The Journal of Animal & Plant Sciences, 25 (3 Suppl. 1) 2015 Special Issue Page: 128-133
ISSN: 1018-7081

SSR PRIMER SELECT AND ANALYSIS IN *Malus* Mill GENETIC RELATIONSHIP RESEARCH

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ABSTRACT

Thirty pairs of polymorphism SSR primers were used for cluster analysis on 18 Xinjiang wild apple clones, 23 edible apple varieties, and 7 apple rootstock varieties. Forty eight varieties were classified into three groups: edible apple, Xinjiang wild apple, and rootstock apple, which do not interlace with each other. Varieties with genetic relationships were closely clustered inside the groups, consistent with the traditional family tree. This paper classified the primers into three types. The relevant analysis of the comprehensive cluster results of all primers indicated the second primer group had the best correlation, with the coefficient of 0.957, whereas the first premier group had the lowest correlation, with the coefficient of 0.871. The second primer group had better distinguishing ability for edible apple and rootstock apple; the third primer group had poor distinguishing ability for rootstock apple; the first primer group can be applied to distinguish Xinjiang wild apple. The cluster results of the combination of 6 highly-effective primers on 48 apple samples had the correlation coefficient of 0.940 with the comprehensive cluster results of 30 pairs of primers.

Key words: *Malus* Mill; SSR marker; primer selection; primer efficiency.

INTRODUCTION

The genetic resources of *Malus* Mill are abundant (Dong *et al*., 2013) with various types including numerous wild species, semi-wild species, and cultivated species having high economic value in fruit growing, production, and breeding. Various germplasms of apples, such as species, semi-wild species, cultivated species, and virus-resistant germplasm, are abundant, and have an important role in the study of the origin of apples, relationship evolution of germplasm resources, and other aspects.

The simple sequence repeat (SSR) marker has good mark stability, high polymorphism, strong primer specificity, and other features. In addition, the SSR marker has been an important way of exploring plant genetic diversity and relationship, screening the core primer of polymorphism, and establishing stable reaction system, which is the key to correct SSR analysis (Zietkiewicz *et al*. 1994; Guarino *et al*. 2006). Previous researchers have disregarded the screening of SSR primer and took primer polymorphism as the sole criterion of primer screening. In addition, the amount and representativeness of the primers have seldom been considered. Different cluster results have been obtained, leading to the offset and confusion of cluster results and the unconformity of origin or traditional classification, thereby decreasing the reliability of the results. Several studies on plant genetic diversity and genetic relationship have adopted SSR. However, only few studies on the analysis and comparative studies on primers have been carried out. Taking apple as an example, the current study aims to explore the genetic relationship among *Malus* Mill, analyze the differences between SSR markers, explore the reasons for SSR clustering offset, and provide reference for future SSR analysis and prime screening.

MATERIALS AND METHODS

Experimental materials: The 23 edible apple varieties used in this study were from Xiongyue Germplasm repository of Liaoning Institute of Pomology. The 18 Xinjing wild apple were new clones (numbered Y1-Y18), were selected from 600 wild single plants in Xinyuan County, Tianshan, Xinjiang, and were grafted and planted in Mancheng County Nursery Garden in Hebei. The 7 rootstock varieties were from the Garden of Hebei Agricultural University. The sources of each variety and clone were shown in Table 1. In spring of 2013, 10 cm branch with new tender leaves from each variety was clipped and stored in a refrigerator at −70°C.

DNA extraction: Improved CTAB method (Han *et al*. 2009) was adopted to extract genome DNA of each series. First, the tissues and cells were smashed, and the soluble matter in the cell was then removed through dissolution and centrifugation. Through purification and dissolution, Nanodrop 2000 nucleic acid analyzer was used to detect quality and diluted to 30 ng·μL for PCR reaction.

