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Full Length Research Paper

Genetic analysis of wild apple resources in Shandong province based on inter-simple sequence repeats (ISSR) and sequence-specific amplification polymorphism (S-SAP) markers

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Apple (*malus domestica* Borkh.), which is a widely cultivated, important and economic fruit crop with nutritive and medicinal importance, has emerged as a model horticultural crop in this post-genomic era. Wild apple resources are important and they develop gradually in apple industry and genetic diversity. In this study, two molecular markers (inter-simple sequence repeats, ISSR and sequence-specific amplification polymorphism, S-SAP) were evaluated for genetic analysis of 31 wild apple resources in Shandong. A total of 20 ISSR primers were used and 110 polymorphic bands were amplified. Five S-SAP primer sets yielded a total of 496 bands, of which 201 (40.52%) were polymorphic. The similarity coefficient between resources ranged from 0.70 to 0.94 for ISSR analysis and from 0.66 to 0.95 using the S-SAP methodology. This study indicates that the results obtained based on the dendrograms constructed using unweighted pair-group using arithmetic average (UPGMA) cluster analysis were significantly correlated. The ISSR and S-SAP markers were found to be useful for wild apple resources identification and assessment of phonetic relationships.

Key words: Apple, wild resource, inter-simple sequence repeats (ISSR), sequence-specific amplification polymorphism (S-SAP).

INTRODUCTION

Apple is an important economic fruit crop widely cultivated in temperate and sub-tropical climate. It belongs to the rose family (Rosaceae) of order Rosales and class Magnoliopsida (Bhatti and Jha, 2010). The cultivated apple is *Malus domestica*, while its wild relatives are *Malus sieversii* and *Malus sylvestris* (Coart et al., 2006). With the ever-growing requirements for environmental protection and food safety in the production of high quality apples, the modern apple breeding becomes more and more dependent on resistant gene resources from the wild genetic resources in the genus (Crosby et al., 1992). Therefore, information on the origin of apple and its phylogenetic relationship with the closely related species will become more and more important for future apple industry.

Several molecular markers studies on apple have been published using techniques such as restriction fragment length polymorphism (RFLP) (Nybom and Schaal, 1990; Watillon et al., 1991), random amplified polymorphic DNA (RAPD) (Koller et al., 1993; Dunemann et al., 1994;

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Abbreviations: AFLP, Amplified fragment length polymorphism; **ISSR,** inter-simple sequence repeats; **LTR,** long terminal repeat; **PCR,** polymerase chain reaction; **RAPD,** random amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **S-SAP,** sequence-specific amplification polymorphism; **SSR,** simple sequence repeats.

Gardiner et al., 1996; Goulão and Oliveira, 2001), AFLP (amplified fragment length polymorphism) (Goulão and Oliveira, 2001) and SSR (simple sequence repeats) (Guilford et al., 1997; Gianfranceschi et al., 1998; Goulão and Oliveira, 2001), ISSR (inter-simple sequence repeats) (Goulão and Oliveira, 2001).

Many different marker types are available and markers based on insertional polymorphism of transposable elements have become an increasingly popular option. LTR-retrotransposons provide particularly useful markers for the analysis of polymorphism between individuals (Waugh et al., 1997; Ellis et al., 1998; Porceddu et al., 2002; Tam et al., 2005) and their insertions are irreversible and therefore stable over millions of years (Schulman et al., 2004; Vitte et al., 2004; Jing et al., 2005). The sequence-specific amplification polymorphism (S-SAP) approach is one of the most popular transposon-based molecular marker methods presently (Waugh et al., 1997; Schulman et al., 2004). S-SAP is an anchored PCR approach derived from AFLP (Vos et al., 1995), which amplifies the region between a transposon insertion and an adjacent restriction site. The factor limiting the deployment of S-SAP technology to new crops has been the need for sequence information from the LTR terminal region of the mobile element, in order to design the primers required for the method (Waugh et al., 1997). This obstacle has been reduced considerably by the development of rapid and efficient methods for accessing LTR terminal sequences by direct experiment (Pearce et al., 1999) or by searching the increasing amount of DNA sequence information available in genomic databases. In the last several years, S-SAP markers have been developed and applied for linkage and diversity analysis in barley (Gribbon et al., 1999), pea (Ellis et al., 1998), oat (Yu and Wise, 2000), maize (Casa et al., 2000), medicago (Porceddu et al., 2002), wheat (Queen et al., 2004), apple (Venturi et al., 2006; Zhao et al., 2010) and vicia (Sanz et al., 2007).

