysis

Frequent cutter *Mse* I and rare cutter *EcoR* I endonucleases source of purchase were used for restriction digestion of genomic DNA, restriction and ligation were then carried out together. Restriction and ligation mixture were carried out in a total volume of 20 μL: 2 μL of 10×AFLP digest-ligation buffer, 1.8 μL of AFLP digest-ligation enzyme mix, 1 μL of *Eco*R I adaptor (10 μM), 1μL of *Mse* I adaptor (10 μM), 4 μL of DNA template and 10.2 μL of ddH_2O; 25 °C 5 h.

The total amount of pre-amplification was 20 μL: 10μL of 2×PCR Mix, 1 μL of E00 (20 μM), 1μL M00 (20 μM), 4 μL of restriction-ligation products and 4 μL of ddH_2O. The DNA amplification was performed using the following program: 94 °C for 3 min for the initial denaturing step, followed by 30 cycles at 94 °C for 30 s, 50 °C for 35 s, and 72 °C for 1 min for denaturing, annealing, and extension, respectively. The quality and concentration of pre-amplification PCR products were determined using 1% agarose gels and a NanoPhotometer® spectrophotometer (Implen, CA, USA).

Selective amplification were carried out in a 20 μL mixture: 10 μL of 2×PCR Mix, 1 μL of E primer (20 μM), 1 μL of M primer (20 μM), 5 μL Pre-PCR products (Pre-PCR products were diluted 20-fold and used as DNA template for selective amplification) and 3 μL ddH_2O. Selective amplification was carried out using the following touchdown program: 95 °C for 5 min, 95 °C for 35 s, 60 °C for 35 s, 72 °C for 1 min, followed by 12 cycles of each with 0.7 °C lowering of annealing temperature and finally 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min.

Table 1. Names, sources and parentage of *P. pyrifolia* 'Nakai' varieties

Code	Name of varieties	Sources	Percentage
Z01	Chuxiang	China	Laiyangcili×Ejima
Z02	Cuiguan	China	Kousui×(hangqing×Shinseiki)
Z03	Deshengxiang	China	Landrace
Z04	Eli 1	China	Jinshuisu×Fuli
Z05	Eli 2	China	Zhongxiang×(Fuli×Beurre Giffard)
Z06	Ninomiyahuri	Japan	Yali×Zhenyu
Z07	Housui	Japan	Bayun×Cuixing
Z08	Huali 1	China	Syounan×Ejima
Z09	Huangguan	China	Sheinseiki×Xuehuali
Z10	Whangkeumbae	Korea	Nijisseik×Niitaka
Z11	Jinshui 2	China	Ejima×Chojuro
Z12	Jinshui 3	China	Syouna×Ejima
Z13	Jinshuiqiu	China	Okusankichi×Laiyangcili
Z14	Jinshuisu	China	Jinshui 1×Xinglongmali
Z15	Jingli 1	China	Landrace
Z16	Laiyangcili	China	Landrace
Z17	Lvbaoshi	China	Sheinseiki×Zaosu
Z18	Qingxiang	China	Sanhua×Sheinseiki
Z19	Qiuyue	Japan	Kousui×(Niitaka×Kousui)
Z20	Wuzili	China	Yali×Rushanbali
Z21	Xizilv	China	(Bayun×Hangqing)×Sheinseiki
Z22	Niitaka	Japan	Imanuraaki×Amanokawa
Z23	Xueqiu	China	Landrace
Z24	Yulv	China	Taibai×Laiyangcili
Z25	Yuxiang	China	Jinshuisu×Fuli
Z26	Wonhwang	Korea	Waseaka×Okusankichi
Z27	Zaomeisu	China	Zaosu×Shinseiki
Z28	Zaosu	China	qianliang×Pingguoli
Z29	Chojuro	Japan	Landrace
Z30	Zhongxiang	China	Landrace