Table 1. Apple varieties and clones for the tests

No.	Variety name	Type	Parentage or origin
1	Alps Otrome	Edible	Japan, Fuji×Jonathan
2	Tsugaru	Edible	Japan, Gold Delicious×Jonathan
3	Korin	Edible	Japan, Fuji×Unknown
4	Sekaiichi	Edible	Japan, Delicious×Gold Delicious
5	Yueshuai	Edible	StarkingRed×Gold Delicious
6	Sansa	Edible	Japan, Gala×Jonathan
7	Gala	Edible	New Zealand (Delicious× Kidd'sOrange Red)×Gold Delicious
8	Ralls	Edible	America, Unknown
9	Wangshanhong	Edible	Liaoning, China, Fuji, Bud sport
10	Maypole	Edible	England, Ornamental small apple
11	Braeburn	Edible	Japan, Fuji, Bud sport
12	Jinshayilamu	Edible	Xinjiang, China
13	Jonathan	Edible	America, Esopus Spitzenburg seedling
14	Yueping	Edible	Unknown
15	Laomu50	Edible	Unknown
16	Hanfu	Edible	Liaoning, China, Toko×Fuji
17	Beniaika	Edible	Japan, Fuji seedling
18	Dongxiang	Edible	Unknown
19	Red Chief Delicious	Edible	America, Delicious, Bud sport
20	Mollies Deli	Edible	America, Delicious×Edgewood
21	TengMu 1	Edible	America, Unknown
22	GrannySmith	Edible	Australia, Unknown
23	Unknow	Edible	Unknown
24-41	Y1-Y18	Wild	Xinjiang, China, clones
42	M9	Rootstock	England
43	M26	Rootstock	England, M9×M16
44	MM106	Rootstock	England, M1×Unknown
45	SH9	Rootstock	Ralls×Henanhaitang
46	SH38	Rootstock	Ralls×Henanhaitang
47	SH40	Rootstock	Ralls×Henanhaitang
48	CG24	Rootstock	M8×Unknown

SSR marker primer: SSR primer sequences were based on papers reported by Guilford *et al*.(1997), Hokanson *et al*.(1998, 2001), Gianfranceschi *et al*.(1998), and inquired on NCBI. Fifty pairs of SSR primers were selected from 17 chromosome linkage groups and through gel electrophoresis analysis. Thirty pairs of SSR primers with high polymorphism, clear band, sound repeatability, and even distribution were selected for experimental analysis. Primers were synthesized by Shanghai Sangon Biotech Engineering Company (shown in Table 2).

SSR-PCR: The PCR reaction was carried out in a 10 μL system. Approximately 8% polyacrylamide gel was used for electrophoresis. After silver staining Photos were taken and records were kept.

Table 2. Primer Selection

No	Primer	Repeats	Annealingtemperature Tm (°C)	ChromosomeLinkage group
1	HI02^{c}07	$(GA)_{29}$	57.8	LG1
2	CH02^{b}10	$(CT)_{19}$	55.1	LG2
3	CH02^{c}02^{a}	$(AG)_{27}$	56.6	LG2
4	CH03^{g}07	$(TC)_{12}$	54.5	LG3
5	MS14^{h}03	$(TC)_{24}$	59.6	LG3
6	CH02^{h}11^{a}	$(GA)_{14}$	57.8	LG4
7	CH04^{e}02	$(GA)_{10}$	57.8	LG4
8	CH03^{a}09	$(CT)_{21}$	61.9	LG5
9	CH05^{f}6	$(CT)_{17}$	60.1	LG5
10	CH03^{d}12	$(TC)_{22}$	58.2	LG6
11	CH03^{d}07	$(CT)_{20}$	52.2	LG6
12	CH02^{a}04	$(TC)_{18}$	55.8	LG7
13	CH04^{e}05	$(AG)_{19}$	56.3	LG7
14	CH01^{c}06	$(AG)_{30}$	57.8	LG8
15	CH01^{f}03^{b}	$(AG)_{24}$	55.8	LG9
16	CH01^{h}02	$(TC)_{17}$	57.8	LG9

17	CH02B03[b]	$(CT)_{22}$	57.1	LG10
18	CH04[g]07	$(TC)_{19}$	57.8	LG11
19	CH04[d]07	(AG)13	57.8	LG11
20	CH04[d]02	(CT)19	57.8	LG12
21	CH01F02	(AG)22	58.0	LG12
22	CH03[a]08	(CT)19	56.3	LG13
23	CH05[c]04	(CT)28	56.3	LG13
24	CH03[d]08	(TC)19	58.2	LG14
25	CH02[c]09	(CT)18	56.8	LG15
26	CH02[d]11	(AG)24	59.6	LG15
27	CH02[a]03	(TC)22	57.6	LG16
28	CH05[a]04	(GA)24	53.7	LG16
29	CH01[h]01	(TC)25	59.8	LG17
30	CH05[g]03	(TC)21	58.2	LG17

