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Assessing genetic variation using arbitrary oligonucleotide markers system in apple genotypes

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ABSTRACT

A core collection of 50 apple genotypes was taken into consideration to access the genetic diversity and phylogenetic relationship using arbitrary oligonucleotide RAPD and ISSR markers system. The level of polymorphism across the subjected genotypes was 67.26% and 64.56% by RAPD and ISSR markers, respectively. Dendrogram grouped the subjected apple genotypes among three major clusters with similarity coefficient ranging from 0.20 to 0.94 using RAPD and among four major clusters with similarity coefficient ranging from 0.40 to 0.94 using ISSR markers, respectively. Pooled RAPD and ISSR along with UPGMA clustering based on Jaccard's similarity coefficient were estimated with a view to assess efficiency of the marker system in apple genotypes. The genotypes 'Royal Delicious' and 'Snow Drift' were found to be most distantly related to each other as revealed from pooled dendrogram analysis of both the markers. Estimates of pooled similarity coefficient ranging from 0.35 to 0.94 indicated a broad genetic base promising for application to future molecular screening, map construction, mining of desirable genes for genetic improvement of fruiting cultivars and comparative genomic studies, etc. Therefore, the obtained results in the present study confirmed the potential of subjected markers as a reliable, rapid and inexpensive discrimination method that could be helpful in framing more extensive studies on the determined apple genotypes.

Key words: *Malus* × *domestica*, genetic diversity, phylogenetic relationship, RAPD, ISSR.

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) is an important temperate fruit of family Rosaceae with basic chromosome no. 17 (n) and is grown in areas where winters are cold, springs are frost free, and summer is mild. Apple is one of the most widely distributed perennial fruit crops. Originally domesticated in central Asia (near the Tian Shan mountain range), *M.* × *domestica* is now grown in temperate areas of the northern and southern hemispheres. The progenitor species for domesticated apple include *Malus sieversii* M. Roem, *Malus orientalis* Uglitzk., and *Malus sylvestris* (L.) Mill. (Cornille *et al.* 6). Apple provides an excellent study system for understanding the genetic effects of perennial plant domestication, due to its relatively small diploid genome and the extensive germplasm collections available around the world (Gross *et al.* 10).

Understanding genetic relationship of frequently used germplasm is vital to any breeding programme attempting to increase the genetic diversity of new cultivars. An accurate knowledge of the origin and parentage of parental germplasm may also lead to better understanding of the inheritance of important genetic traits. Assessment of genetic diversity to recognize groups with similar genotypes is very important to conserve, evaluate and utilize the genetic resources, for studying the diversity of the germplasm as potential basis of genes that may be capable to improve the performance of cultivars and for determining the distinctness and uniqueness of the phenotypic and genetic information of genotypes with the purpose of protecting the intellectual property rights of the breeder (Nemera *et al.* 17). Diversity studies would also be desirable for the purpose of better management and conservation of the genetic resources and for planning the breeding strategies.

Species identification based on morphological characteristics is often difficult, since most of these characteristics are under the influence of environmental factors (Kimura *et al.* 14). Introduction of DNA-based markers has provided a large number of markers independent of environmental influences and are suitable for genetic typing at very early stages of development. Polymerase chain reaction (PCR) and non-PCR based molecular markers have become increasingly popular in the characterization and identification of genetic resources because they are not influenced by environmental factors and are more polymorphic. Non-PCR based restriction fragment length polymorphism (RFLP) and PCR

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based amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), sequence specific amplification polymorphism (S-SAP), simple sequence repeat length polymorphism (SSRLP), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), cleaved amplified polymorphic sequence (CAPS), and direct or directed amplification of minisatellite region DNA amplified using the polymerase chain reaction (DAMD-PCR) have been exploited for the identification of genotypes or accessions at the taxonomic level, assessment of the relative diversity or similarity within and between species, and selection of diverse accessions with desirable traits for breeding purposes.

RAPD and ISSR markers have a number of advantages for use in the detection of genetic variation such as technical simplicity, rapidity of assay, minimal DNA requirements, and low assay cost. In addition, no prior knowledge about the sequence under investigation is required (Izzatullayeva, 11). The use of random amplification of polymorphic DNA (RAPD), the first PCR-based technique, underscored the advantages of accessing genetic diversity in different fruit crops (Bhatt, 3). Inter-simple sequence repeat (ISSR) has also been used in species identification, genetic mapping, gene locating, phylogeny and evolution in different fruit crops. Both RAPD and ISSR markers have been successfully employed in our research laboratory for characterizing peach germplasm (Sharma and Sharma, 19). The capability and efficiency of these markers in determination of genetic diversity provides a strong base for framing the present study by their use in estimation of genetic variation prevailing among different apple genotypes growing in Indian Himalayan region for future breeding programmes. Considering these facts, the objective of our study was to provide markers that can identify all prominent cultivars, test their efficiency in discriminating closely related cultivars, and evaluate future respective marker applications for maintenance of apple germplasm.

