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Genetic diversity analysis of indigenous and exotic apple genotypes using inter simple sequence repeat markers

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ABSTRACT

The domesticated apple (*Malus* x d*omestica* Borkh) is one of the most widely cultivated fruit crop of the temperate world. Large number of commercial cultivars and varieties are available which need an accurate characterization to be used in the breeding programmes. Keeping this thing in mind, the genetic diversity among 46 genotypes was investigated using Inter Simple Sequence Repeat (ISSR) markers. A total of 130 amplification products were detected with 21 ISSR primers of which 130 (100%) were reported to be polymorphic. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more polymorphic bands. The number of bands generated by 21 ISSR primers ranged from 4(UBC 840) to 10 (ISSR 3) with a mean of 6.19 bands per primer. The band size ranged from 300-1700bp. The Jaccard's similarity coefficient ranged from 0.435 to 0.822 suggesting that the 46 genotypes used in the study were highly divergent. Cluster analysis based on the UPGMA method and bootstrap analysis separated all the genotypes into three major clusters. Maximum Resolving Power (Rp) was computed to be 5.696 (ISSR 3) while the minimum was 1.174 (UBC 850). The highest Polymorphism Information Content (PIC) was recorded to be 3.946 (ISSR 3) while the lowest was 1.700 (UBC 850). The overall Heterozygosity Averages (H_{av}) values of primers were 0.068 and 0.717 respectively for ISSR and UBC series. The present study has successfully distinguished morphologically similar genotypes that emphasize the use of molecular markers to the taxonomists.

Key words: Apple, ISSR-PCR, DNA fingerprinting, genetic diversity, cluster analysis.

INTRODUCTION

The domesticated apple *Malus* x d*omestica* (Borkh) is a complex hybrid of several *Malus* species including *M. sieversii, M. roemer, M. orientalis, M. sylvestris, M. baccata, M. mandshurica* and *M. prunifolia*. Its domestication has not been yet fully documented. *M. sieversii* from Central Asia is thought to be a major species contributor, while *M. orientalis* and *M. sylvestris* are the potential minor species contributors (Buttner, 1; Watkins, 2). Human domestication and incorporation of *M. orientalis* into other genetic backgrounds may have occurred during its westward movement from Armenia and Transcaucasus into the area of ancient greek civilization (Buttner, 1; Ericisli, 3). It is economically the most important fruit tree crop of the temperate zone and a high number of commercial cultivars are available as the result of open pollinated seedlings, controlled crosses in the breeding programmes and exploitation of the naturally or induced somatic mutations in the adapted varieties and cultivars. Several molecular markers studies on apple have been published using the techniques such as RFLP (Restricted Fragment length Polymorphism) (Nymbom and Schall, 16; Watillon *et al.,* 20), RAPD (Randomly amplified Polymorphic DNA) (Gardiner *et al.,* 3; Goulao and Cristina, 5, Dantas *et al.,* 2; Landry *et al.,*12), AFLP (Amplified Fragment Length polymorphism) (Hokanson *et al.,* 7), SSR (Simple Sequence Repeats (Kenis *et al.,* 10, Volk *et al.,* 19; Lamboy, 11; Liebhard *et al.,* 13; Zhou *et al*., 24; Yamamoto *et al.,* 23) and ISSR (Inter Simple Sequence Repeats) (Hokanson *et al.,* 7).

Evaluations of genetic diversity of wild Malus population have provided useful assessments of germplasm diversity in the U.S. Department of agriculture (USDA), Agriculture research service (ARS), National Plant Germplasm System (NPGS) collection (Volk, 19, Lodhi *et al.,* 14). For example *M. siversii* population in Kazakhstan was shown to have regional structure with most of the diversity represented within half sib families. *Malus siversii* wild apples are generally larger and sweeter than the apples of *Malus orientalis* and Chinese species of wild apples. This may be a result

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of widespread movement and selection during the domestication of apples by humans over thousand of years. In order to assess the genetic diversity in apple, PCR based DNA markers are among the best tools. A range of DNA markers, *viz*. AFLP, RAPD, SSR and ISSR have already been used for exploring the diversity of the global apple germplasm pool. Among these ISSR (Inter simple Sequence Repeat) is a reproducible semi arbitrary primed PCR method that uses simple sequence repeats as primers, combining most of the advantages of the microsatellites and Amplifies Fragment Length Polymorphism (AFLP) to the universality of Randomly Amplified Polymorphic DNA (RAPD). ISSRs offer a greater probability than any other PCR marker system in the repeat regions of the genome, which are most potent regions for producing cultivar specific markers. This is also the attribute of ISSRs, which renders them useful as a supplementary system to any of the random dominant marker systems. Automated PCR base makes ISSRs the markers of choice for screening the genotypes. Consequently, we choose the ISSR marker system to assess the genetic diversity among 46 apple genotypes growing in the Himalayan region of India.

