

Genetic Diversity of 41 Apple Rootstocks Based on Simple Sequence Repeat Markers

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ABSTRACT. Molecular markers are valuable tools in evaluating genetic diversity and fingerprinting plant germplasm. In this report, simple sequence repeat (SSR) markers were used for assessing genetic diversity in 41 dwarf and semidwarf and early flowering apple (*Malus* sp.) rootstocks. Sixty-two of 112 pairs of SSR primers generated multiple, scorable fragments. The total number of scored bands was 4138 with the polymorphic frequency ranging from 22.0% to 68.6% with a mean value of 58.5% in 737 alleles. The number of alleles per locus ranged from 6 to 19 with an average of 11.9 alleles. Polymorphic information content per locus was ranged from 0.176 to 0.885 with an average value of 0.606. These results suggested a complex genetic background and genetic diversity in these apple rootstocks. Based on three principal components and unweighted pair group mean average (UPGMA) of SSR data, the 41 apple rootstocks were divided into five groups. Group I contained *M. xiaojinensis* 'Xiaojinhaitang'. Group II consisted of *M. hupehensis* var. *pingyiensis* 'Pingyitiancha'. Group III contained *M. baccata* 'Shandingzi' and its offspring. Group IV was composed of 16 apple rootstocks, including Malling and Malling Merton series from Great Britain; 'Budagovski 9' from Russia; 'Polish 22' from Poland; 'Cornell-Geneva 24' from the United States; and 'GM.256', 'Nei Meng 11', 'MD.001', '7734', and '7848' from China. Group V consisted of 16 Shao series rootstocks, which were offspring of *M. honanensis* × *M. domestica* 'Ralls Genet'. This research suggests that the breeding can achieve best performance with more robust rootstock if crosses were performed among these five major groups of germplasms rather than within the major groups.

Apple (*Malus ×domestica*) is one of the major economically important fruit tree species in the world. The past 20 years saw rapid development of intensive and efficient cultivation technology in dwarf rootstocks in Europe, North America, Australia, and New Zealand, which leads to better global apple production industry. Similar trends have occurred in China in recent years (Fideghelli et al., 2003; Han et al., 2005). This cultivation technology bears many advantages such as early fruiting, easy tree management and machinery handling, labor-efficiency, high yielding, and high quality. Many dwarf and semidwarf apple rootstocks have been developed since the 1960s. For example, the Malling (M) and Malling Merton (MM) series were developed by the East Malling Experiment Station, U.K. (Kamboj et al., 1999); the Polish (P) series was selected by the Horticulture Institute, Poland (Zagaja, 1980); and the Jork (J) series from Germany (Kosina, 2002), the

Ottawa (O) series from Canada (Nelson, 1976), the Budagovski (B) series from Russia (Seemüller et al., 2008), and the Mark and Cornell-Geneva (CG) series from the United States (Norelli et al., 2003). Apple dwarf rootstock breeding has been steadily increasing since the introduction of M and MM series in the 1960s to 1980s from the United Kingdom. China is the largest apple production country, but its apple dwarf rootstocks are seldom known (Han et al., 2005). The M and MM series were used as parent materials of rootstock breeding. The apple dwarf rootstocks '7734' and '7848', which had cold and drought resistance and early flowers and fruit, were selected from M series (Li et al., 2000). The apple dwarf rootstocks 'NM.11', 'GM.256', and 'MD.001' had strong cold resistance and their lineages were thought to have M or MM heritage (Han et al., 2005). Wild apple species such as *M. baccata*, *M. hupehensis*, *M. xiaojinensis*, and *M. honanensis* were used as apple rootstocks or parent materials in China. For example, 'Shandingzi' (*M. baccata*) is found in Shanxi, Gansu, Liaoning, and Heilongjiang provinces in China and has good adaptability to cold and drought stresses. The dwarf rootstock Yang (Y) series originated from a seedling of 'Shandingzi' and they bear flowers and fruits early (Yang et al., 2006). *Malus honanensis* is a dwarf wild apple rootstock and used as rootstocks or parent material (Liu et al., 2006). The Shao (SH) series of dwarf and