MATERIALS AND METHODS

For molecular analysis, 50 different apple genotypes were sourced from Regional Horticulture Research Station, Mashobra, Shimla (Dr YS Parmar University, Nauni, Solan) and National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Phagli, Shimla, Himachal Pradesh (Table 1). Genomic DNA was extracted from young leaf tissue using CTAB method. RNA contaminants in all the samples were digested with 100 µg/ml RNaseA for 30 minutes at 37 ^{0}C. DNA concentration and purity was measured using UV/VIS spectrophotometer at 260 nm and 280 nm absorbance, respectively.

Polymerase chain reaction was used to study the genetic diversity among selected genotypes. PCR was carried out in a 15 µl reaction volume for each marker analysis containing Taq DNA polymerase (3U/reaction, Genei India), Taq DNA polymerase buffer (1X, Genei, India) with 1.5 mM $MgCl_2$ (Genei, India), primers (10 pmol/reaction, Genei, India), deoxynucleotide triphosphate (dNTPs) (25 mM, Genei, India) and template DNA (50 ng/reaction). A total of 52 RAPD and 44 ISSR primers at their appropriate annealing temperatures were used to characterize 50 apple genotypes. The following programme comprised of an initial cycle of 4 min at 95 °C followed by 35 cycles of 1 min at 94 °C, annealing temperature depending upon T_m value of primer for 1 min, elongation step of 2 min at 72 °C, and a final extension step of 8 min at 72 °C followed by a 4 °C soak until recovery was run on thermocycler (Applied Biosystems, USA). PCR Products were analysed by electrophoresis on different agarose (GeNei, India) concentrations i.e., 1.6% for RAPD and 2.0% for ISSR in 1X TAE buffer containing ethidium bromide (10 mg/ml), respectively and images were taken using Gel Documentation Unit (Syngene, UK). The size of the amplified product was determined by co-electrophoresis of 100 bp standard molecular weight markers (Genei, India). Only those primers which produced bands with all the samples were used to score for polymorphism.

Marker index for each molecular marker was calculated by calculating polymorphism information content (PIC) values in order to characterize the capacity of each primer to detect polymorphic loci among different genotypes as described by Nie *et al.* (16). The data on band position on agarose gel was recorded by assigning '0' for the absence of band and '1' for presence of band. The similarity matrix generated using Jaccard coefficient was used for unweighted pair-group method based on arithmetic average (UPGMA) using software package NTSYS-PC ver.2.02i. The output data was graphically represented as a dendrogram using NTSYS and neighbour-joining tree analysis along with bootstrap values on the branches using with DARwin5 ver.5.0.158 software packages, respectively.

RESULTS AND DISCUSSION

Of the 52 random RAPD primers used, only 40 were able to amplify the genomic DNA (Table 2). These 12 primers failed to amplify the genomic DNA uniformly and were not included into further analysis.

All random primers were found to be polymorphic that might be due to the length differences in the amplified sequence between primer annealing sites and variation in the primer annealing sites. For a total of 40 primers, the number of bands varied from 4 (primers OPB-01, OPC-08, OPE-03 & OPL-18) to 12 (primers OPB-17, Fig. 1), respectively with amplicon size ranging from 100-1600 bp (approx.) for all the informative primers. Per cent polymorphism was observed 67.26% with an average amplified fragments 6.93, repectively. The RAPD primers OPA-10, OPB-18, OPE-03 and OPH-04 showed the highest (100%) polymorphism, while RAPD primer OPB-12 showed the lowest (40%) polymorphism. In the present study, high level of polymorphism among the targeted apple genotypes was observed that has also been recorded by Muzher *et al.* (15) with 82.70% level of polymorphism among some Syrian local apple cultivars using the same RAPD markers strategy. While, Erturk and Akcay (8) examined low level of genetic variability among few accessions of 'Amasya' apple cultivars. Of the total 441 RAPD

Table 1. Apple genotypes used in current study.