MATERIALS AND METHODS

Leaf samples of 46 genotypes of apple (*Malus* x *domestica* Borkh) representing different species and varieties (exotic and indigenous) were collected from Regional Research Station of Indian Agricultural Research Institute, Amartara, Shimla (Himachal Pradesh) in the present investigation (Table 1). Young leaves were collected from the single tree of each species and variety and frozen in liquid nitrogen immediately. Leaf samples were stored at -80°C till DNA was isolated. Total genomic DNA was extracted from the green leaves using the cetyl trimethyl ammonium bromide (CTAB) method with minor modifications (Rohlf, 17). DNA was quantified in a TKO 100 fluorometer (Hoefer, San Fransisco, CA).

Forty ISSR primers (15-16mer oligos UBC: University of British Columbia) were synthesized (Microsynth Gmbh, Switzerland) and were tested for PCR amplification on 26 randomly selected genotypes to identify the primers that were giving good, scorable and polymorphic amplification products. In order to estimate experimental reproducibility, two independent amplifications were carried out for the selected primers over the same set of genotypes and the primers that showed a clear and reproducible band pattern were chosen for further studies on 46 apple genotypes (Table 1). Amplifications were carried out with 10 mm Tris-HCL (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.1% Triton X-100, 2% formamide, 200 nM primer, 1 unit of *Taq* DNA Polymerase (Operon Technologies, Almada, CA, USA) and 25 ng of genomic DNA. Amplifications were carried out using a 96 thermal cycler (Perkin-Elmer, USA) programmed for 35 cycles as follows: an initial denaturation was for 5 min. at 94°C, 1 min. at 52°C, 2 min. at 72°C and final extension for 7 min at 72° C. The amplification products were stored at 4° C until loading. PCR products were resolved at 60 volts for 3 h on 1.6% agarose gel prepared in 1X TBE buffer. Gel was photographed using Gel Documentation System (Gel Doc Mega, biosystematica, UK).

Table 1. List of 46 apple genotypes used in the study.

Sl. No.	Genotype	Sl. No.	Genotype
1.	*Cydonia oblonga*	24.	*Malus pumila*
2.	M-26	25.	Liberty
3.	Maruba	26.	*Malus eseltine*
4.	*Malus baccata* Khrot	27.	*Malus baccata* Dhak
5.	Top Red	28.	Golden Delicious-5
6.	*Malus kindsomer*	29.	*Malus micromalus*
7.	*Malus orientalis*	30.	*Malus baccata* Rohru
8.	Golden Delicious-3	31.	Tydeman's EarlyWorcester
9.	STRD-1	32.	EC-115820
10.	M-7	33.	E. M. Wilson
11.	Vance Delicious	34.	EC-768527
12.	*Malus baccata* Kashmir	35.	*Prunus cerasoides*
13.	Royal x Prima	36.	*Malus baccata* Shillong
14.	Red Fuji	37.	*Malus baccata* Kinnaur
15.	DR-382	38.	STRD-2
16.	*Malus mandshurica*	39.	STRD-4
17.	*Malus prunifolia*	40.	Michael
18.	Golden Delicious-2	41.	STRD-3
19.	Schlomit	42.	Early Strawberry
20.	Golden Delicious-1	43.	*Malus sargentii*
21.	*Malus simcoe*	44.	Golden Delicious-4
22.	Red Delicious	45.	STRD-5
23.	MM-106	46.	*Malus purpurea*

Each band was treated as one ISSR marker. Scoring of bands was done from the photographs. Homology of bands was based on their migration distance in the gel. The presence of a band was scored as '1' and absence of a band as '0'. All the amplifications were repeated twice and only reproducible bands were considered for analysis. The amplified fragments were named by the primer and their size in base pairs (bp). Each band or the amplification product was considered as ISSR marker. All the numerical taxonomic analysis were conducted using the software NTSYS-PC version 2.11 (Exeter Software, New York (Jaccards, 9). The robustness of the nodes in the dendrogram was tested by bootstrap analysis using Winboot Program developed by IRRI, Philippines. Jaccard's similarity coefficient (J)

was used to calculate the similarity between the paired accessions as J = y / n – d where, y was the number of bands common to sample a and b, n was the total number of bands present in all samples and d was the number of bands not present in a or b, but present in other samples (Rohlf,17). The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated

RESULTS AND DISCUSSION

Forty ISSR primers (thirty primers of University of British Columbia and ten microsynth primers (Gmbh, Switzerland) were tested for PCR amplification on apple genotypes. Out of 40 primers used, 21 primers showed amplification whereas 19 primers did not amplify DNA of any of the genotype. The primers that produced satisfactory, scorable and polymorphic amplification products (Table 2) were selected for molecular characterisation of apple genotypes. In order to estimate experimental reproducibility, two independent amplifications were carried out for the selected primers over the same set of genotypes and the primers that showed a clear and reproducible band pattern were chosen for further study on 46 genotypes of apple (Fig. 1). The present investigation generated a total of 130 bands from 21 informative ISSR primers. All the bands revealed 100% polymorphism. The number of bands generated by 21 ISSR primers ranged from 4 (Primer UBC-840) to 10 (Primer ISSR-3) with a mean of 6.19 bands per primer. ISSR analysis using 28 apple genotypes showed the 100% polymorphism with Jaccard's similarity ranging from 0.435 (between Golden Delicious-4 and C*ydonia oblonga*) to 0.853 (between DR-382 and *Malus prunifolia*) with a mean of 0.644.

Using the band data from only four primers, an identification key could be generated (Table 2). Based on the absence and the presence of the bands from the profiles obtained with the primers ISSR-1, ISSR-3, ISSR-6, ISSR-7, and UBC-808 all the genotypes except *Cydonia oblonga* and early Strawberry could be identified

Table 2. Total number of bands, polymorphic bands and percent polymorphism generated from the ISSR primers in 46 apple genotypes.

S.No.	Primer	Primersequence(5'-3' end)	Total No. of bands	Total No. of polymor-phicbands	Percent polymo-rphism	Band size (bp)	Amplification temperature (Ta)^{O}C
1	ISSR-1	CACACACACACACACARY	6	6	100	300-850	49.0
2	ISSR-2	CAGAGAGAGAGAGAGAYT	5	5	100	300-800	49.0
3	ISSR-3	GAGAGAGAGAGAGAGAYC	10	10	100	300-1500	49.0
4	ISSR-4	AGAGAGAGAGAGAGAGYT	8	8	100	300-1000	49.0
5	ISSR-5	AGAGAGAGAGAGAGAGYC	8	8	100	400-1500	51.0
6	ISSR-6	AGAGAGAGAGAGAGAGYG	9	9	100	250-1000	49.0
7	ISSR-7	ACACACACACACACACYC	8	8	100	200-1100	49.0
8	ISSR-8	CACACACACACACACA	5	5	100	400-800	47.5
9	UBC-808	AGAGAGAGAGAGAGAGC	5	5	100	300-8000	47.5
10	UBC-829	TGTGTGTGTGTGTGTGC	6	6	100	300-900	49.7
11	UBC-840	GAGAGAGAGAGAGAGACTC	4	4	100	300-1200	47.5
12	UBC-841	GAGAGAGAGAGAGAGACTC	5	5	100	300-900	49.7
13	UBC-848	CACACACACACACACAAGG	5	5	100	400-900	47.5
14	UBC-850	GTGTGTGTGTGTGTGTCTC	6	6	100	300-600	49.7
15	UBC-854	TCTCTCTCTCTCTCTCCAGG	7	7	100	300-1600	49.7
16	UBC-855	ACACACACACACACACCTT	5	5	100	300-700	52.0
17	UBC-876	GATAGATAGACAGACA	6	6	100	300-800	47.5
18	UBC-880	GGAGAGGAGAGGAGA	6	6	100	300-1200	47.5
19	UBC-886	ACGAGTACGCTCTCTCTCTCTCT	5	5	100	300-1500	49.7
20	UBC-890	ACGACTACGGTGTGTGTTTGTGT	6	6	100	300-1700	49.7
21	UBC-894	TGGTAGCTCTTGTCAGGCAC	5	5	100	300-1200	49.7