Table 2. Sequences of 17 pairs of primers used in SSR marker

Code	Locus name	Primer pairs sequences (5'-3')	Locus name	Primer pairs sequences (5'-3')
P1	NH013a-F	GGTTTGAAGAGGAATGAGGAG	NH013a-R	CATTGACTTTAGGGCACATTTC
P2	BGT23b-F	CACATTCAAAGATTAAGAT	BGT23b-R	ACTCAGCCTTTTTTTCCCAC
P3	CH03g12-F	GCGCTGAAAAAGGTCAGTTT	CH03g12-R	CAAGGATGCGCATGTATTTG
P4	NH011a-F	GGTTCACATAGAGAGAGAGAG	NH011a-R	TTTGCCGTTGGACCGAGC
P5	CH04g09-F	TTGTCGCACAAGCCAGTTTA	CH04g09-R	GAAGACTCATGGGTGCCATT
P6	CH03d12-F	GCCCAGAAGCAATAAGTAAACC	CH03d12-R	ATTGCTCCATGCATAAAGGG
P7	CH04e05-F	AGGCTAACAGAAATGTGGTTTG	CH04e05-R	ATGGCTCCTATTGCCATCAT
P8	CH01h10-F	TGCAAAGATAGGTAGATATATGCCA	CH01h10-R	AGGAGGGATTGTTTGTGCAC
P9	CH05c07-F	TGATGCATTAGGGCTTGTACTT	CH05c07-R	GGGATGCATTGCTAAATAGGAT
P10	NH017a-F	CAGAAAGGAGAGGGCTACAG	NH017a-R	CCCTCACCCAATCAAAACTC
P11	CH03d02-F	AAACTTTCACTTTCACCCACG	CH03d02-R	ACTACATTTTTAGATTTGTGCGTC
P12	CH01f02-F	ACCACATTAGAGCAGTTGAGG	CH01f02-R	CTGGTTTGTTTTCCTCCAGC
P13	NH009b-F	CCGAGCACTACCATTGA	NH009b-R	CGTCTGTTTACCGCTTCT
P14	NH004a-F	AGGATGGGACGAGTTTAGAG	NH004a-R	CCACATCTCTCAACCTACCA
P15	CH02d11-F	AGCGTCCAGAGCAACAGC	CH02d11-R	AACAAAAGCAGATCCGTTGC
P16	NH007b-F	TACCTTGATGGGAACTGAAC	NH007b-R	ATAGTAGATTGCAATTACTC
P17	NH015a-F	TTGTGCCCTTTTTCCTACC	NH015a-R	CTTTGATGTTACCCCTTGCTG

Table 3. Sequences of oligonucleotide adapters and primer combinations used in AFLP

No.	Primer combinations and adapters	Sequences (5'-3')	Primer combinations and adapters	Sequences (5'-3')
	E00	GACTGCGTACCAATTC	M00	GATGAGTCCTGAGTAA
	*Eco*R I adapter1	CTCGTAGACTGCGTACC	*Eco*R I adapter2	AATTGGTACGCAGTCTAC
	Mse I adapter1	GACGATGAGTCCTGAG	*Mse* I adapter2	TACTCAGGACTCAT
	*Eco*R I primer		*Mse* I primer	
A1	E75	GACTGCGTACCAATTCGTA	M62	GATGAGTCCTGAGTAACTT
A2	E83	GACTGCGTACCAATTCTCG	M50	GATGAGTCCTGAGCGGCAT
A3	E32	GACTGCGTACCAATTCAAC	M66	GATGAGTCCTGAGTAAGAT
A4	E77	GACTGCGTACCAATTCGTG	M49	GATGAGTCCTGAGTAACAG
A5	E84	GATGAGTCCTGAGTAATCC	M62	GATGAGTCCTGAGTAACAG
A6	E85	GACTGCGTACCAATTCGTG	M50	GATGAGTCCTGAGCGGCAT
A7	E37	GACTGCGTACCAATTCACG	M66	GATGAGTCCTGAGTAAGAT
A8	E59	GACTGCGTACCAATTCCTA	M60	GATGAGTCCTGAGCGGCTC
A9	E86	GATGAGTCCTGAGTAATCT	M49	GATGAGTCCTGAGTAACAG
A10	E42	GACTGCGTACCAATTCCTA	M51	GATGAGTCCTGAGCGGCAT

Data analysis

Data was analyzed using GeneMarker 2.2 software to display the fragment sizes as electropherograms and binary data. The NTSYSpc 2.11 software package was used to calculate the individual similarity coefficient (ISC) and cluster analysis was performed by UPGMA method. The genetic diversity of the samples was analyzed by Popgene and NTSYS software. Structure 2.3.4 software was used to analyze the data of SSR and AFLP to obtain the group structure diagram of SSR and AFLP (Evanno *et al*., 2005; Falush *et al*., 2007).

Results

Amplification of SSRs and AFLPs

For SSR analysis, 17 pairs of SSR primers were tested in four *P. pyrifolia* 'Nakai' varieties ('Eli 2', 'Housui', 'Jingli 1' and 'Wonhwang') in order to identify candidate primers. The results showed that polymorphism of clear bands was generated among the total analyzed sample (Fig. 1). Finally, 10 pairs of SSR polymorphic primers were used to amplify *P. pyrifolia* 'Nakai' samples, and a total of 90 alleles were detected (Table 4). The number of alleles detected by each pair of primers was between 5.0 and 12.0, with an average of 9.0 (Table 4). The actual number of alleles detected by each primer was 5.0686. The polymorphism information content indexes (PIC) were between 0.5578 and 0.8423, with an average of 0.7585, indicating that the polymorphism information content indexes of the primers were quite different.