S. No.	Name of genotype	Origin	S. No.	Name of genotype	Origin
1	Royal Delicious	USA	26	Top Red	USA
2	Red Delicious	USA	27	Vance Delicious	USA
3	Stark Spur Golden	USA	28	Hardeman	USA
4	Tydeman	UK	29	Bright N Early	UK
5	Red Spur	USA	30	Real Mecoy	USA
6	Criterion	USA	31	Ambred	India
7	Well Spur	USA	32	Ambrich	India
8	Silver Spur	USA	33	Ambroyal	India
9	Starkrimson Delicious	USA	34	Ambstarking	India
10	Golden Spur	USA	35	Margrate	UK
11	Red Baron	USA	36	Wagenar	USA
12	Yellow Newton	USA	37	Lodi Early Golden	USA
13	Red Baldwin	USA	38	Worcester Pearmain	UK
14	Gravenstein	Denmark	39	Red Gold	USA
15	Red Fuji	Japan	40	Grand Alexander	Ukraine
16	Cox's Orange Pippin (Kesari)	UK	41	Pomon Chinese	China
17	Summer Golden Pippin	UK	42	Pink Superior	USA
18	Wealthy Double Red	USA	43	Directeur *Van De Plassche*	Netherland
19	Ingrid Marie	Denmark	44	Memory of Manchurian	USA
20	James Grieve	UK	45	Chinese Cinnamon	China
21	Mutsu	Japan	46	Survovets	USA
22	Turley Winesap	USA	47	Dessert of Isaac	UK
23	Grimes Golden Double Life	USA	48	Summer Queen	USA
24	Golden Delicious	USA	49	Red Flush	USA
25	Gale Gala	New Zealand	50	Snow Drift	USA

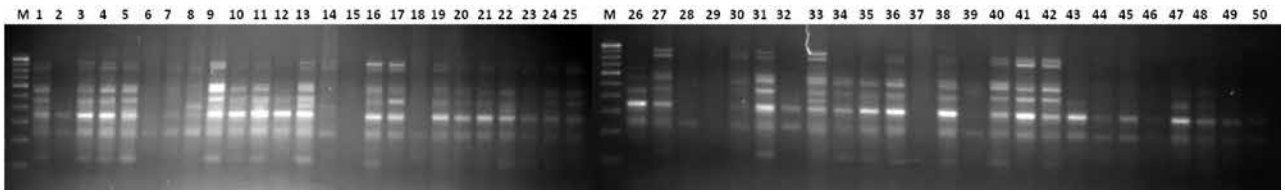

Fig. 1. DNA profiling obtained using RAPD (OPB-17) marker in apple genotypes.

Table 2. Primer sequences, annealing temperature, size of amplicons, number of amplified bands, percent polymorphism and polymorphic information content values of RAPD markers studied among apple genotypes.

S. No.	Primer	Sequence (5'-3')	Ta (°C)	Size of amplicons (bp)	No. of amplified bands	Polymorphism (%)	PIC (%)
1.	OPA-01	CAGGCCCTTC	32	200-1200	07	71.42	0.33
2.	OPA-04	AATCGGGCTG	32	350-1300	06	66.66	0.35
3.	OPA-05	AGGGGTCTTG	30	200-1200	05	60.00	0.36
4.	OPA-09	GGGTAACGCC	32	300-1100	07	71.43	0.36
5.	OPA-11	CAATCGCCGT	37	200-1600	08	100.00	0.42
6.	OPA-14	TCTGTGCTGG	30	200-1400	06	83.33	0.35
7.	OPA-18	AGGTGACCGT	30	150-1200	09	66.67	0.32
8.	OPA-20	GTTGCGATCC	32	250-900	05	60.00	0.30
9.	OPB-01	GTTTCGCTCC	32	350-900	04	75.00	0.32
10.	OPB-07	GGTGACGCAG	32	200-1100	08	50.00	0.25
11.	OPB-12	CCTTGACGCA	32	300-900	05	40.00	0.19
12.	OPB-17	AGGGAACGAG	32	150-950	12	55.55	0.40
13.	OPB-18	CCACAGCAGT	30	200-900	09	100.00	0.44
14.	OPC-01	TTCGAGCCAG	32	100-800	07	57.14	0.24
15.	OPC-03	GGGGGTCTTT	30	250-850	05	60.00	0.26
16.	OPC-07	GTCCCGACGA	32	100-950	08	62.50	0.36
17.	OPC-08	TGGACCGGTG	32	250-750	04	75.00	0.42
18.	OPD-04	TCTGGTGAGG	30	200-900	06	66.67	0.33
19.	OPD-05	TGAGCGGACA	32	150-800	05	60.00	0.32
20.	OPD-11	AGCGCCATTG	30	200-1200	08	62.50	0.37
21.	OPD-12	CACCGTATCC	32	100-1100	10	80.00	0.40
22.	OPE-03	CCAGATGCAC	32	250-800	04	100.00	0.43
23.	OPE-07	AGATGCAGCC	30	100-850	07	71.43	0.32
24.	OPE-14	TGCGGCTGAG	32	150-900	07	57.14	0.23
25.	OPE-15	ACGCACAACC	30	250-900	05	80.00	0.36
26.	OPE-18	GGACTGCAGA	32	300-1250	10	60.00	0.32
27.	OPF-17	AACCCGGGAA	30	200-1050	07	57.14	0.21
28.	OPG-01	CTACGGAGG	30	250-1000	08	62.50	0.32
29.	OPG-04	AGCGTGTCTG	32	300-1100	06	83.33	0.25
30.	OPH-04	GGAAGTCGCC	32	200-1200	08	100.00	0.38
31.	OPH-14	ACCAGGTTGG	32	300-1350	08	75.00	0.25
32.	OPL-12	GGGCGGTACT	32	200-1200	10	70.00	0.32
33.	OPL-18	ACCACCCACC	32	350-950	04	50.00	0.34
34.	OPP-02	TCGGCACGCA	32	250-1400	08	62.50	0.26
35.	OPP-12	AAGGGCGAGT	30	400-900	05	60.00	0.27
36.	OPU-01	ACGGACGTCA	32	200-1250	07	57.14	0.33
37.	OPU-11	AGACCCAGAG	32	300-1500	06	83.33	0.20
38.	OPU-20	ACAGCCCCCA	32	250-1200	08	62.50	0.37
39.	OPY-07	AGAGCCGTCA	30	200-1000	09	55.55	0.36
40.	OPY-16	GGGCCAATG	30	100-1000	11	75.00	0.41
Mean					6.93	67.26	0.32