It is believed to have originated from central Asia and from there spread to the rest of the world (Harris et al., 2002). China is one of the largest centre of origin and genetic diversity of genus *Malus* in the world (Jiang, 1986; Li, 1989). It was observed that the first and second largest annual yield and cultivated area of apple were from Shandong province respectively in China. A large number of apple accessions were found in Shandong province. The purpose of this work was to find new evidence at the molecular level for the origin and evolution of apple accessions in Shandong province and to infer the phylogenetic relationships among the closely related species of apple accessions in Shandong province by ranking the rich genetic resources.

MATERIALS AND METHODS

A diverse set of 31 wild apple accessions were selected for genetic diversity study from Shandong province (Table 1).

DNA extraction

Total genomic DNA was extracted from fresh leaves following a modified CTAB method (Ma et al., 2008). DNA quality was checked by 0.8% agarose gel electrophoresis. The purified DNA was quantified at 260 nm using a spectrophotometer, diluted to a working concentration of 100 ng/µL in sterile water and stored at -20°C.

ISSR analysis

A total of 20 primers were used to screen for polymorphisms in 31 wild apple resources from Shandong province (primer sequences) (Table 2). ISSR analyses were carried out in 20 µl volume containing 1 U *Taq* DNA polymerase (Tiangen, China), 10 x Buffer, 50 ng of genomic DNA, 200 µM dNTPs, 10 pM of each oligonucleotide primer. DNA amplification was performed in a PTC-200 DNA Engine Tetrad (MJ Research) under the following conditions: one step of 94°C for 30 min; 38 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 1 min; a final step of 72°C for 10 min. The reaction products were separated on 1.5% agarose gels and the bands were visualized with ethidium bromide.

S-SAP analysis

LTRs used in this study were from CTcrm1 (GenBank accession no. FJ705357) and CTcrm2 (GenBank accession no. FJ705356) retrotransposons (Zhao et al., 2010). The LTR primer sequences employed were ACGAGGAGGGAATTCTAACAA (LTR1) and ATGGGCATTTAATTTCACGAG (LTR2) (in direction 5'-3').

The S-SAP procedure was essentially performed as described by Waugh et al. (1997) with the following modifications. All restriction digestions and ligation were performed using NEB buffers and enzymes. Between 300 and 500 ng genomic DNA was digested overnight with the EcoR1 and Mse1 restriction enzyme at 37°C in a final volume of 25 µl. The digested DNA samples were incubated at 80°C for 20 min to inactivate the restriction enzymes. This was followed by the ligation of adapters (EcoRI: 5′-CTCGTAGACTGCGTACC-3′ and 5′-AATTGGTACGCAGTCTAC-3′; MseI: 5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) to the restriction fragments. Ligations were performed for 3 h at room temperature in a final volume of 32 µl with 1× NEB T4 ligation buffer, 25 µl digested DNA, 50 pM EcoR1 and Mse1 adapters and 200 U T4 DNA ligase (NEB). The ligation mixture was again incubated at 65°C for 10 min to inactivate the enzyme. The adapter-ligated DNA was used for pre-amplification with a primer pair (EcoRI: GACTGCGTACCAATTC; MseI: GATGAGTCCTGAGTAA) based on the sequences of the EcoR1 and Mse1 adapters with no selective nucleotides, in a final volume of 25 µl containing 10 pM of each primer, 200 µM dNTPs, 1 U *Taq* DNA polymerase (Tiangen, China) and 5 µl ten-fold diluted (digested and ligated) DNA in a PTC-200 DNA Engine Tetrad (MJ Research) at 95°C for 1 min followed by 25 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for min, with a final extension of 7 min at 72°C.