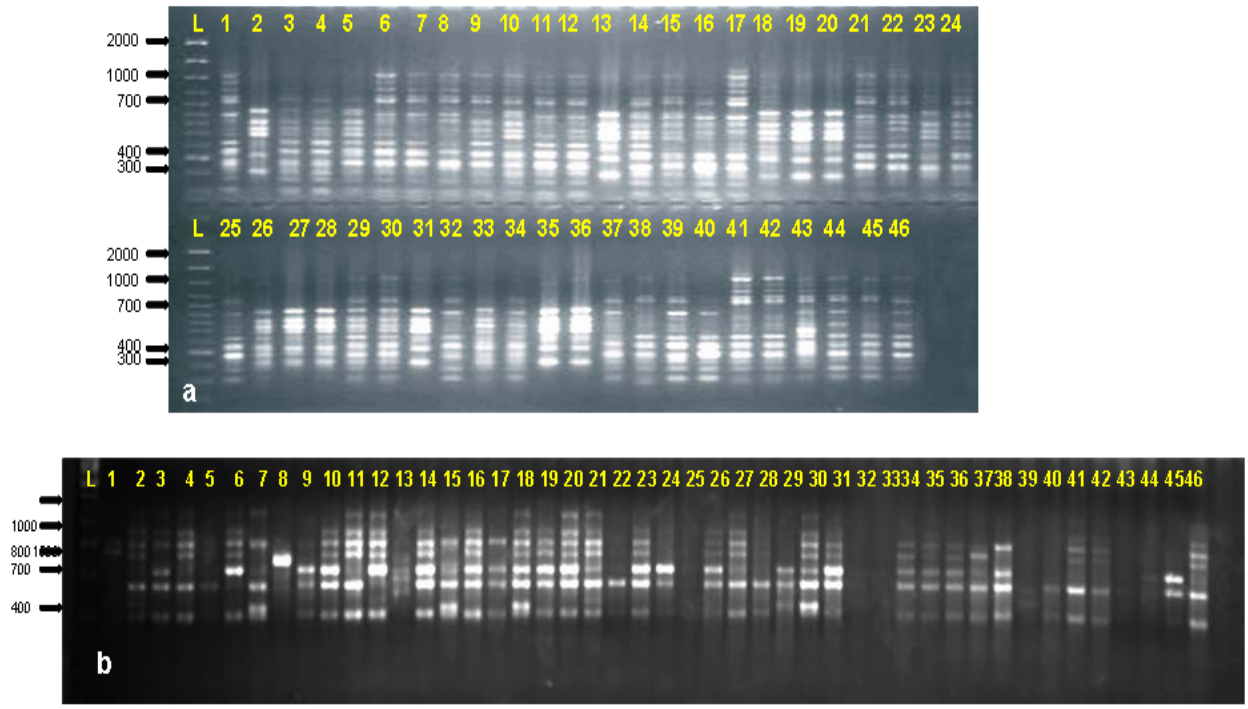

Fig. 1. Amplification profile of apple genotypes employing primer (a) ISSR-1 and (b) UBC-854, ladder (100 bp).

(Figs. 2) The present study using ISSR markers reliably distinguishes the morphologically similar genotypes, e.g. based on the morphological traits Golden Delicious 1, 2, 3, 4 are different strains of Golden Delicious and *Malus baccata* Shilling, *M.baccata* Rohroo, *M.baccata* Dhak, *M. baccata* Kinnaur are the different strains of *Malus baccata* collected from different areas of cultivation, all are reported to be genetically different and thus shared different clusters.

In the ISSR analysis, Jaccard's similarity coefficient values between the pairs of genotypes ranged from 0.435 (between Golden Delicious-4 and C*ydonia oblonga*) to 0.853 (between DR-382 and *Malus prunifolia*) with an average similarity of 0.644 (Fig. 3). The jaccard's similarity coefficient values for the different genotypes used in the study are presented in Table 4. The higher similarity values obtained by ISSR analysis could be attributed to the relatively less variable inter- repeat regions of the genome. Karihaloo (9) attributed the high genetic similarity to a narrow gene pool from which the cultivated forms arose, while Xu and Korban (22) suggested intense selection efforts and/or a narrow genetic background to be the reason.