Ta: annealing temperature, bp: base pairs

loci that were amplified by 38 random primers, 180 were found to be polymorphic with 39.98% diversity in commercial cultivar of apple produced in Turkey.

The PIC value provides an estimate of the discriminatory power of a locus or loci, by taking into account not only the number of alleles that are expressed, but also relative frequencies of these alleles. Referring to PIC value recorded for all the informative RAPD primers, the PIC vary from a minimum of 0.19 for OPB-12 and maximum of 0.44 for OPB-18 with an average of 0.32 (Table 2). The genotypes 'Bright N Early' and 'Royal Delicious' were found to be highly diversified among all subjected apple genotypes. The results obtained by Erturk and Akcay (8) in case of apple genotypes (0.04 to 1.00) and Orhan *et al*. (18) in quince genotypes (0.42 to 0.96) holds good with the present investigation while estimating genetic diversity in temperate pome fruit crops. Therefore, RAPD fingerprinting confirmed certain molecular markers that might be associated with certain commercial characteristics. Our results holds good with the findings of Erturk *et al*. (9) on the characterization of wild-grown blackthorn plants by using RAPD markers which allows interpreted information for future studies on the appropriate use of these cultivars in breeding programs, proper biodiversity assessment and better conservation of germplasm resources.

In the present study, molecular characterization of apple genotypes was further analyzed using 44 ISSR primers, out of which 32 gave informative and reproducible results (Table 3). For a total of 32 primers, the number of bands varied from 3 (primers ISSR-808, ISSR-819 & ISSR-842) to 8 (primer ISSR-847, Fig. 2), respectively with amplicon size ranging from 150-1500 bp (approx.) for all the informative primers. Average per cent polymprphism was observed 64.54% with an average amplified fragments 5.03. Similarly, Kashyap *et al*. (12) also observed that 21 ISSR primers were able to amplify a total of 130 amplification product of apple genotypes, of which 130 (100%) were reported to be polymorphic. These results were in accordance with other reports on temperate pome fruit crops such as apple (Wang *et al.* 20) and pear (Carrasco *et al.* 4) where higher polymorphism was revealed with ISSR primers from closely related cultivars. Referring to PIC value recorded for all the informative ISSR primers, the PIC vary from a minimum of 0.19 for ISSR-873 and maximum of 0.48 for ISSR-847 with an average of 0.36 (Table 3). Similar results were also observed by Khajuria *et al.* (13) in apple genotypes with similarity coefficient values ranged from 0.10 to 0.80 indicating very high level of diversity with an average of 0.45. Likewise, Dehbashi *et al*. (7) in quince genotypes determined similarity coefficient values ranging from 0.28 to 0.86 using Jaccard's coefficient thus, indicated substantial diversity present in the germplasm.