The 10 pairs of the selected AFLP combined primers were used for PCR amplification of the *P. pyrifolia* 'Nakai' DNA samples. As shown in Table 5, the average amplification results of each primer combination could detect 105 bands, 169 bands at most. In the currently reported AFLP technology based on silver staining, the number of bands amplified by most primer combinations was approximately 50, and the maximum number was not more than 100. Our results showed that the number of bands detected by AFLP in this experiment was significantly more than that reported in the literatures (Bao *et al*., 2008; Monte-Corvo *et al*., 2000), which confirmed the abundance of genetic diversity of pear varieties and enhances the reliability of this technique in analyzing such diversity. Moreover, the detection rate of polymorphism was also quite high. The polymorphic bands of 10 pairs of primer combinations among *P. pyrifolia* 'Nakai' varieties were 1,046 with an average polymorphic percentage of 86.46%. The primer combination with the highest polymorphism is E84M62, with a polymorphism percentage of 92.24%. Therefore, specific genetic differences exist among *P. pyrifolia* 'Nakai' varieties.

Cluster analysis

The results of SSR analysis showed that the genetic distance or genetic similarity coefficient between these four varieties ('Deshengxiang', 'Eli 1', 'Yuxiang', and 'Zhongxiang') and other varieties is 0. Thus, the results of SSR cluster and population structure maps do not include these four varieties. As shown in Fig. 2, when the genetic similarity coefficient was 0.30, the results of SSR dendrogram analysis showed that 26 varieties can be divided into cluster I (SSR-I), cluster II (SSR-II), and cluster III (SSR-III). SSR-I covered 19 materials, accounting for 73% of the total materials, including 12 domestic varieties ('Chuxiang', 'Yulv', 'Huali 1', 'Xueqiu', 'Jinshui 3', 'Cuiguan', 'Jinshui 2', 'Eli 2', 'Jingli 1', 'Xizilv', 'Qingxiang', and 'Huangguan') and 7 foreign varieties ('Ninomiyahuri', 'Qiuyue', 'Housui', 'Wonhwang,' 'Whangkeumbae', 'Chojuro', and 'Niitaka'). SSR-II contained 3 domestic varieties ('Jinshuiqiu', 'Laiyangcili', and 'Jinshuisu'), accounting for 12% of the total varieties. SSR-III covered 4 domestic varieties ('Lvbaoshi', 'Zaosu', 'Zaomeisu', and 'Wuzili'), accounting for 15% of the total varieties.

According to the data of genetic similarity coefficient, when the population similarity coefficient exceeded 0.72, the genetic differentiation between different varieties becomes larger and larger, and was divided into three clusters (Fig. 3). *P. pyrifolia* 'Nakai' varieties can be divided into cluster I (AFLP-I), cluster II (AFLP-II), and cluster III (AFLP-III). AFLP-I consisted of 11 varieties ('Chuxiang', 'Whangkeumbae', 'Jingli 1', 'Cuiguan', 'Niitaka', 'Chojuro', 'Jinshui 3', 'Huali 1', 'Yuxiang', 'Laiyangcil'I, and 'Ninomiyahuri'), accounting for 37% of the total varieties. This cluster included most Chinese varieties and a few foreign varieties. AFLP-II consisted of 13 varieties ('Deshengxiang', 'Yulv', 'Jinshui 2', 'Eli 1', 'Eli 2', 'Housui', 'Wonhwang', 'Huangguan', 'Jinshuiqiu', 'Zaosu',

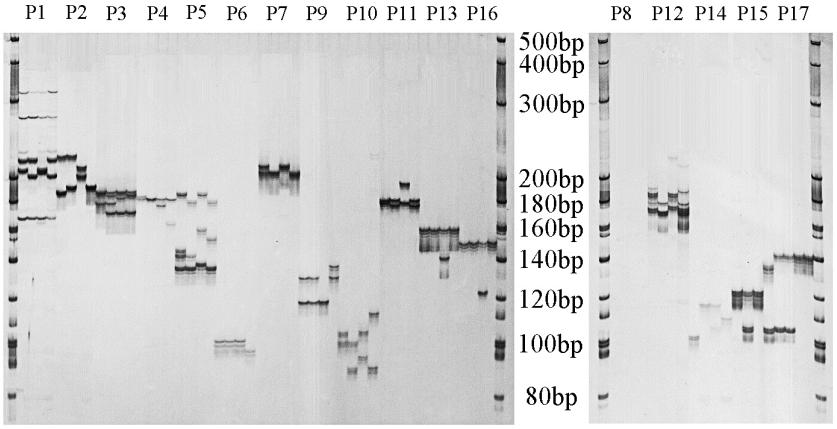

Fig. 1. Four *P. pyrifolia* 'Nakai' varieties ('Eli 2', 'Housui', 'Jingli 1' and 'Wonhwang') were amplified by 17 SSR primer pairs