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semidwarf rootstocks were mostly selected by cross-breeding from *M. honanensis* × *M. domestica* ‘Ralls Genet’ by Shanxi Academy of Agricultural Sciences in China (Shao et al., 1988, 1991). They had cold-hardiness, drought resistance, and early flowers and fruits in the scion cultivar. Other wild *Malus* species, which have been used in rootstock breeding, included the apomictic triploid ‘Pingyitiancha’ (*M. hupehensis* var. *pingyiensis*) and the apomictic tetraploid ‘Xiaojinhaitang’ (*M. xiaojinensis*) (Cheng et al., 2000; Shi et al., 2008; Wang et al., 2002, 2008). ‘Pingyitiancha’ is resistant to apple powdery mildew (*Podosphaera leucotrich*) and woolly apple aphid (*Eriosoma lanigerum*) (Wang et al., 2009). ‘Xiaojinhaitang’ has resistance to iron deficiency, low temperature, and drought stress (Cheng et al., 2000; Shi et al., 2008).

DNA molecular marker technology has been widely used to reveal the genetic variations among individuals, between

groups, and trace the genetic histories with a high degree of specificity and reliability (Benora et al., 2008; Cantini et al., 2008; Wang et al., 2011). SSR marker technology is an ideal molecular tool for analysis of genetic diversity because of its stability, transferability, ease of performance, and its locus richness (Benora et al., 2008; Liebhard et al., 2002; Silfverberg-Dilworth et al., 2006). SSR markers have been widely used in apple for cultivar fingerprinting, characterization of the genetic diversity, and preservation of core genetic resources (Iannaccone et al., 2007; Richards et al., 2009). Zhang et al. (2007) had characterized 109 *M. sieversii* accessions from four geographical populations located in Xinjiang province, China, by SSR markers and found that the genetic structure and diversity of populations in these ecogeographical populations were relatively independent. Oraguzie et al. (2005) used seven SSR markers to identify 66 apple rootstocks from different

Table 1. The apple rootstocks used in this study of genetic diversity.

Accession no.	Parentage	Country of origin	Specific characteristics
SH.1	<i>Malus honanensis</i> × <i>Malus</i> × <i>domestica</i>	China	Dwarf
SH.3	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Semidwarf
SH.6	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.9	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.12	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.17	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Semidwarf
SH.18	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.19	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.28	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Semidwarf
SH.29	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Semidwarf
SH.38	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Extreme dwarf
SH.39	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Extreme dwarf
SH.40	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Extreme dwarf
SH.41	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Extreme dwarf
SH.a	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.b	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.c	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.f	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
SH.g	<i>M. honanensis</i> × <i>M. xdomestica</i>	China	Dwarf
M.7	<i>Malus pumila</i>	UK	Semidwarf
M.9	<i>M. pumila</i> var. <i>paradisiaca</i>	UK	Dwarf
M.26	<i>M. pumila</i>	UK	Semidwarf
MM.106	<i>M. pumila</i>	UK	Semidwarf
Mark	M.9 seedling selection	U.S.	Extreme dwarf
GM.256	M.9 seedling selection	China	Dwarf
CG.24	unknown	U.S.	Semidwarf
B.9	M.9 seedling selection	Russia	Semidwarf, cold-resistant
P.22	M.9 seedling selection	Poland	Extreme dwarf
MD.001	unknown	China	Dwarf
NM.11	unknown	China	Dwarf
7848	M.9 seedling selection	China	Dwarf
7734	M.9 seedling selection	China	Semidwarf
Shandingzi	<i>Malus baccata</i>	China	Cold- and drought-resistant
Y.a003	‘Shandingzi’ seedling selection	China	Early flowering
Y.a017	‘Shandingzi’ seedling selection	China	Early flowering
Y.b009	‘Shandingzi’ seedling selection	China	Early flowering
Y.b029	‘Shandingzi’ seedling selection	China	Early flowering
Y.c002	‘Shandingzi’ seedling selection	China	Early flowering
Y.d002	‘Shandingzi’ seedling selection	China	Early flowering
Xiaojinhaitang	<i>Malus xiaojinensis</i>	China	Apomictic, iron deficiency-resistant
Pingyitiancha	<i>Malus hupehensis</i> var. <i>pingyiensis</i>	China	Apomictic

countries. The results showed that these apple rootstocks could be differentiated effectively as a result of the high level of genetic diversity.