For pooled RAPD and ISSR studies (Fig. 3), the similarity coefficient was as low as 0.35 to as high as 0.94 with a mean value of 0.65 indicated substantial diversity present in the selected genotypes. The highest similarity coefficient 0.88 was observed between 'Top Red' and 'Hardeman' genotypes while lowest 0.35 was observed in 'Snow Drift' with rest of the genotypes. While, Khajuria *et al*. (13) described low pooled similarity coefficient range of 0.10 to 0.80 with a mean value of 0.45 in apple genotypes than reported in the present study. In the present study, dendrogram obtained after pooled RAPD and ISSR analysis showed that the genotypes were broadly divided into two major and four minor clusters branched at similarity coefficient value of 0.35 (Fig. 3). First major cluster comprising of 16 genotypes merged at 0.65 similarity coefficient contained two sub-cluters grouping 13 and 3 genotypes, respectively. Genotypes 'Top Red' and 'Hardeman' showed as high as 0.94 similarity coefficient. Four Indian hybrids named 'Ambstarking', 'Ambred', 'Ambroyal' and 'Amrich' had 0.78 similarity coefficient. The genotypes 'Lodi Early Golden' and 'Worcester Pearmain' truncated at 0.81 similarity coefficient. Under this sub-cluster, 'Royal Delicious' and 'Grimes Golden Double Life' were found to be highly diversified merged at 0.65 similarity coefficient. Similarlily, second major cluster truncated at 0.68 similarity coefficient grouped 11 genotypes. Among these, 'Red Fuji' and 'Gale Gala' showed as high as 0.89 similarity coefficient. Genotype 'Pink Superior'

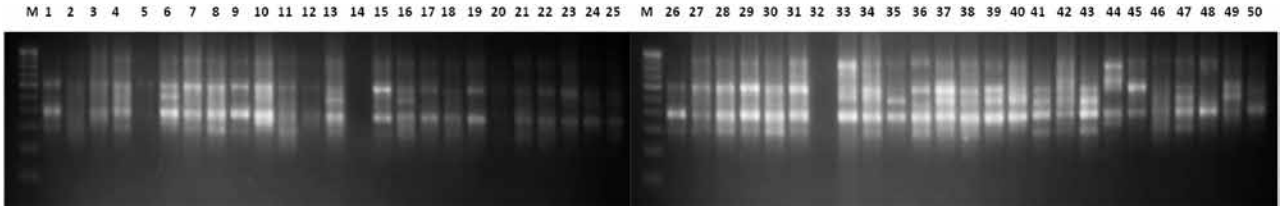

Fig. 2. DNA profiling obtained using ISSR (ISSR-847) marker in apple genotypes.

Table 3. Primer sequences, annealing temperature, size of amplicons, number of amplified bands, percent polymorphism and polymorphic information content values of ISSR markers studied among apple genotypes.

