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RESEARCH ARTICLE

Inter Simple Sequence Repeat (ISSR) markers based genetic characterization of selected Delicious group of apple cultivars

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Abstract

Present study report genetic characterization in selected Delicious group of apple cultivars from Uttarakhand (West Himalaya) using Inter Simple Sequence Repeat (ISSR) markers. The samples were collected from three different cultivars (i.e. Red, Royal and Golden Delicious) representing six different locations/orchards at 1771 to 2780 m asl. Of the 45 screened ISSR primers, 14 produced 129 clear and reproducible fragments ranging from 220 to 1400 bp in size with an average of 7.92 fragments per primer. The ISSR markers revealed 8.26% to 52.89% polymorphism (Pp) in selected cultivars highest in Red Delicious from Khabrar location and lowest from Naugaoun location. Similarly, the expected heterozygosity (He) was in the range of 0.035 to 0.186 with highest in Royal Delicious from Khabrar and lowest from Naugaoun location. Analysis of molecular variance revealed 27% to 47% variance among cultivars and 53 to 73% within cultivars.

Introduction

The domesticated apple (*Malus×domestica* Borkh.) is one of the most important fruit crops of the colder and temperate parts of the world (Zohary et al., 2000). Apple is widely consumed fruit and reported to reduce risk of lung cancer, asthma, type-2 diabetes, thrombotic stroke, ischemic heart disease, and proliferation activities (Knekt et al., 1997; Knekt et al., 2002; Boyer and Liu, 2004). All these properties are linked to the presence of wide array of dietary antioxidants in the fruit. In addition, this fruit crops have been subjected to heavy selection and breeding (Zhang et al., 2011). A number of apple cultivars are available all over the world, which have been originated as a result of open pollinated seedlings, controlled crosses in breeding programme, and exploitation of naturally or induced somatic mutations (Goulao and Oliovera, 2001). All these cultivars varied in their morphological and genetic attributes and therefore, detail investigation on genetic characterization is required. It has been reported that the cultivars with higher genetic diversity have greater chances to bear good fruit quality with high productivity (Zhang et al., 2011). Studies also revealed that knowledge on genetic diversity and of genetic relationships among breeding materials has a great impact on crop improvement (Ganesh and Thangavelu, 1995). Moreover, maintenance and management of apple germplasm are labour intensive, costly, and require commitment of land resources for germplasm conservation efforts; therefore, determining genetic identity and genetic relatedness among accessions is reported to accelerate efficiency and utilization of germplasm collections in breeding programme (Kreosvich and McFerson, 1992; Russell et al., 1997). A number of approaches are available to determine the genetic diversity in crop species i.e., morphological and agronomic characteristics, biochemical and DNA based markers (Liu et al., 1997). Besides morphological markers, molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) are largely been used for germplasm characterization in order to assist and complement phenotypic assessments (Bretting and Widrlechner, 1995). Among these, ISSR markers are highly

reproducible and have been demonstrated in a wide range of applications in molecular biology and found suitable for genetic diversity analysis (Powell et al., 1996; Nagaoka and Ogihara, 1997; Guilford et al., 1997).

In India, apple is largely grown in Himalayan states and mostly in Jammu and Kashmir, Himachal Pradesh, and Uttarakhand. The variation in microclimatic conditions and wide altitudinal range in the Himalayan region are responsible for greater diversity in the morphology, fruit quality, production, size and shape of the apple. In addition, source of mother plants, root stock and type of cultivars are also responsible for such variations. Therefore, evaluation of genetic characterization and identification of promising cultivars for breeding and cultivation is essentially required. In view of this, an attempt aimed to evaluate and analyze the genetic attributes within and among selected apple cultivars collected from diverse localities of Uttarakhand (West Himalaya), India. The results of this study can be used for identification of promising cultivars for mass multiplication and further apple breeding programs.

Material and Methods

Site selection

Different cultivars of apple i.e., Red Delicious, Royal Delicious and Golden Delicious were collected from 6 different sites/orchards of Uttarakhand, India during February 2012. These cultivars considered most popular in the region for their fruit quality and production. The collection sites represented from an altitudinal range of 1771 to 2780 m asl (Table 1).

Plant material

The leaves of three to five accessions from each group (Red, Royal and Golden Delicious) were collected in plastic bags separately and immediately placed on ice packs and transported to laboratory to protect them from heat, air and light exposure. The leaf samples were then stored in -20 ºC till DNA extraction. All the experiments were performed in Biodiversity Conservation and Management Laboratory at G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora.