In this study, SSR molecular markers were used for fingerprinting the 41 apple rootstocks to 1) reveal genetic diversity and relatedness of rootstocks originated from Europe, North America, and less characterized ones from China; and to 2) provide a baseline for making full use of diverse apple rootstocks in breeding of advanced apple rootstocks.

Materials and Methods

PLANT MATERIALS, DNA EXTRACTION, AND AMPLIFICATION. A total of 41 apple rootstocks were analyzed in this experiment. They were collected from the germplasm centers of Shanxi Academy of Agricultural Sciences, Taigu, China, Changping Fruit Research Institute, Beijing, China, and Institute of Forestry and Pomology, Beijing Academy of Agricultural and Forestry Sciences, Beijing, China. Most of them were dwarf or semidwarf rootstocks. Their genetic background, country of origin, and specific characteristics are listed in Table 1.

Total genomic DNA was extracted from 250 mg fresh young leaves using the CTAB protocol described by Thomas et al. (1993). A total of 112 pairs of SSR primers were synthesized by Sangon (Shanghai) Co. (Shanghai, China) according to the sequences reported by Liebhard et al. (2002) and Silfverberg-Dilworth et al. (2006). The amplification reactions were conducted in a polymerase chain reaction (PCR) thermocycler (Techne TC312; Bibby Scientific, Stone, U.K.) in 20- μ L volume containing 2 μ L of $10 \times$ buffer, 2 μ L of Mg^{2+} (25 mmol), 1 μ L of dNTPs (10 mmol), 1 μ L of each primer (10 pmol), 2.5 U of *Taq* polymerase [Sangon (Shanghai) Co.], and 3 μ L of genomic DNA (100 ng). The PCR amplification conditions were: one cycle at 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 50–62 °C for 30 s, 72 °C for 30 s, and a final cycle at 72 °C for 10 min. After amplification, 4 μ L of PCR products were loaded in a 6% polyacrylamide gel. Gel staining was performed with silver nitrate as described by Xu et al. (2006).

DATA ANALYSIS. The products of SSR fragments visualized in the gel were recorded depending on the presence (scored as 1) or absence (scored as 0) of bands to obtain a binary similarity matrix calculated using the DICE coefficient formula (Wang et al., 2006). The polymorphic SSR bands generated by viable 62 primer pairs were calculated for polymorphism information content (PIC) based on Hurtado's formula, which measures gene diversity (Hurtado et al., 2008). The formula is: $PIC = 1 - \sum_{i=1}^n f_i^2$ where f_i is the frequency of the i^{th} allele. Meanwhile, principal component analysis (PCA) was conducted based on Zhao et al. (2007), which is a metric multidimensional classical scaling method. A clustering analysis was performed using the UPGMA method and the software NTSYS-pc Version 2.1 (Esselink et al., 2003) was used to generate the dendrogram.

Results and Discussion

POLYMERASE CHAIN REACTION AMPLIFICATION. Of the 112 SSR primer pairs, 62 primer pairs amplified discrete polymorphic bands with the fragment sizes ranging from 100 to 300 bp as reported by Liebhard et al. (2002) and Silfverberg-Dilworth et al. (2006), indicating the success of the SSR reactions with reliable data for statistical analysis (an example gel is shown in Fig. 1). For a specific SSR primer pair, genetically related individuals sharing the same band type or a similar SSR band pattern were generated among the materials that shared a similar genetic background, whereas different SSR band patterns were generally produced between those rootstocks bearing different genetic backgrounds.