S. No.	Primer	Sequence (5'-3')	Repeat Motifs	Ta (°C)	Size of amplicons (bp)	No. of amplified bands	Polymorphism (%)	PIC (%)
1.	ISSR-808	AGAGAGAGAGAGAGAGC	(AG)8C	48	250-1100	03	66.67	0.40
2.	ISSR-810	GAGAGAGAGAGAGAGAT	(GA)8T	48	200-1300	05	75.00	0.32
3.	ISSR-811	CACCACACACACACAAT	(GA)8C	48	250-1000	06	60.00	0.37
4.	ISSR-814	CTCTCTCTCTCTCTCTTG	(CT)8TG	52	200-1000	05	80.00	0.36
5.	ISSR-815	CTCTCTCTCTCTCTCTGT	(CT)8GT	52	250-1100	05	60.00	0.31
6.	ISSR-818	CACACACACACACAC	(CA)8G	50	300-1200	07	57.14	0.32
7.	ISSR-819	GTGTGTGTGTGTGTGT	(GT)8A	48	200-900	03	66.67	0.43
8.	ISSR-822	TCTCTCTCTCTCTCTAC	(TC)7TAC	48	200-1000	06	50.00	0.27
9.	ISSR-823	TCTCTCTCTCTCTCTCC	(TC)8C	50	200-1000	05	80.00	0.46
10.	ISSR-824	TCTCTCTCTCTCTCTCG	(TC)8G	48	350-800	04	50.00	0.46
11.	ISSR-825	ACACACACACACACACT	(AC)8T	48	150-1000	04	50.00	0.21
12.	ISSR-826	ACACACACACACACACC	(AC)8C	50	250-900	05	50.00	0.30
13.	ISSR-827	ACACACACACACACACGG	(AC)8GG	52	250-750	04	100.00	0.44
14.	ISSR-830	TGTGTGTGTGTGTGTGG	(TG)8G	50	200-1000	06	50.00	0.38
15.	ISSR-840	GAGAGAGAGAGAGAGAGT	(GA)8GT	52	300-1000	05	60.00	0.36
16.	ISSR-842	GAGAGAGAGAGACCCGGG	(GA)6(C)3(G)3	58	300-700	03	66.67	0.43
17.	ISSR-847	CACACACACACACACAGC	(CA)8GC	52	300-900	08	100.00	0.48
18.	ISSR-849	GTGTGTGTGTGTGTGAA	(GT)7GAA	48	275-1300	06	50.00	0.26
19.	ISSR-851	GTGTGTGTGTGTGTGTCG	(GT)8CG	52	250-750	05	60.00	0.32
20.	ISSR-856	ACACACACACACACACG	(AC)8G	50	300-700	04	50.00	0.36
21.	ISSR-857	ACACACACACACACACGGTC	(AC)8GGTC	56	200-1500	06	83.33	0.35
22.	ISSR-873	GACAGACAGACAGACA	(GACA)4	48	200-1000	05	40.00	0.19
23.	ISSR-880	GGAGAGGAGAGGAGAGT	(GGAGA)3T	52	200-700	06	66.67	0.46
24.	UBC-809	AGAGAGAGAGAGAGAGG	(AG)8G	52	300-900	04	75.00	0.47
25.	UBC-829	TGTGTGTGTGTGTGTGC	(TG)8C	50	300-1000	05	60.00	0.30
26.	UBC-835	AGAGAGAGAGAGAGAGCC	(AG)8CC	54	250-1100	06	66.67	0.40
27.	UBC-841	GAGAGAGAGAGAGAGACC	(GA)8CC	52	200-900	05	80.00	0.34
28.	UBC-848	CACACACACACACACAAGG	(CA)8AGG	48	275-1200	06	50.00	0.27
29.	UBC-855	ACACACACACACACACCTT	(AC)8CTT	52	200-850	04	75.00	0.30
30.	UBC-868	GAAGAAGAAGAAGAAGAA	(GAA)6	48	200-1000	06	66.67	0.35
31.	UBC-874	CCCTCCCTCCCTCCCT	(CCCT)4	56	250-1100	05	60.00	0.34
32.	UBC-880	GGAGAGGAGAGGAGA	(GGAGA)3	48	300-1000	05	60.00	0.24
Mean						5.03	64.54	0.36

Ta: annealing temperature, bp: base pairs

showed 0.74 similarity coefficient with 'Survovets' and 'Directeur Van De Plassche' which in turn both were 0.85 similar to each other.

Likewise, third cluster comprising of five genotypes, 'Wealthy Double Red', 'Real Mecoy', 'Turley Winesap', 'James Grieve' and 'Margrate' grouped at 0.62 similarity coefficient. Among these, 'Wealthy Double Red' and 'Real Mecoy' showed as high as 0.80 whereas, 'James Grieve' and 'Margrate' genotypes showed 0.72 similarity coefficients, respectively. Likewise, fourth cluster grouped at 0.63 comprising of four genotypes named