Selective amplification was performed with primer pairs containing two or three selective nucleotides on the adapter primer (EcoRI or MseI) and one retrotransposon-based primer and to LTR2, EcoRI-agg and MseI-agc all produced high quality and reliable banding patterns (Figure 2). So, these primers combination were selected for the final S-SAP analysis in 31 wild apple accessions. Example of S-SAP silver staining result of these retrotransposon primers combinations, with LTR1/EcoRI-act for comparison, is shown in Figure 3, and the polymorphism data were summarized in Table 2. Five primer pair combinations generated 496 amplified bands, of which 201 were polymorphic between accessions, and approximately 40.52% (Table 3). In all, 496

Table 1. Wild apple accessions used for ISSR and S-SAP analysis.

Group	Number	Name
M. mricromalus Makino	1	Zibohaitang
	2	Laiwuhuahong
	3	Zibochangbasuan
	4	Zibofengukzhi
	5	Zibohongmiguo
	6	Zibofukui
	7	Yishuihaitang
	8	Yishuifukui
	9	Yishuibanzi
	10	Yishuiqiufengmi
	11	Laiwuchaguozhi
	12	Laiwuhongguozhi
	13	Laiwunanyan
	14	Zibodongchaguo
	15	Yishichaguo
	16	Ziboaobuqihaitang
	17	Ziboyizhuomao
	18	Laiwuxifuhaitang
	19	Yishuidahuahong
	20	Yishuiwukou
	21	Yishuiyiwufeng
	22	Yishuixiaohuahong
	23	*Malus robusta* (carr.) Rehd.
M. hupehensis (Pamp.) Rehd.	24	Mengshantiancha1#
	25	Mengshantiancha2#
	26	Mengshantiancha3#
	27	Mengshantiancha4#
	28	*Malus hupehensis* var. pingyiensis
	29	Qingzhen
	30	*Malus hupehensis* var. taishanensis
M. baccata (L.) Borkh.	31	*M. baccata* (L.) Borkh.

amplification products were scored and the relationship tree deduced from these data is shown in Figure 4. The results reveal that 31 wild apple accessions from Shandong province were grouped into four cluster based on cutoff value of the average similarity at 0.76. Cluster I included 21 *M. mricromalus* Makino accessions except for Yishuixiaohuahong and *Malus robusta* (carr.) Rehd. Cluster II was comprised of Yishuixiaohuahong and *Malus robusta* (carr.) Rehd. Cluster III consisted of seven *M. hupehensis* (Pamp.) Rehd. accessions. Cluster IV included *M. baccata* (L.) Borkh..

Similarity matrices were calculated independently from ISSR and S-SAP data (Figure 5). The cophenetic matrices compared with the original similarity data showed significant correlation of 73.12% (p = 1.0000, t = 11.7153) in both cases, and a good fit of the cluster analyses was performed.

DISCUSSION

Assessment of the genetic analysis within a cultivated crop had important consequences in plant production and the conservation of genetic resources. It is particularly useful in the characterization of individual accessions and

Table 2. Primers used for ISSR and S-SAP analysis and number of amplified products scored per primer.

ISSR primer	Number of scored band	Number of polymorphic band
TATATATATATATATAG	8	5
GAGAGAGAGAGAGAGAT	10	4
CTCTCTCTCTCTCTCTG	8	3
GTGTGTGTGTGTGTGTA	12	4
TCTCTCTCTCTCTCTCC	13	7
ACACACACACACACACG	4	3
ATATATATATATATAYC	11	6
AGAGAGAGAGAGAGAGYC	9	4
TATATATATATATATARC	12	8
GAGAGAGAGAGAGAGAYC	8	2
CTCTCTCTCTCTCTCTRG	10	6
GTGTGTGTGTGTGTGTYC	15	10
TGTGTGTGTGTGTGTGA	13	8
TCTCTCTCTCTCTCTCRT	9	6
ACACACACACACACACYG	7	3
TGTGTGTGTGTGTGTGRA	12	9
AGTAGTAGTAGTAGTAGT	8	4
CTCCTCCTCCTCCTCCTC	10	6
TGCTCGTGCTGCTGCTGC	6	2
AGAGTTGGTAGCTCGTGATC	12	9
S-SAP primer set		
LTR1/EcoRI-act	108	45
LTR1/EcoRI-acc	76	52
LTR2/EcoRI-agg	113	44
LTR1/MseI-tca	92	36
LTR2/MseI-agc	107	24