POLYMORPHISM ANALYSIS OF SIMPLE SEQUENCE REPEAT MARKERS. The 62 pairs of SSR primers yielded a total of 4138 discrete bands. A total of 737 alleles were identified, averaging 11.9 alleles per locus. The number of alleles for each SSR locus varied from 6 to 19. The polymorphic frequency of the 62 SSR primer pairs ranged from 22.0% to 68.6% with an average of 58.5%. The SSR locus CH2h11a had the highest PIC value of 0.885, and the SSR locus CH04c03 had the lowest PIC value of 0.176. The distribution of PIC value of 62 SSR loci is shown in Figure 2 with the average PIC value being 0.606.

ANALYSIS OF THE GENETIC DIVERSITY OF 41 APPLE ROOTSTOCKS. With PCA analysis of the SSR data from 41 apple rootstocks, the first principal component contributed 33.1% to the variation; the second contributed 16.1%; and the third 8.9%. The total of the three principal components was 58.1% of the genetic diversity. Based on the PCA distribution map of these three principal components (Fig. 3), the 41 apple rootstocks were broadly classified into five groups, namely *M. xiaojinensis*, *M. hupehensis*, *M. baccata* and its offspring, SH series, and M and MM series. Furthermore, the PCA distribution map showed that rootstocks in the SH series class distribution were relatively concentrated, suggesting relatively small genetic variations among this group; the distribution of apple rootstocks was quite dispersed within 'Shandingzi' and its offspring as well as in M and MM series, suggesting relatively large genetic variations among those rootstock groups.

The results of the SSR data analyzed using NTSYS software show a similarity coefficient of 0.36, and based on the results, the 41 accessions were divided into five major groups

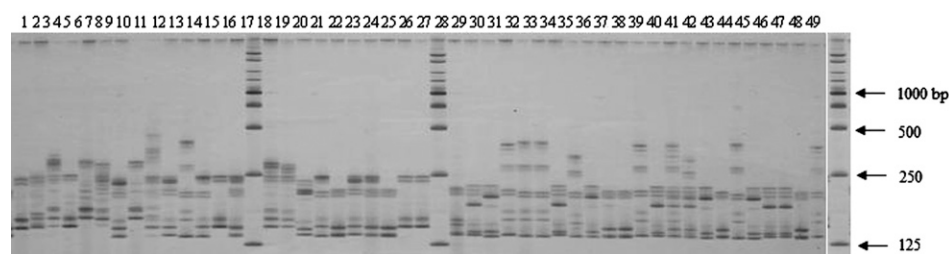


Fig. 1. Simple sequence repeat band pattern of the 41 apple rootstocks generated by the primer pair CH04d02. Lanes 1 to 49: (1) 'Pingyitiancha', (2) 'Xiaojinhaitang', (3) 'Y.d002', (4) 'Y.c002', (5) 'Y.b029', (6) 'Y.b009', (7) 'Y.a017', (8) 'Shandingzi', (9) 'Y.a003', (10) 'Shandingzi' (repeat), (11) '7734', (12) '7848', (13) 'NM.11', (14) 'MD.001', (15) DNA marker, (16) 'P.22', (17) 'B.9', (18) 'CG.24', (19) 'GM.256', (20) 'Mark', (21) 'MM.106', (22) 'M.26', (23) 'M.9', (24) 'M.7', (25) 'M.7' (repeat), (26) DNA marker, (27) 'SH.g', (28) 'SH.f', (29) 'SH.c', (30) 'SH.b', (31) 'SH.a', (32) 'SH.a' (repeat), (33) 'SH.41', (34) 'SH.40', (35) 'SH.39', (36) 'SH.38', (37) 'SH.3' (repeat), (38) 'SH.1' (repeat), (39) 'SH.6' (repeat), (40) 'SH.29', (41) 'SH.28', (42) 'SH.19', (43) 'SH.18', (44) 'SH.17', (45) 'SH.12', (46) 'SH.9', (47) 'SH.6', (48) 'SH.3', and (49) 'SH.1'.