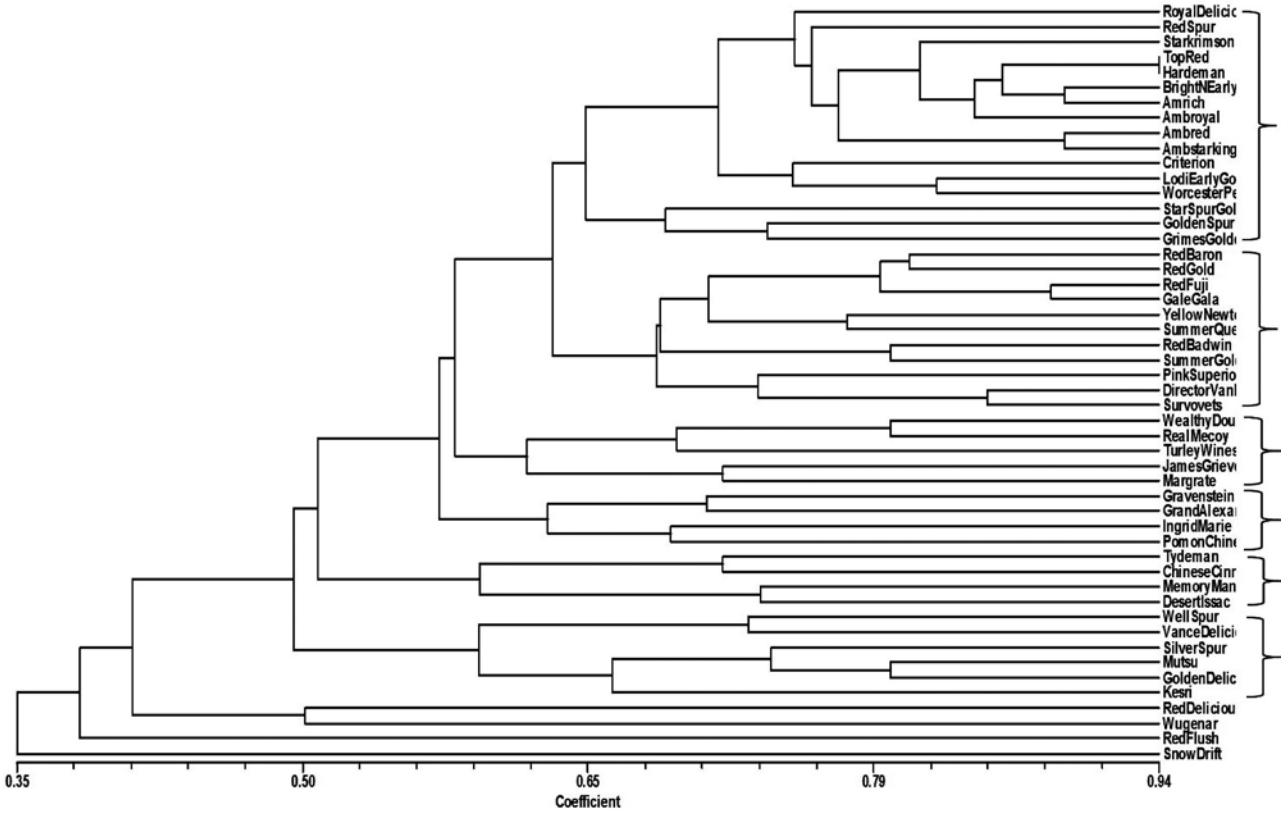

Fig. 3. Pooled dendrogram obtained after RAPD and ISSR analysis in apple genotypes.

'Gravenstein', 'Grand Alexander', 'Ingrid Marie' and 'Pomon Chinese'. Among these, Gravenstein' and 'Grand Alexander' showed 0.72 and 'Ingrid Marie' and 'Pomon Chinese' genotypes showed 0.69 similarity coefficients, respectively. Likewise, fifth cluster truncated at 0.60 similarity coefficient grouped four genotypes named 'Tydeman', 'Chinese Cinnamon', 'Dessert of Isaac' and 'Memory of Manchurian'. Among these, 'Tydeman' and 'Chinese Cinnamon' showed 0.70 and 'Dessert of Isaac' and 'Memory of Manchurian' showed 0.73 similarity coefficients, respectively. Sixth cluster grouped six apple genotypes 'Well Spur', 'Vance Delicious', 'Silver Spur', 'Mutsu', 'Golden Delicious' and 'Kesri' which merged at 0.60 similarity coefficient. Among these, 'Mutsu', 'Golden Delicious' genotypes showed maximum 0.80 similarity coefficient. Alternatively, two genotypes named 'Red Delicious' and 'Wugenar' truncated at 0.40 similarity coefficient from all the other genotypes which were grouped under six major and minor clusters. The similarity coefficient between these two genotypes was found to be 0.50. In the present study, two wild apple genotypes named 'Red Flush' and 'Snow Drift' truncated at 0.38 and 0.35 similarity coefficient independently without grouping themselves in any clusters. These both genotypes were found to be distantly related with other apple genotypes taken into consideration.

The neighbor-joining cluster analysis with boot strap support values of apple genotypes obtained after pooled molecular marker analysis revealed high diversity among 50 genotypes subjected under present study (Fig. 4). The majority of genotypes were grouped in two major groups as same as reported using NTSYS software. Similarly, the wild genotypes 'Red Flush' and 'Snow Drift' were found to be distant among all the apple genotypes. This clearly revealed the precise genetic analysis of genotypes taken into consideration in the present study. Similar results were reported by Coart *et al.* (5) in which boot strap support value clustered the apple genotypes in five major groups with value ranging from 77% to 100%, separating ornamental and edible cultivars. Further neighbour joining tree generated using UPGMA by Bhatt *et al.* (3) clustered pear genotypes into two main groups I and II having 5 and 6 genotypes, respectively. Our result also holds well with Bao *et al.* (1), Bassil *et al.* (2) and Sharma and Sharma (19) in characterizing apple, pear and peach germplasm, respectively. Therefore,