Table 4. Analysis of polymorphic bands of 10 pairs of SSR primers

Code	Locus name	Na	Ne	I	Ho	He	Fst	PIC
P1	NH013a	9	5.4516	1.8892	0.8462	0.8326	0.4819	0.7931
P2	BGT23b	9	4.3613	1.7680	0.7692	0.7858	0.5010	0.7460
P4	NH011a	10	4.8828	1.8946	0.8000	0.8114	0.5255	0.7750
P7	CH04e05	11	6.5631	2.0787	0.9231	0.8643	0.4555	0.8302
P9	CH05c07	8	3.5767	1.5286	0.6538	0.7345	0.5462	0.6781
P10	NH017a	8	5.8276	1.8836	0.7692	0.8446	0.5357	0.8067
P11	CH03d02	12	6.9691	2.1755	0.9231	0.8733	0.4611	0.8423
P14	NH004a	10	6.2304	1.9867	0.8462	0.8560	0.4960	0.8198
P16	NH007b	5	2.5177	1.1629	0.6154	0.6146	0.4896	0.5578
P17	NH015a	8	4.3057	1.6909	0.7308	0.7828	0.5241	0.7362
Mean		9	5.0686	1.8059	0.7877	0.8000	0.5009	0.7585

Note: Na-Observed number of alleles; Ne-Effective number of alleles; I-Shannon's Information; Ho-Observed heterozygosity; He-Expected heterozygosity; Fst-Genetic differentiation coefficient; PIC-The percentage of polymorphic loci.

Table 5. Analysis of polymorphic bands of 10 pairs of AFLP primers

No.	Primer combination	Na	Ne	Ho	I	P1	P2 (%)
A1	E75M62	1.9118	1.5569	0.3232	0.4820	93	91.18
A2	E83M50	1.5854	1.3586	0.2087	0.3108	72	58.54
A3	E32M66	1.8759	1.5712	0.3283	0.4859	120	87.59
A4	E77M49	1.9217	1.5546	0.3253	0.4869	106	92.17
A5	E84M62	1.8895	1.5212	0.3151	0.4753	169	88.95
A6	E85M50	1.9224	1.6145	0.3556	0.5248	107	92.24
A7	E37M66	1.8939	1.6138	0.3516	0.5167	118	89.39
A8	E59M60	1.8796	1.5826	0.3388	0.5007	95	87.96
A9	E86M49	1.8842	1.6002	0.3426	0.5039	84	88.42
A10	E42M51	1.8817	1.6123	0.3486	0.5112	82	88.17
Mean		1.8646	1.55859	0.3238	0.4798	104.6	86.46

Note: P1-Polymorphic bands, P2-Percentage of polymorphic bands

'Zaomeisu', 'Jinshuisu', and 'Zhongxiang'), accounting for 43% of the total varieties. AFLP-III covers 6 varieties' ('Lvbaoshi', 'Qingxiang', 'Qiuyue', 'Wuzili', 'Xueqiu', and 'Xizilv'), accounting for 20% of the total varieties.

SSR and AFLP data were combined for UPGMA clustering analysis. The genetic similarity coefficient were 0.72, which was expressed in cluster I (SSR+AFLP)-I, cluster II (SSR+AFLP)-II, and cluster III (SSR+AFLP)-III (Fig. 4). The first major cluster (SSR+AFLP)-I included 6 varieties, accounting for 20% of the total varieties, which included 5 domestic varieties ('Chuxiang', 'Zaosu', 'Huali 1', 'Jingli 1', and 'Jinshui 3') and 1 Korean variety ('Whangkeumbae'). The second cluster (SSR+AFLP)-II contained 18 varieties, accounting for 60% of the total varieties, including 13 domestic varieties ('Cuiguan', 'Zaomeisu', 'Laiyangcili', 'Huangguan', 'Jinshuiqiu', 'Zhongxiang', 'Jinshuisu', 'Yuxiang', 'Jinshui 2', 'Deshengxiang', 'Yulv', 'Eli 1', and 'Eli 2') and 5 imported varieties ('Niitaka', 'Chojuro', 'Ninomiyahuri', 'Wonhwang', and 'Housui'). The third cluster (SSR+AFLP)-III contained 6 varieties, accounting for 20% of the total varieties, covering 5 Chinese varieties ('Lvbaoshi', 'Qingxiang', 'Wuzili', 'Xueqiu', and 'Xizilv') and 1 Japanese variety ('Qiuyue').