(Fig. 4). Group I consisted of ‘Xiaojinhaitang’; Group II consisted of ‘Pingyitiancha’; Group III included seven accessions ‘Shandingzi’, in which ‘Y.a003’, ‘Y.a017’, ‘Y.b009’, ‘Y.b029’, ‘Y.c002’, and ‘Y.d002’ were seedling selections despite relatively divergent male parentage. Group IV contained 16 accessions, all of which were derived from M or MM series. These included M and MM series from the United Kingdom; ‘B.9’ from Russia;

‘P.22’ from Poland; ‘CG.24’ from the United States as well as Chinese rootstocks ‘GM.256’, ‘NM.11’, ‘MD.001’, ‘7734’, and ‘7848’. In addition, ‘SH.3’, ‘SH.18’, and ‘SH.39’ of SH series were also placed in this group. ‘SH.3’, ‘SH.18’, and ‘SH.39’ may have been mislabeled in the process of production or distribution (Jin et al., 2010). Group V was comprised of 16 accessions of most of the SH series. The genetic similarity coefficient in the group was between 0.51 and 0.95. Some had higher genetic similarity such as ‘SH.12’ with ‘SH.29’ and ‘SH.9’ with ‘SH.19’ with a similarity coefficient of 0.95 and 0.94, respectively. Those rootstocks that were classified into Group V have high genetic similarity, in general.

The choice of SSR primers is a vital step for effective amplification among samples with diverse genetic background. Wang et al. (2005) screened 10 of 20 SSR primer pairs for effective identification of 25 apple cultivars. Based on 12 of 20 SSR primer pairs, Gao et al. (2007) divided 59 *Malus* taxa into three large clusters, which were consistent with their traditional pedigrees. However, both reports used a limited number of markers and the coverage of these markers in the genome is limited; therefore, they may not fully represent the genetic variation at the whole genome level. In this work, we evaluated 112 SSR markers and found 62 of them yielded polymorphic bands. These 62 SSR markers are well distributed over all 17 linkage groups (Celton et al., 2009; Liebhard et al., 2003; N’Diaye et al., 2008); therefore, they are quality markers for assessing the genetic diversities. The 41 rootstock taxa used in this work are mostly dwarf and semidwarf, and this is the first report to analyze the collection of dwarf and semidwarf rootstocks. The high average PIC value 0.606 of SSR markers also confirms that the SSR markers contain a large amount of genetic information.

Based on both PCA and UPGMA analysis on the 62 polymorphic markers, the 41 apple rootstocks can be classified into five groups, which are also in agreement with the variation of their different general backgrounds such as ‘Xiaojinhaitang’, ‘Pingyitiancha’, and ‘Shandingzi’. The apple rootstocks ‘Mark’, ‘B.9’, ‘P.22’, ‘CG.24’, ‘GM.256’, ‘NM.11’, ‘MD.001’, ‘7734’, and ‘7848’ were selected from crosses of M or MM series, which were selected from *M. pumila*. The SH series of rootstocks were mostly derived from *M. honanensis* and *M. domestica* ‘Ralls Genet’. The three rootstocks originally labeled ‘SH.3’, ‘SH.18’, and ‘SH.39’ have been placed in Group IV (the M and MM series) rather than in Group V (*M. honanensis* and *M. domestica* ‘Ralls Genet’), confirming earlier suspicions that they might have been mislabeled in the process of production or distribution (Jin et al., 2010).

The genetic analysis of these 41 apple rootstocks can be applied for further breeding of dwarf and semidwarf rootstocks to improve the apple industry in various counties. Also, the breeding can achieve best performance with more robust rootstock if crosses were performed among these five major groups of germplasms rather than within the major groups.