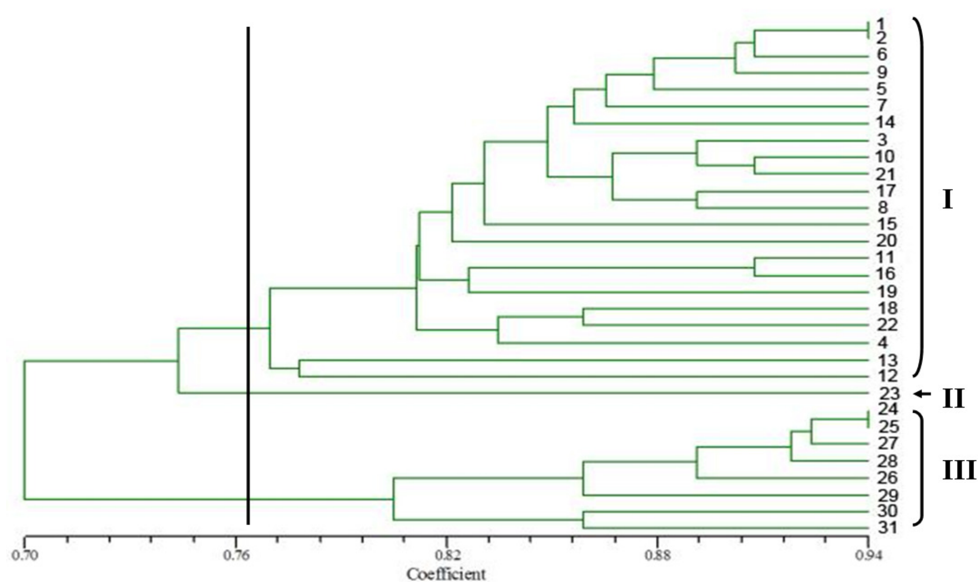

Figure 1. Cluster analysis of 31 wild apple accessions based on polymorphic bands obtained with ISSR marker.

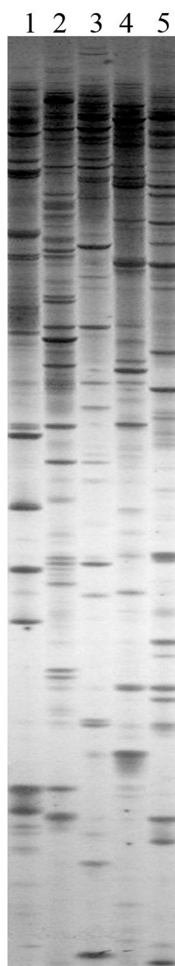

Figure 2. S-SAP marker optimizes primer pairs. Lanes 1-5 correspond to primer pairs LTR1/EcoRI-act, LTR1/EcoRI-acc, LTR1/MseI-tca, LTR2/EcoRI-agg and LTR2/MseI-agc, respectively.

cultivars, in detecting duplications of genetic material in germplasm collections, and as a general guide in the choice of parents for breeding hybrids (Goulão and Oliveira, 2001). Several molecular markers were widely used for genetic analysis assessment and cultivar identification in a large number of species (Loerz and Wenzel, 2004). In this study, we reported the use of ISSR and S-SAP for wild apple accessions genetic analysis.

Initially, the level of polymorphism and discrimination capacity was estimated. Our study demonstrates that accessions from *M. mricromalus* Makino and *M. hupehensis* (Pamp.) Rehd. groups were clustered into genotypes. Both ISSR and S-SAP marker methods were positively correlated and only slight differences were detected in some of the closer branches of the dendrograms constructed by cluster analysis.