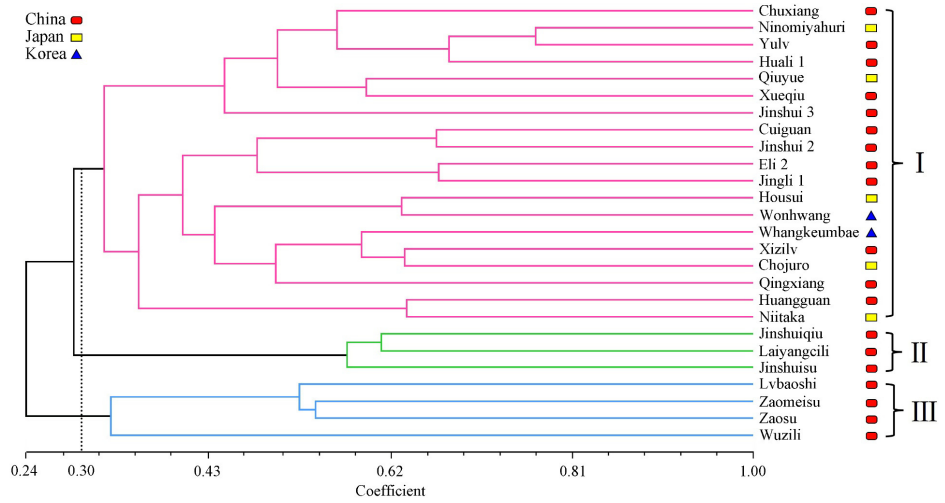

Fig. 2. Dendogram obtained by cluster analysis based on SSR data

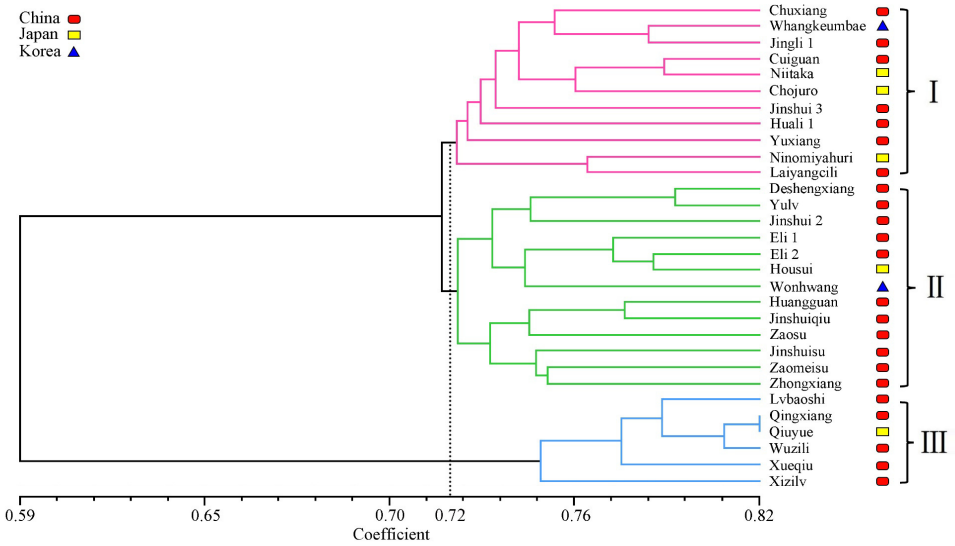

Fig. 3. Dendogram obtained by cluster analysis based on AFLP data

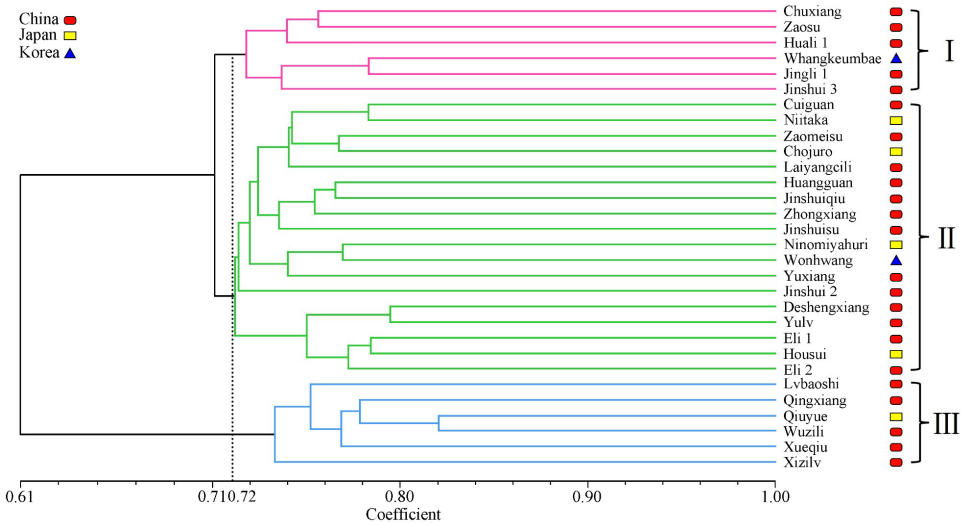

Fig. 4. Dendogram obtained by cluster analysis based on SSR and AFLP data

Genetic structure

Structure 2.3.4 software was used to analyze the data of SSR and AFLP, and the optimal K values of both SSR and AFLP were 2 (Fig. 5). In other words, both SSR and AFLP populations contained two genotypes, which were expressed by PK and PA. Each of the two genotypes in the SSR population structure diagramed account for half (Fig. 6), which was consistent with Fst in SSR genetic variation analysis (Table 4). Both were heterozygotes in the population, and no pure breed exists. SSR-I contained two genotypes, PK and PA, of which the proportion of the PK genotype was slightly larger than that of the PA genotype. However, the proportion of PA in *P. pyrifolia* 'Nakai' varieties in SSR-II and SSR-III was much larger than that in PK.