Statistical analysis: All statistics of binary data were collected based on the presence of a ladder. When a ladder exists, it will be denoted as 1; otherwise, it is denoted as 0. DPSv7.05 and EXCEL were adopted for data processing. Percentage of polymorphic loci of each group and primers (*p*), *Nei's* genetic diversity (*h*), *Shannon's* Index (*I*), and effective number of alleles (*Ne*) were collected. *Nei* and *Li*, the dissimilarity coefficient, were used for MPGMA cluster analysis using the group average method. The cluster dendrogram was then established. Cluster analysis was applied on each pair of primers; the correlation coefficient in pairs for dissimilarity coefficient matrix was then computed; thus, the correlation coefficient matrix was established. Finally, influences of differences between primers on cluster results were explored.

RESULTS

Genetic relationship analysis on *Malus* Mill variety and clones: As shown in Figure 1,all test samples were completely separated and clearly classified into three types. The first type included 23 edible apple varieties; the second included all 18 Xinjiang wild apple clones; and the third included 7 rootstock varieties. The tie line was at 0.32, which clustered the first and second types together. This finding indicated that the genetic relationship between wild apples and edible apples was closer. At the molecular level, the correlation between Xinjiang wild apple as the source of East Asia gene center and many edible varieties in origin and evolution was verified. However, rootstock apples were mostly filial generation of apple and *Malusspectabilis*, which differed a lot from the former two types of apple groups in gene structure. In the edible apple group, Maypole and Tengmu No.1 are separately clustered together and are separate from other Fuji apples. This finding indicated that the genetic relationship between varieties from different regions was far. Tsugaru (Gold Delicious× Jonathan), Yueshuai (Gold Delicious × Starking Red), and Sekaiichi (Gold Delicious × Marshal) are offsprings of Gold Delicious. Their cluster indicated that these varieties mostly inherited the genetic factors of Gold Delicious. Fuji series and relevant varieties, such as, Korin (Fuji × unknown), Wangshanhong (Fuji bud mutation), Hongjiangjun (Fuji bud mutation), Ralls (Ralls × Marshal → Fuji), Red Chief Delicious (Marshal bud mutation), and Hangu (Fuji × Toko), were closely clustered. Gala (Kidd's Orange Red × Gold Delicious) and Sansa (Gala × Jonathan), filial generation of Gala, were also closely clustered. M9 and M26 (M9 × M16) were clustered together. Three varieties of SH series (Ralls × Henan Malusspectabilis) were clustered together. The cluster results of this study were consistent with the origin of each variety and genetic relationship, indicating the reliability of the study results.