Many different marker types are available and markers based on insertional polymorphism of transposable elements have become an increasingly popular option. LTR-retrotransposons provide particularly useful markers for the analysis of polymorphism between individuals (Waugh et al., 1997; Ellis et al., 1998; Porceddu et al., 2002; Tam et al., 2005) and their insertions are irreversible and therefore stable over millions of years (Schulman et al., 2004; Vitte et al., 2004; Jing et al., 2005). The S-SAP approach is one of the most popular transposon-based molecular marker methods presently (Waugh et al., 1997; Schulman et al., 2004). S-SAP-based polymerphism may result from transpositional activity of retroelements and/or a restriction site polymorphism, as is the case for AFLP and some restriction fragment length polymorphisms. The ideal marker method should access a very large number of polymorphisms which are broadly distributed within the genome under analysis. Retrotransposon-based S-SAP markers fulfill the criteria very well. The data quality for S-SAP closely resembles that of AFLP. One advantage of S-SAP over AFLP is that higher levels of polymorphism are accessible (Waugh et al., 1997; Ellis et al., 1998). Another advantage of S-SAP over AFLP is that it per-forms better in the analysis of genetic diversity. Retro-transposon markers generate taxonomic data that are more consistent with geographical and morphological criteria than AFLP-based markers (Ellis et al., 1998).

S-SAP was found to be formative in revealing genetic variations both at the intra- and inter-cultivar levels. Although, the exact sources of this genetic variation are difficult to predict, a multitude of factors may be responsible for maintaining intra- and inter-cultivar variation. These may include breeding practices, spontaneous mutations and induction of transposition system. The ubiquitous nature of retrotransposons in plant genomes (Flavell et al., 1992; Kumar and Bennetzen, 1999; Suoniemi et al., 1999) and their broad genomic distribution makes retrotransposons-based markers ideal tools for a wide range of applications in plants, including measurements of biodiversity, genome evolution, linkage analysis and mapping. Once a retrotransposon is stably integrated into a genomic location, it behaves in a Mendelian fashion. Therefore, integration sites shared between two plants are likely to have been present in their last common ancestor (Soleimani et al., 2005). Here, 31 wild apple accessions were reasonably differentiated using S-SAP marker based on five primer pair combinations. The results obtained with S-SAP demon-