The results of population structure analysis showed that the PK genotype was introgression in the two pear varieties because a small amount of PK gene was doped in ancestral parents of the two pears during the breeding process. The SSR population structure map showed the rich background of genetic diversity of *P. pyrifolia* 'Nakai' varieties we have studied, thereby further verifying the PIC value of SSR polymorphism band analysis.

The population structure results analyzed by AFLP were presented in Fig. 7. The proportion of PK in the population was much larger than PA, which was consistent with the gene diversity index Ho in AFLP polymorphism analysis (Table 5). Overall, the *P. pyrifolia* 'Nakai'varieties included 18 purebreds, of which 14 varieties ('Cuiguan', 'Whangkeumbae', 'Jinshui 3', 'Laiyangcili', 'Chuxiang', 'Deshengxiang', 'Housui', 'Huangguan', 'Jinshui 2', 'Jinshuiqiu', 'Qingxiang', 'Xizilv', 'Qiuyue', and 'Xueqiu') only contained PK. According to the genetic background, almost all parents were Japanese pears or local varieties. The genotype of the other four varieties ('Yuxiang', 'Wonhwang', 'Zaomeisu', and 'Zaosu') was PA. Except 'Yuxiang', the three other varieties were clustered on AFLP-II, which further verified the clustering accuracy of AFLP.

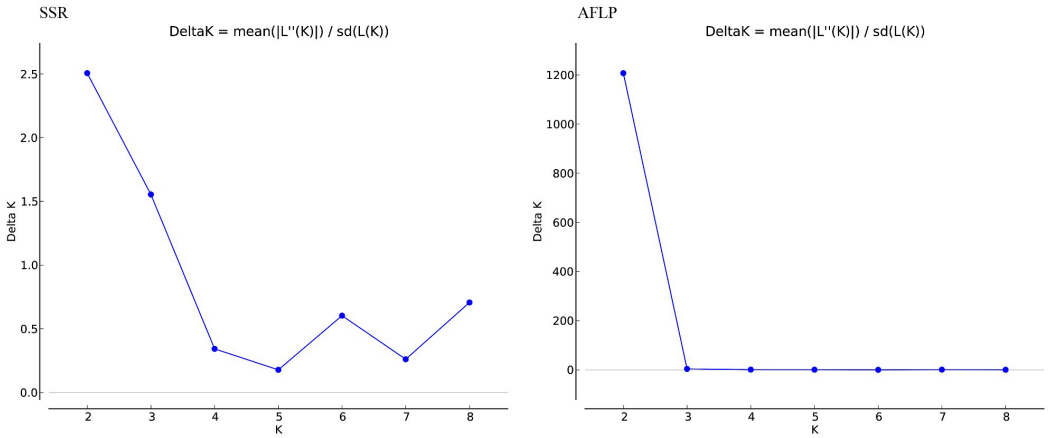

Fig. 5. Estimation of the most probable K value for the *P. pyrifolia* 'Nakai' cultivars, based on the method of Evano (2005)