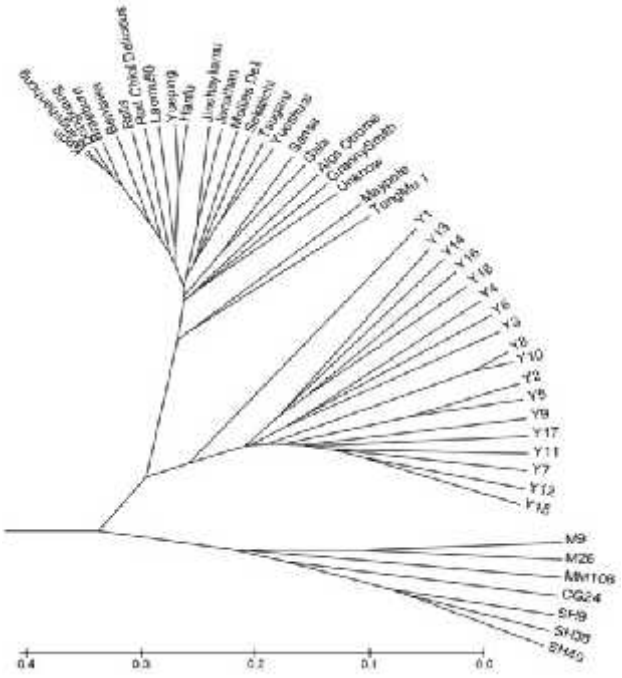

Fig. 1 Dendrogram of 48 apple varieties based on SSR data

Primer variety and representational difference analysis: Each pair of primers was used to carry out diversity analysis on 48 apple samples, calculate and compare the percentages of polymorphic loci (*p*), *Nei's* genetic diversity (*h*), *Shannon's* Index (*I*), and effective number of alleles (*Ne*). Results are shown in Table 3. The genetic diversity of the different primers varied and the numbers of polymorphic loci fell between 4 and 12; primer 1 was the maximum, whereas primers 6, 11, 18, 19, and 30 were 12. The variation range of effective number of alleles (*Ne*) was between 1.138 and 1.385; primer 9 was the maximum and primer 5 was the minimum. The variation range of *Nei's* genetic diversity (*h*) was between 0.111 and 0.246; primer 9 was the maximum and primer 5 was the minimum. *Shannon's*

Index (*I*) was between 0.206 and 0.339; primer 9 was the maximum and primer 5 was the minimum. The variation range of mean polymorphism information amount was between 0.347 and 0.843; primer 1 was the minimum and primer 6 was the maximum.

Table3. Genetic diversity parameter of 30 pairs of SSR primers

No.	LG	*p*	(*Ne*)	(*h*)	(*I*)	*PIC*	Correlation coefficient
1	1	4	1.235	0.170	0.288	0.347	0.506***
2	2	9	1.221	0.149	0.258	0.701	0.374***
3	2	10	1.264	0.166	0.270	0.748	0.547***
4	3	8	1.160	0.120	0.219	0.559	0.639***
5	3	7	1.138	0.111	0.206	0.508	0.281***
6	4	12	1.201	0.142	0.254	0.843	0.766***
7	4	7	1.338	0.201	0.325	0.508	0.837***
8	5	6	1.370	0.213	0.339	0.640	0.852***
9	5	5	1.385	0.246	0.391	0.650	0.337***
10	6	8	1.279	0.177	0.294	0.697	0.421***
11	6	12	1.331	0.207	0.332	0.831	0.385***
12	7	10	1.325	0.204	0.327	0.781	0.484***
13	7	8	1.295	0.210	0.351	0.814	0.530***
14	8	6	1.276	0.180	0.303	0.577	0.375***
15	9	10	1.232	0.160	0.279	0.783	0.657***
16	9	5	1.339	0.215	0.348	0.535	0.343***
17	10	9	1.288	0.202	0.337	0.822	0.532***
18	11	12	1.217	0.142	0.241	0.770	0.535***
19	11	12	1.163	0.123	0.225	0.828	0.536***
20	12	10	1.213	0.146	0.252	0.724	0.492***
21	12	6	1.362	0.228	0.370	0.690	0.669***
22	13	9	1.261	0.178	0.303	0.803	0.767***
23	13	8	1.262	0.170	0.283	0.673	0.457***
24	14	7	1.249	0.168	0.283	0.612	0.505***
25	15	8	1.308	0.196	0.312	0.726	0.715***
26	15	8	1.285	0.188	0.311	0.745	0.400***
27	16	6	1.355	0.218	0.348	0.642	0.773***
28	16	11	1.204	0.137	0.240	0.752	0.543***
29	17	9	1.254	0.161	0.268	0.724	0.603***
30	17	12	1.180	0.131	0.237	0.772	0.606***

Note: Correlation coefficient refers to the correlation between each pair of primers and the distance matrix of 48 cluster varieties and the ones between and the distance matrix of 30 pairs of separate cluster primers. *** refers to extremely significant correlation ($P<0.001$), n=1128 and $r_{\alpha}=0.097847$.

Thirty pairs of primers were used for cluster analysis on 48 apple samples solely; the cluster results presented a significant difference. As shown in Table 3, all primers reached extremely significant positive correlation. Primers 8 and primer 7 were the maximum and their correlation coefficients were 0.852 and 0.837, respectively; whereas primer 5 was the minimum, with correlation coefficient of 0.281.