UPGMA dendrogram generated from the pooled data (Fig. 2.) grouped the genotypes in three major clusters (1, 2 and 3) with 3 sub clusters (2a, 2b and 2c) and two ungrouped genotypes. There were two ungrouped genotypes, *i.e. Cydonia oblonga* and Early Strawberry. *Cydonia oblonga* was the most distinct ungrouped genotype sharing 52% similarity coefficient with other genotypes. A low chill cultivar Early Strawberry did not grouped with any other genotype in cluster 2. It shared 61% similarity with sub-cluster 2 b. Cluster 1 comprised of 6 genotypes including 3 species (*Malus baccata* Khrot, *M. kindsomer* and *M. orientalis*) and three varieties (M-26, Maruba and Top Red). Cluster 2 had majority of genotypes comprising of 13 *Malus* species and 23 varieties including *Prunus cerasoides*. The sub-cluster 2 a had 13 genotypes *M. baccata* Kashmir, *M. mandshurica*, *M. prunifolia*, Golden Delicious-3, STRD-1, M-7, Vance Delicious, Royal x Prima, Red Fuji, DR 382, Golden Delicious-2, Schlomit and Golden Delicious-1. In this sub-cluster, Red Fuji and Royal x Prima shared the highest similarity (85%) among all the genotypes used in the study. In the sub-cluster 2 b there were 21 genotypes. They were *M. simcoe*, *M. pumila*, *M. eseltine*, *M. micromalus*, *Prunus cerasoides*, *M. baccata* Dhak, *M. baccata* Rohru, *M. baccata* Shillong, *M. baccata* Kinnaur, Red Delicious,MM-106, Liberty, Golden Delicious-5, Tydeman's Early Worcester, EC-115820, E.M. Wilson, EC-768527, STRD-2, STRD-4, Michael and STRD-3. The highest similarity (83%) in this sub-cluster was shared by Tydeman's Early Worcester and EC-115820, and *M. baccata* Kinnaur and STRD-2 though grouping in different sub-sub clusters. The sub-cluster 2 c had two genotypes viz., *M. sargentii* and Golden Delicious-4.

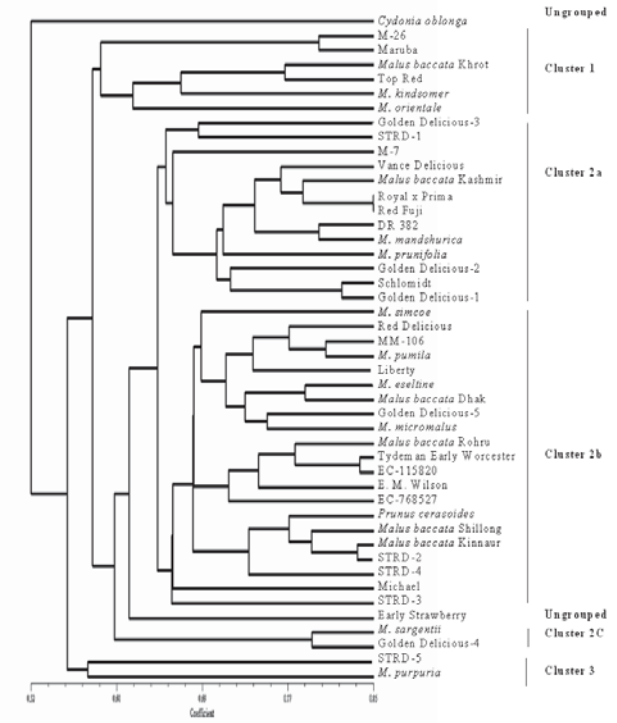

Fig. 2. Dendrogram derived from cluster analysis using Jaccard's coefficient and UPGMA of apple genotypes.

All the genotypes except *Cydonia oblonga* and Early Strawberry could be identified (Fig. 2) in the present study using ISSR markers reliably distinguishes the morphologically similar genotypes e.g. based on the morphological traits Golden Delicious 1, 2, 3, 4 are different strains of Golden Delicious and *Malus baccata* Shillong, *Malus baccata* Rohroo, *Malus baccata* Dhak, *Malus baccata* Kinnaur are the different strains of *Malus baccata* collected from different areas of cultivation, all are reported to be genetically different and thus shared different clusters. The genotypes Royal x Prima, Red Fuji, Vance Delicious, Schlomidt and DR-382 were earlier reported to be different morphologically although with insignificant differences but in the present study they shared the same Cluster 2.

The study has shown that the ISSR markers can effeciently be employed for elucidating the genetic diversity in apple genotypes (Goulao *et al.*, 5; Lodhi *et al.*, 14, Leibhard *et al*., 13; Goulao *et al.*, 5; Guilford *et al.*, 6). It has been shown that apple is highly cross pollinated in nature. The cluster and sub-cluster formation did not show any distinct grouping of the species/genera/ hybrids/chance seedlings/mutants confirming high degree of out crossing and cross compatability in the available gene pool.The results also suggested that apple being a highly heterozygous species coupled with high rate of outbreeding resulted in wide variation in evolution of new/superior genotypes. As evident the different genera did not grouped with *Malus* species though being a member of group Pomoideae sharing about 50% similarity with the genotype germplasm selected in the study. Based on the overall band statistics and genetic diversity study the highly informative primers were primer ISSR-1, primer ISSR-3, primer ISSR-6, primer ISSR-7, primer UBC-808, primer UBC-829, primer UBC-854 and primer UBC-886.

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