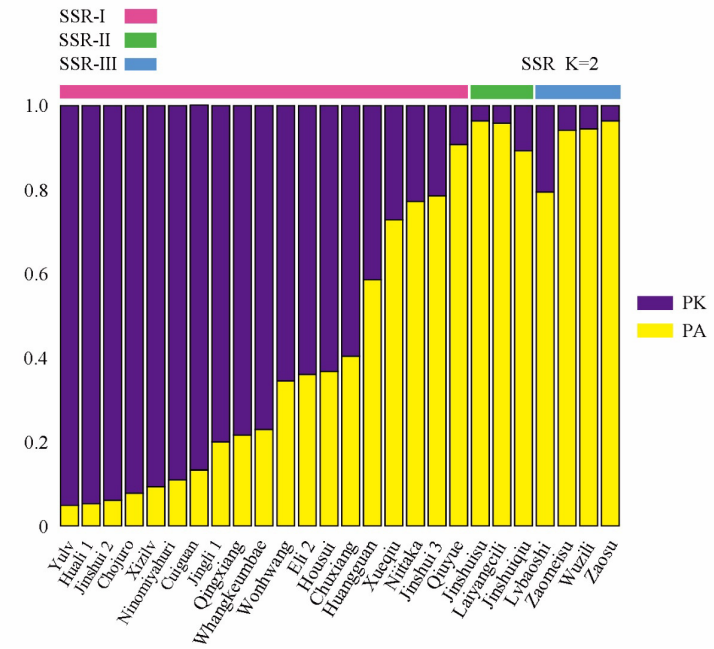

Fig. 6. Genotype class assignment of 26 *Pyrus pyrifolia* 'Nakai' varieties based on the software Structure2.3.4 using SSR

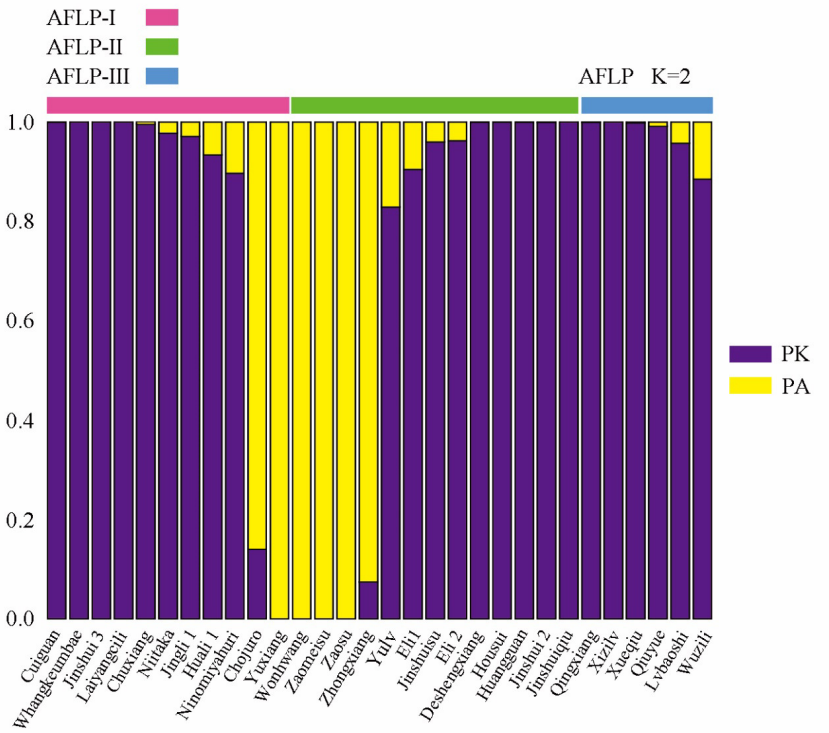

Fig. 7. Genotype class assignment of all *Pyrus pyrifolia* 'Nakai' varieties based on the software Structure 2.3.4 using SSR

Discussion

SSR and AFLP have been used in variety identification and genetic diversity determination of various fruit trees. Therefore, this study combined SSR and AFLP marker technology to analyze the genetic diversity among pear varieties. Here, SSR (75.85%) and AFLP (86.46%) in selected *P. pyrifolia* 'Nakai' varieties have shown high polymorphism similar to that of the markers in other fruit tree studies: 75.24% in 8 mango genotypes (384 SSRs) (Song *et al*., 2015), 71.00% in 9 coconut genotypes (171 SSRs) (Loiola *et al*., 2016), 76.30% in 27 papaya genotypes (378 SSRs) (Sengupta *et al*., 2014), 87% in 27 plum accessions (134 SSRs) (Pop *et al*., 2018), 73.50% in 162 pineapple genotypes (377 AFLPs) (Kato *et al*., 2005), 78.34% in 137 *Adansonia digitata* 'Linn'. genotypes (217 AFLPs) (Assogbadjo *et al*., 2006), and 53.00% in 16 banana genotypes (148 AFLPs) (Loh *et al*., 2000). The above results showed that both SSR and AFLP were ideal molecular markers, which could effectively evaluate the genetic diversity of *P. pyrifolia* 'Nakai' varieties.