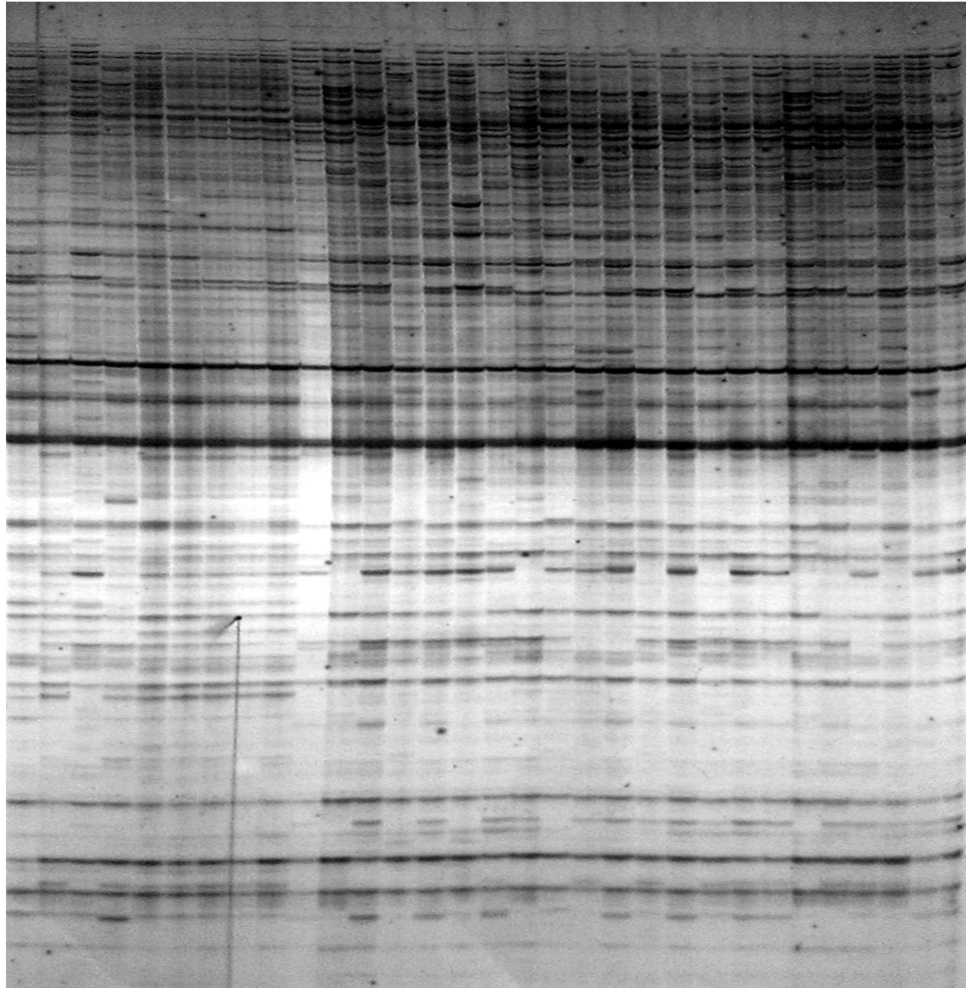

Figure 3. S-SAP marker profiles for LTR1/ EcoRI-act primer pairs in a diverse set of 31 wild apple accessions DNA. Lanes from left to right correspond to plant samples 1 to 31 respectively, in Table 1.

Table 3. Comparison of the results obtained with the ISSR and S-SAP methodologies.

PCR methodology	ISSR	S-SAP
Number of primer set/primer used	20	5
Total number of bands scored	199	496
Number of bands per primer set/primer	9.95	99.2
Number of polymorphic bands	110 (55.28%)	201 (40.52%)
Range in DNA diversity (% similarity)	0.70-0.94	0.66-0.95

strate the reliability of ISSR and S-SAP markers and potential use of both markers in genome assessments for fingerprinting, mapping and diversity study. So, use of retrotransposon-based markers can be a valuable tool for apple breeders. The S-SAP technique can be used for a more complete genome study.

In conclusion, wild apple accessions of Shandong were successfully developed with genetic analysis based on ISSR and S-SAP markers, which are useful in apple industry and genetic diversity.

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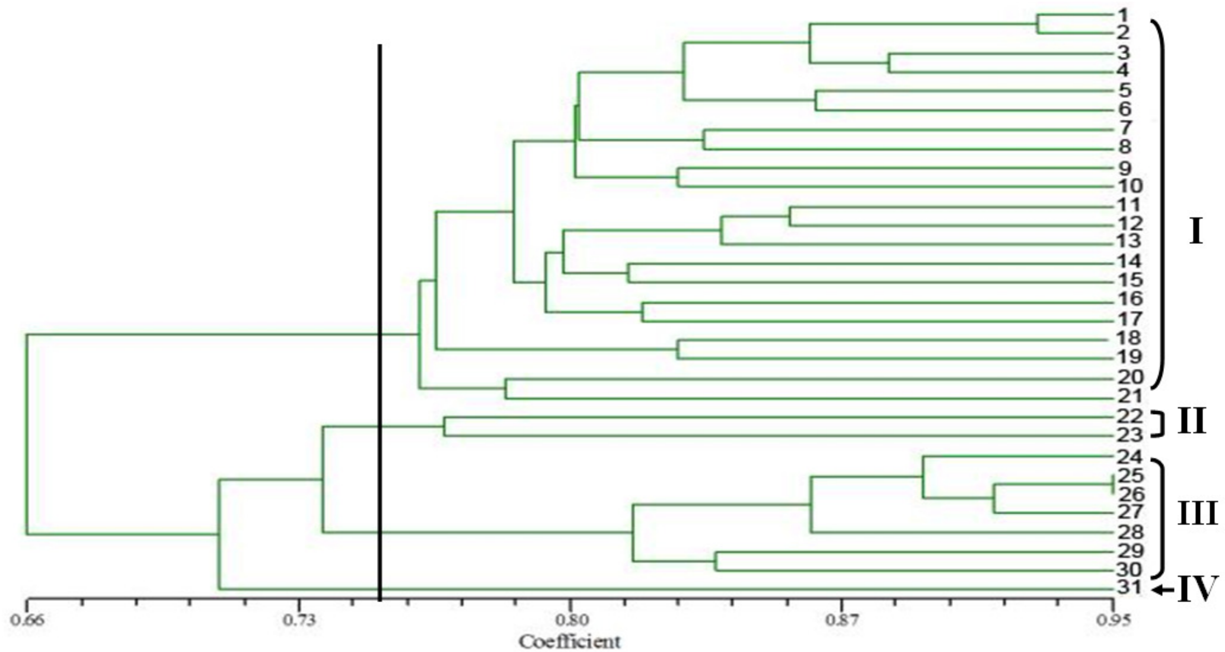