DNA extraction and PCR amplification

Leaf samples (1g) of each individual were homogenized in liquid nitrogen for DNA extraction. Genomic DNA was extracted from leaves using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications. PCR amplification was performed in total reaction volume of 20 µl with 2 µl of 10 X buffer, 2 µl of $MgCl_2$, 0.2 µM of each dNTPs, 0.2 µM of primer, 1 U of *Taq* polymerase (Hi media, India) and 20 ng of DNA template. The Thermocycler was programmed as 5 min at 95 ^{0}C followed by 35 cycles at 94 ^{0}C for 1 min, annealing at 52 ^{0}C and 55 ^{0}C for different primers (Table 2), extension at 72 ^{0}C for 2 min and final extension cycle of 7 min at 72 ^{0}C. A total of 45 primers were tested (Merck biosciences, Germany; and University of British Columbia; series 800-900). The amplification product were separated by 2% agarose gel and visualized under the UVI Pro Platinum Gel Imaging System (version 11.9, Cambridge, UK). Gene ruler TM DNA ladder mix (Genetix, India) was used as DNA fragment size marker.

Statistical analysis

Reproducible bands were scored as '0' and '1' in each accession respectively and a binary matrix was constructed. For assessing genetic diversity, the percentage of polymorphic loci (Pp), observed number of alleles (Na), effective number of alleles (Ne), Nei's (1973) gene diversity (He) and Shannon's information index (I) were calculated using GenALEx software version 6.1 (Peakall and Smouse, 2006). Relationships among cultivars were established by constructing a dendrogram using PHYLIP version 3.68. The binary matrix data was converted into PHYLIP input file with the help of CONVERT version 1.31 (Glaubitz, 2004). Distance matrices were produced using GENEDIST programme of the PHYLIP package (Felsenstein, 1995). Unrooted phylogenetic trees were constructed by using the neighbour-joining method (Saitou and Nei, 1987) using Neighbour component of the PHYLIP package. The statistical significance of the groups obtained was assessed by bootstrapping (10,000 replicates), using the programmes SEQBOOT, GENEDIST, NEIGHBOR, and CONSENSE (Felsenstein, 1995). An analysis of molecular variance (AMOVA) was performed to partition genetic variation among orchard using GenALEx software version 6.1 (Peakall and Smouse, 2006). Pearson correlation coefficient through SPSS version 16 was used to determine the relationship among the various parameters.

Results and Discussion

A total of 14 primers yielded a total of 129 bands ranging from 220 to 4000 bp in size with an average of 9.21 fragments per primer, of which 98.4% were polymorphic (Table 3). In Golden Delicious, the Pp, with a mean of 30.30%, ranged from 10.74% (Shitlakhet) to 45.45% (Chaubatiya). He ranged between 0.043 (Shitlakhet) and 0.178 (Chaubatiya) having a mean of 0.114. In Red Delicious, the Pp ranged from 25.62% (Mukhwa) to 52.89% (Khabrar), mean of 36.64%. He ranged from 0.101 (Naugaoun) to 0.184 (Khabrar) with mean 0.140. In Royal Delicious, the Pp ranged between 8.26% (Naugaoun) to 51.24% (Khabrar). Similarly, He was in the range of 0.035 (Naugaoun) to 0.186 (Khabrar). Nei's unbiased measure of genetic distance revealed maximum distance in Golden Delicious cultivars and the maximum genetic diversity was found in the Chaubatiya and Shitlakhet (0.384), however, lowest in the Naugaoun and Mukhwa (0.082). In Red Delicious, the maximum was found in the Satbunga and Mukhwa (0.249) and lowest in Chaubatiya and Khabrar (0.075). In Royal Delicious, the maximum was found in the Chaubatiya and Naugaoun (0.324) and lowest in the Naugaoun and Mukhwa (0.102).

Table 1. Details of apple sample collection sites

S. No	Location	Altitude (m asl)	Latitude (N)	Longitude (E)
1	Naugaoun	1771	$30^0$46'0.894"	$37^0$88'51.9"
2	Chaubaitya	2040	$29^0$40'11.7"	$79^0$36'50.8"
3	Khabrar	2200	$29^0$26'20.3"	$79^0$36'32.3"
4	Satbunga	2300	$29^0$26'22.9"	$79^0$36'38.7"
5	Shitlakhet	2000	$29^0$35'36.9"	$79^0$32'41.5"
6	Mukhwa	2780	$31^0$2'76.0"	$78^0$46'45.2"

Table 2. List of ISSR primers used for the amplification of delicious group of apple cultivars