Correlation relationship analysis between primers: Correlation analysis was carried out on the distance matrix of the 30 pairs of separately cluster primers to establish the correlation coefficient matrix. According to Euclidean distance, 30 pairs of primers were clustered based on the correlation coefficient matrix, as shown in Figure 2. According to amplification results, primers were classified into three types. The first one included primers 1, primer 4, and other 9 pairs of primers; the second one included primer 6, primer 7, and other 9 pairs of primers; and the third one included primer 21, primer 22, and other 6 pairs of primers.

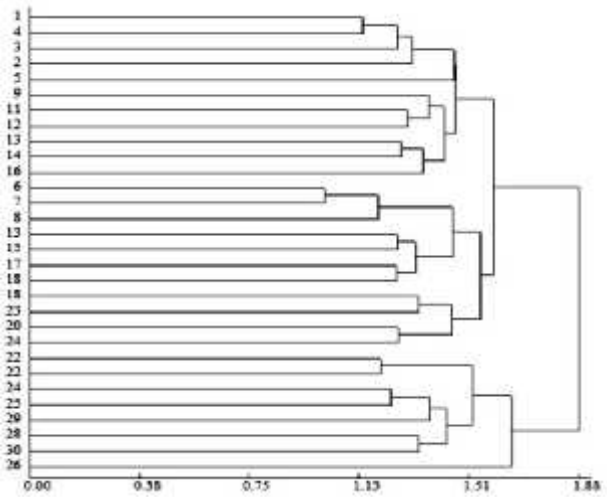

Fig. 2 Dendrogram of 30 pairs of SSR primers

Cluster results and correlation analysis of different primer clusters: According to cluster results of the primers (shown in Figure 2), three types of primers were used for clustering all 48 apple samples. The cluster results of these three primer groups can identify the three apple varieties. However, the cluster results of each apple group presented significant differences, which failed to accurately reflect the genetic relationship between varieties. Cluster analysis was applied on all varieties and three apple groups with 30 pairs of primers and three primer groups; correlation analysis was implemented on the distance matrix of their cluster results, as shown in Table 4. The second primer group had the highest similarity to all primers with integrated cluster results, with correlation coefficient of 0.957, followed by third primer group, with correlation coefficient of 0.934. The similarity of cluster results of the first primer group was the lowest, with correlation coefficient of 0.874. Among the cluster results of each apple group, cluster effects of the second primer group on rootstock apple and edible apple varieties were better. The first primer group had poor effects on three apple groups, especially rootstock apples, with correlation coefficient of only 0.427. The cluster effects of the first primer group on rootstock apple

varieties and wild apple clones were satisfactory. Thus, each primer group had their own optimal application range and different primers may have different cluster results.

Table 4. Correlation coefficient of three primer groups with all primers

Primer group	Edible apples	Xinjiang wild apple	Rootstock apples	All apples
First	0.781***	0.791***	0.901***	0.871***
Second	0.804***	0.696***	0.957***	0.957***
Third	0.732***	0.734***	0.427***	0.934***

Selection of high-efficient primer combination: According to the cluster analysis of primers, 2 pairs with high polymorphism and correlation from three primer groups for different combinations were selected, and a high efficiency primer combination was screened.