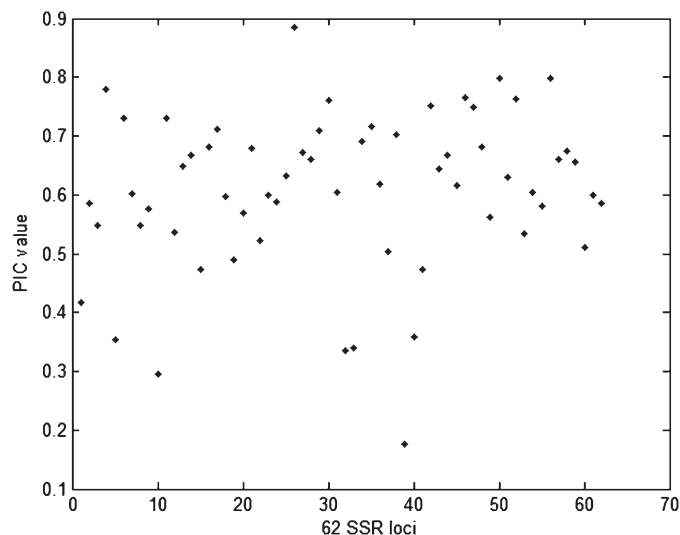


Fig. 2. The distribution of apple polymorphic information content of 62 simple sequence repeat (SSR) loci. The highest polymorphism information content (PIC) value was 0.885 and the lowest PIC value was 0.176, and the average PIC value of 62 SSR loci was 0.606.

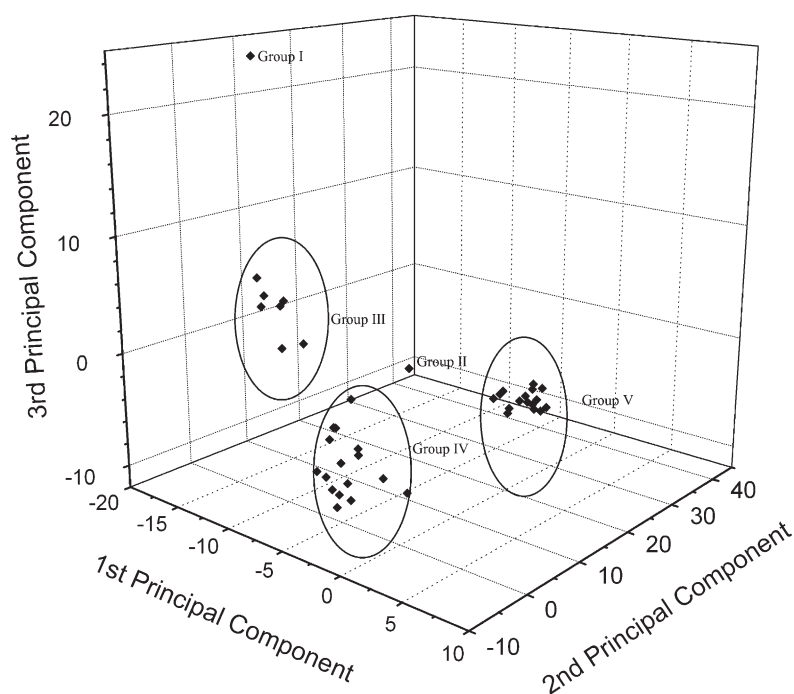


Fig. 3. Distribution of the first, the second, and the third principal component of simple sequence repeat data were from the 41 apple rootstocks. In the principal component analysis (PCA) distribution map, the 41 apple rootstocks were divided into five groups. Group I and Group II contained one accession separately. Group III contained seven apple rootstocks. Group IV was composed of 16 apple rootstocks and Group V consisted of 16 rootstocks.