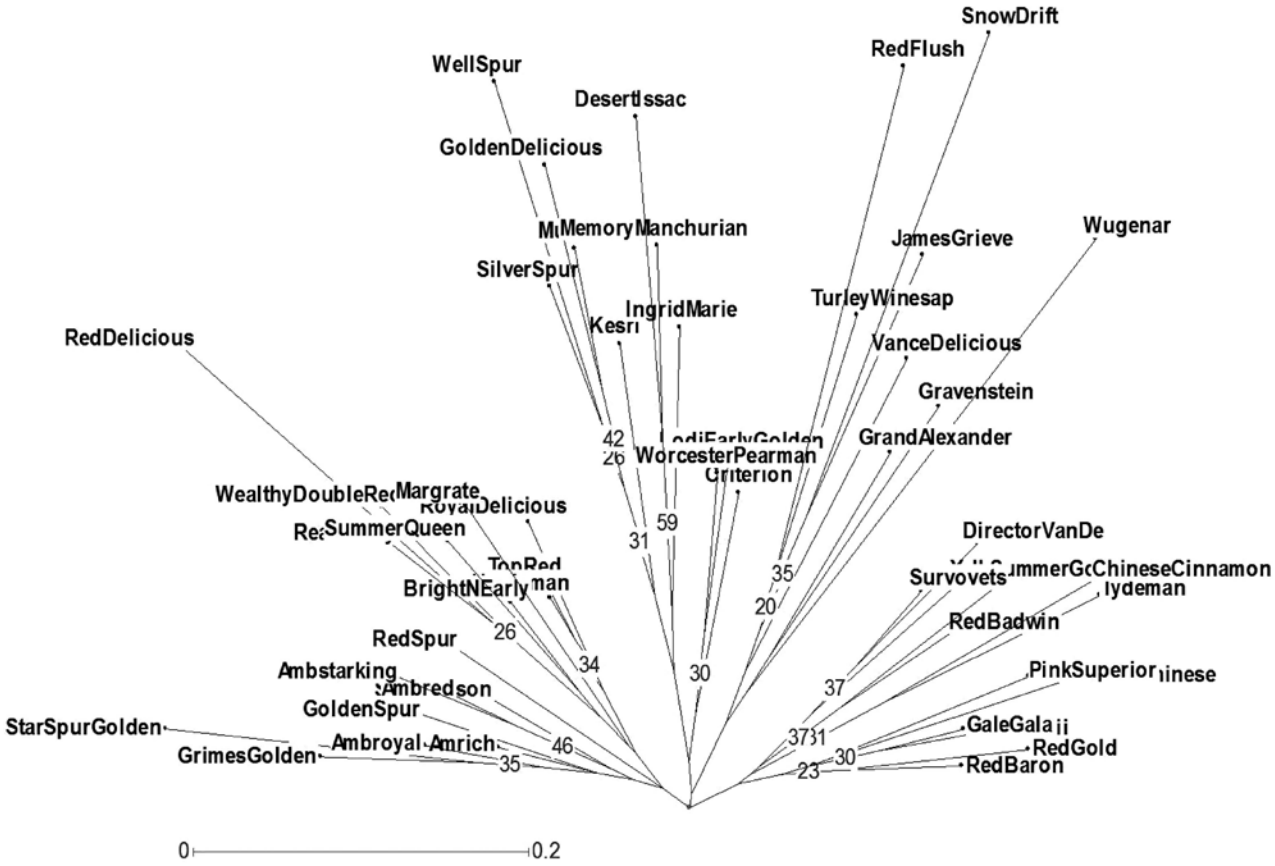

Fig. 4. Neighbour-Joining tree obtained after pooled RAPD and ISSR marker analysis (number on branches are bootstrap values).

the obtained results in the present study confirmed the potential of RAPD and ISSR technology as a reliable, rapid and inexpensive screening method to discriminate apple genotypes. Information on the genetic relationship would be essential for a rationale use of genetic resources, to estimate any possible loss of genetic diversity and to offer evidence of evolutionary forces shaping the genetic diversities etc.

High level of polymorphism indicates their applicability in development of superior progenies, QTL mapping, molecular breeding, investigation of population genetic diversity, comparative mapping, selection of the parents and the combinations among various crop improvement programmes. Therefore, results obtained in the present study could be helpful in framing more extensive studies on the determined apple genotypes.

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