Figure 4. Cluster analysis of 31 wild apple accessions based on polymorphic bands obtained with S-SAP marker.

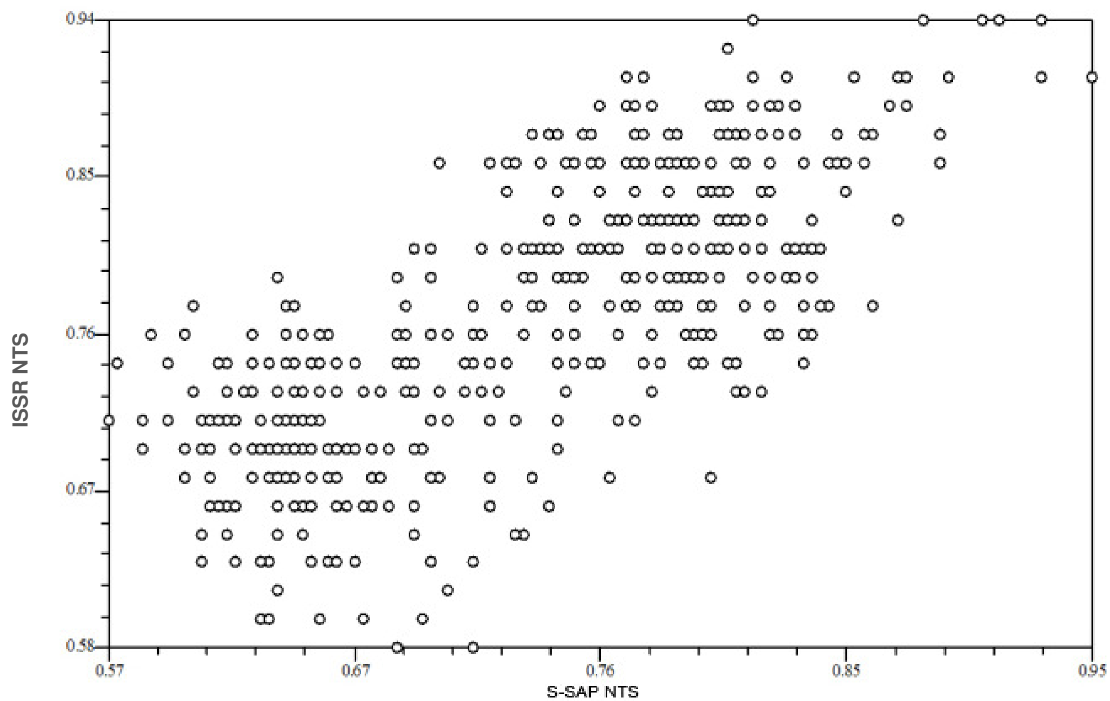

Figure 5. Similarity matrices distribution map for ISSR and S-SAP markers.

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