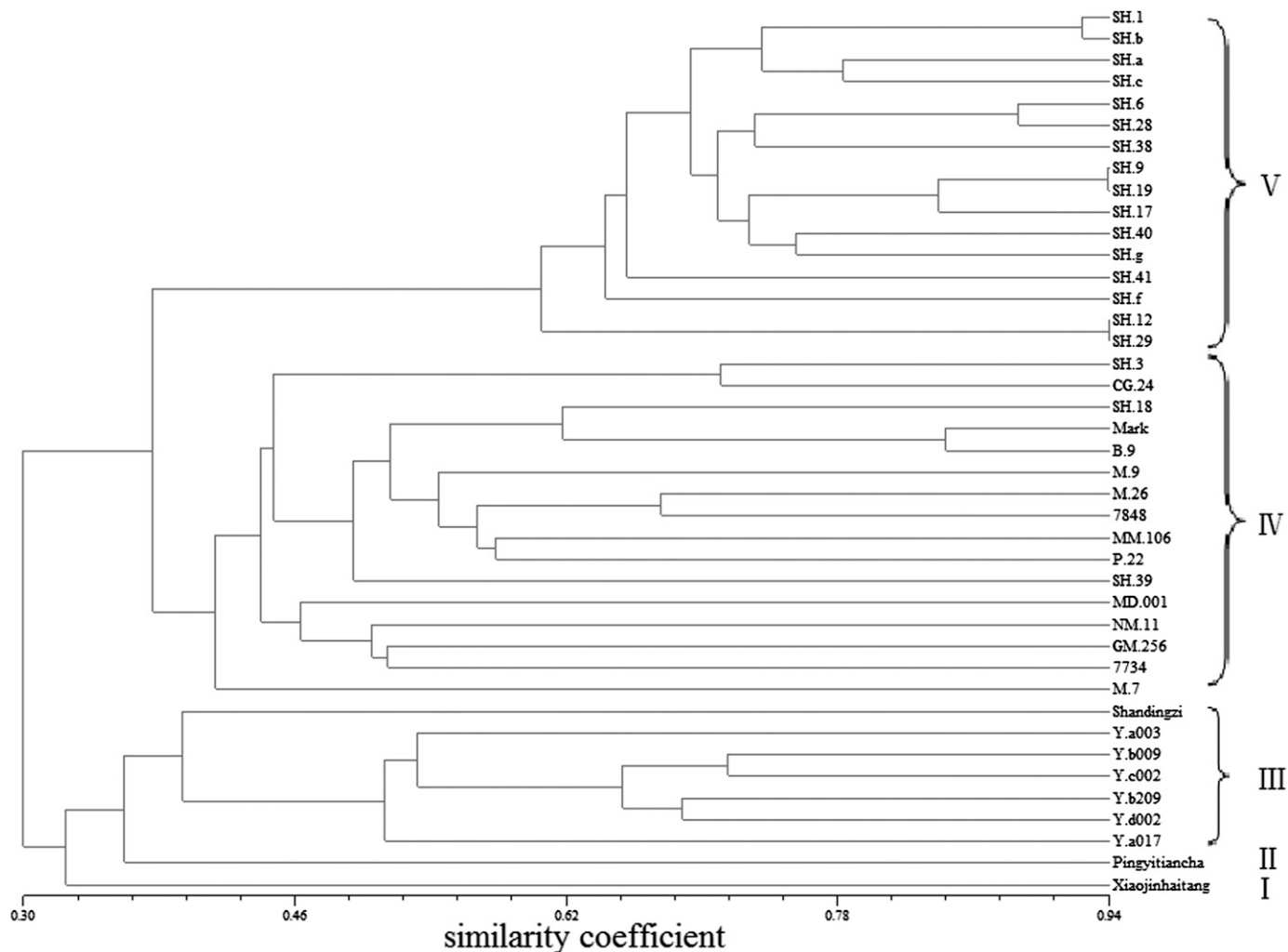


Fig. 4. The cluster dendrogram of the 41 apple rootstocks using unweighted pair group mean average (UPGMA) analysis of simple sequence repeat markers data. The 41 accessions were divided into five major groups in the value 0.36 of similarity coefficient. Group I composed of 'Xiaojinhaitang'. Group II consisted of 'Pingyitiancha'. Group III included seven accessions 'Shandingzi' and its offspring. Group IV contained 16 accessions, including Maling (M) and Malling Merton (MM) series, 'B.9', 'P.22', 'CG.24', 'GM.256', 'NM.11', 'MD.001', '7734', '7848', 'SH3', 'SH18', and 'SH39'. Group V comprised 16 accessions of most of the Shao (SH) series.

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