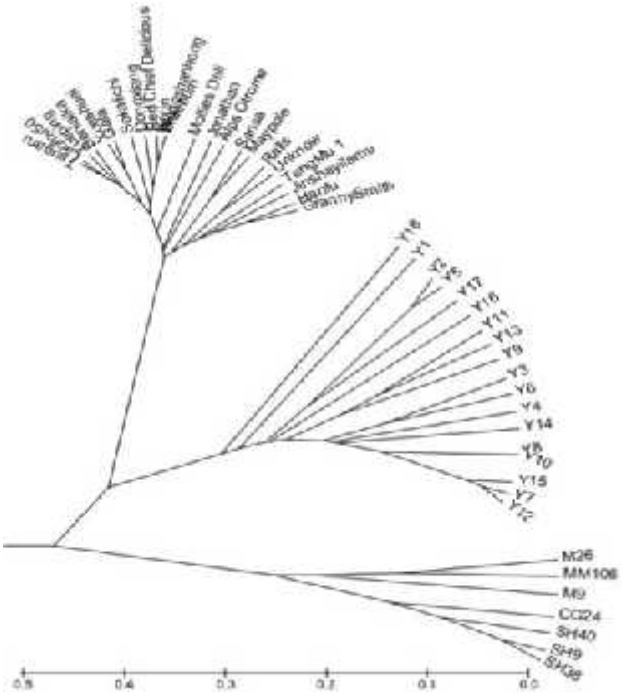

Fig. 3 Dendrogram of six pairs of efficient primers group

The cluster result of the combination of primers 3, 4, 6, 8, 22, and 27 was better (Figure 3), and can classify the three types of apples. Furthermore, most varieties in each group can be classified accurately. This primer combination has relatively high correlation with the integrated cluster results of all 30 pairs of primers, with correlation coefficient as high as 0.940 ($y=0.5934x+0.1940$，$n=1128$).

DISCUSSION

Numerous studies on apple genetic diversity and genetic relationship have been carried out using SSR markers (Hokanson *et al*.1998; Gianfranceschi *et al*. 1998; Hokanson *et al*. 2001;Dilworth *et al*. 2006; Song *et al*. 2006; Chen *et al*. 2007; Gharghani*et al*. 2009; Treuren *et al*. 2010). However, many researchers have disregarded the screening of SSR primers, took primer polymorphism as the sole criterion of primer screening, and seldom considered the amount and representativeness of primers. Significant differences on the efficiency and representativeness of primers have been found through series of comparative analysis on primers. For example, the effective number of alleles (*Ne*), *Nei's* genetic diversity (*h*), and *Shannon's* Index (*I)* of primer 9 are higher than those of the other primers; the representativeness of primers 8 and 7 are highest, whereas the representativeness of primer 5 is lowest. Different primers come from different parts of the chromosome and represent different genetic loci; thus, contents of carried information and reflected intervarietal differences varied. Therefore, some random combinations of different primers obtain different cluster results, which may lead to the offset and confusion of cluster results and the unconformity of origin or traditional classification that may further decrease the reliability of the results. Lu´ısGoulão *et al*. (2001) only adopted seven pairs of primers to study 41 apple varieties. Many varieties failed to separate among the cluster results. Hokanson *et al*. (1998) had a small quantity of primers and blindness of random selection, which had certain limits. Patzak *et al*. (2012) also indicated confusion on some of the results. Varieties with genetic relationship, including Fuji and Ralls, and Gala and Gold Delicious, were not clustered together in the study by Gao *et al*. (2007). Ralls and stark apple with genetic relationship did not cluster together in the study by Wang *et al*. (2005), and Chuqiu, Tsugaru, and Jonagold also failed to show close clusters. Unconformity of origin or traditional classification both occurred in this paper.

This study selected 30 pairs from 50 pairs located on 17 chromosomes and were evenly distributed on each chromosome. The cluster results can completely separate Xinjiang wild apple, edible apples, and rootstock apples without mutual interlace. Moreover, edible apple varieties can be divided according to the closeness of genetic relationship. Different series of rootstock also cluster together. These clusters conform to their origins and verify the reliability of this study. Thus, sufficient number, strong representativeness, even distribution, high polymorphism, and highly efficient primers can accurately reflect the genetic relationship between plants and further obtain accurate results.

Thirty pairs of primers were classified into three groups through the cluster analysis; the groups were used

to cluster each apple group and all varieties, respectively. Significant differences between cluster results were found; among which, the correlation between the second primer group and cluster results of all 30 pairs of primers of 0.957 is the maximum among the groups. Two pairs of primers were selected from three primer groups. Only six out of the 30 pairs of primers have 0.940 correlation with cluster results of all varieties, which proves that few highly efficient primer combinations can obtain well-clustered results. Thus, screening high-efficient SSR primers is important. Only through the primer combination with huge amount of carried information, strong representativeness, and an even distribution can the work of the test be decreased and the plant genetic relationship and genetic diversity be analyzed accurately and rapidly.

Acknowledgments: This research was supported by the Special Research Program for Social and Public Interests of State Forestry Administration of China (No. 201104039).

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