Although the clustering results of *P. pyrifolia* 'Nakai' varieties in different marker results were not completely consistent, the three tree clustering maps generated from SSR, AFLP, and combination data showed approximate similarity. Most of the varieties in (SSR+AFLP)-II correspond to those in SSR-II and AFLP-II, included 8-9 identical domestic varieties. Furthermore, (SSR+SRAP)-III germplasm was highly similar to that in the SSR-III germplasm. SSR-I included cluster I of AFLP or SSR+AFLP dendrogram. Notably, the four varieties ('Chuxiang', 'Eli 1', 'Eli 2', and 'Jingli 1') bred by our research group were in the same cluster as most commercial varieties abroad. However, we also found that some varieties were located in different clusters in the three genetic clustering maps; for example, the domestic variety 'Zaosu' was in SSR-I but in AFLP-III and (SSR+SRAP)-II in the other two genetic clustering maps. On the contrary, some varieties were stable in the same cluster in the three genetic clustering maps. For example, 4 domestic varieties ('Chuxiang', 'Huali 1', 'Jingli 1', and 'Jingshui 3') and Korean variety ('Whangkeumbae') were all located in the first cluster in the three genetic clustering maps, and 'Chuxiang' was ranked first in the first cluster. The second cluster of the three genetic clustering maps covered the domestic varieties of 'Jinshuisu' and 'Jinshuiqiu'. However, the domestic varieties of 'Lvbaoshi' and 'Wuzili' were in the third cluster of three genetic clustering maps, and 'Lvbaoshi' always ranked in the first cluster of the third cluster. By observing the genetic background of *P. pyrifolia* 'Nakai' varieties, we found that 'Nijisseik', ''Ejima, and 'Fuli' were good parent resources. A large amount of *P. pyrifolia* 'Nakai' varieties were bred with these 3 varieties as parents. This discovery can provide theoretical guidance for pear variety breeding and genetic improvement.

In other horticultural plants, genetic diversity analysis was often conducted by combining various types of molecular markers. For example, SSR markers were used to study genetic diversity in tung tree (Zhang *et al*., 2014), AFLP markers were used to study genetic diversity in pomegranate (Nemati *et al*., 2012), SSR and AFLP markers were used to study genetic diversity in watermelon (Hwang *et al*., 2011), SSR and SRAP markers were used to study genetic diversity in lemon (Uzun *et al*., 2011), SSR markers were used to study genetic diversity in wild apricot (He *et*

al., 2007). Although the dendrogram generated by using various molecular markers showed high similarity, some differences exist in cluster analysis obtained from various types of markers. The reason may be the use of different marker systems, different marker quantities or different test groups. Different molecular markers have been developed using different principles. Thus, the expected amplified fragments had different locations in the entire genome and the amount of genome coverage. In the same species, sampling variation may vary depending on the quantities. Therefore, the dendrogram constructed on the basis of the molecular markers with more types and quantities is realistic. Therefore, the results obtained by SSR+AFLP dendrogram can more significantly reflect the genetic diversity among *P. pyrifolia* 'Nakai' varieties in this study.

To a certain extent, most ecological plants were clustered according to their geographical distribution. In this experiment, based on cluster analysis of SSR+AFLP data, 70% of pear varieties from Korea and Japan were combined in cluster II and the remaining 30% were in the other two clusters. The results of this study showed that all Japanese and Korean varieties were not located in a cluster, and no obvious relationship exists between geographical origin and molecular marker clusters, which was consistent with the findings of Johnson *et al*. (2002). The genetic structure diagram showed that *P. pyrifolia* 'Nakai' varieties contained two genotypes, because no significant correlation existed between the geographical origin of varieties and its molecular marker clusters (Yuan *et al*., 2018). The reason may be the diffusion of introduced species, hybridization through geographically different genetic populations as parents, and potential inherent genetic overlaps that may exist in these germplasms (An *et al*., 2017).

Conclusions

It was concluded that the *P. pyrifolia* 'Nakai' varieties shared high genetic similarity and abundant genetic diversity. In addition, the genetic background analysis of *P. pyrifolia* 'Nakai' varieties suggested that 'Nijisseik', 'Ejima', and 'Fuli' were good parent resources among *P. Pyrifolia* 'Nakai' varieties varieties. Due to the communication between varieties of *P. pyrifolia* 'Nakai' in different regions, the genetic diversity of *P. pyrifolia* 'Nakai' varieties increases, which leads to the increase of genetic diversity among varieties of *P. pyrifolia* 'Nakai'. Therefore, these results can provide useful references for further research on molecular identification and conservation of germplasm resources, as well as pear variety breeding and genetic improvement.

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Conflicts of Interest

The authors declare that there are no conflicts of interest related to this article.

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