S. No	Primer	Sequence (5' - 3')	Length (bp)	No. of fragments amplified	Annealing temperature (^{0}C)	Polymorphic band	% polymorphism
1	82974	$(CA)_6GT$	14	8	52	8	100
2	82981	$(CAC)_3GC$	11	13	55	13	100
3	82979	$(GA)_5CC$	14	10	52	10	100
4	82982	$(GAG)_3GC$	11	14	55	14	100
5	82972	$(CT)_8GC$	18	9	55	8	89
6	82977	$(GA)_5GG$	14	10	55	10	100
7	82970	$(CT)_7TG$	18	9	55	9	100
8	82978	$(GT)_5GG$	14	8	55	8	100
9	868	$(CT)_7GC$	18	7	52	7	100
10	880	$(GGAGA)_3$	15	9	52	9	100
11	857	$(AC)_8YG$	18	10	52	9	90
12	887	$DVD(TC)_7$	17	10	55	10	100
13	889	$DBD(AC)_7$	17	7	52	7	100
14	845	$(CT)_8RG$	18	5	52	5	100

Where: Y = (C,T); D = (A,G,T); V = (A,C,G); B = (C,G,T); R = (A,G)

Table 3. Genetic variability of Golden delicious, Red delicious and Royal delicious cultivars

Golden delicious	%P	Na	Ne	I	He	uHe
Chaubatiya	45.45%	1.149	1.316	0.261	0.178	0.194
Khabrar	44.63%	1.058	1.236	0.221	0.144	0.16
Satbunga	39.67%	1.017	1.251	0.215	0.145	0.161
Shitlakhet	10.74%	0.521	1.076	0.063	0.043	0.052
Naugaoun	14.05%	0.504	1.121	0.091	0.064	0.077
Mukhwa	27.27%	0.711	1.195	0.16	0.11	0.132
Mean	30.30%	0.826	1.199	0.169	0.114	0.129

Red delicious						
Chaubatiya	39.67%	1.041	1.243	0.214	0.143	0.163
Satbunga	33.06%	0.934	1.237	0.195	0.133	0.16
Khabrar	52.89%	1.207	1.314	0.277	0.184	0.201
Shitlakhet	42.15%	1.05	1.298	0.247	0.168	0.202
Naugaoun	26.45%	0.702	1.179	0.148	0.101	0.112
Mukhwa	25.62%	0.76	1.205	0.159	0.111	0.133
Mean	36.64%	0.949	1.246	0.207	0.14	0.162
Royal delicious						
Chaubatiya	26.45%	0.752	1.191	0.156	0.107	0.129
Satbunga	23.14%	0.752	1.182	0.142	0.099	0.119
Khabrar	51.24%	1.215	1.327	0.276	0.186	0.203
Shitlakhet	28.93%	0.893	1.232	0.18	0.126	0.151
Naugaoun	8.26%	0.405	1.065	0.05	0.035	0.04
Mukhwa	24.79%	0.62	1.154	0.134	0.09	0.102
Mean	27.13%	0.773	1.192	0.156	0.107	0.124

Pp= the percentage of polymorphic loci; Na= No. of Different Alleles; Ne= No. of Effective number of alleles; I= Shannon's information index; He = Expected Heterozygosity = 2 * p * q; uHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He where for Diploid Binary data and assuming Hardy-Weinberg Equilibrium, q = (1 - Band Freq.)^0.5 and p = 1 - q.

A dendrogram based on Neighbour joining method separated all locations of the studied cultivars (Fig. 1A-C). For instance, Golden Delicious separated in two main group, group I include only Khabrar locations while group II includes remaining one (Fig. 1A). In case of Red Delicious and Royal Delicious, group I include Naugaoun and group II remaining all the other localities (Fig. 1B-C). Analysis of Molecular Variance (AMOVA) revealed variations in the selected cultivars (Table 4). For instance, 55% within and 45% variations among locations in Golden Delicious, 73% within and 27% among location in Red Delicious and 53% within and 47% among locations in Royal Delicious. While analyzing the genetic diversity across altitudinal range, Pearson correlation coefficient revealed no relationship between altitude and genetic diversity parameters i.e., gene diversity (He) and percentage of polymorphism in the selected cultivars viz. Red Delicious, Royal Delicious, Golden Delicious.

Assessment of genetic variability within a cultivated crop has important consequences in plant breeding and the conservation of genetic resources. It is particularly useful in the characterization of individual accessions and cultivars for detecting duplications of genetic material in germplasm collections, and for selection of parents for breeding hybrids (Davilla et al., 1998). Several molecular markers have widely been used for genetic variability assessment and cultivar identification in large number of species like pear, olive and grapes (Monte-corvo et al., 2001). In the present study, assessment of genetic diversity using ISSR markers for Delicious group of apple cultivars viz., Golden, Red and Royal Delicious did not show variation in genetic diversity parameters. These variations are largely attributed to source of mother plants as different material for apple cultivation might be taken from different mother stock. As such, ISSR markers have been used for identification of variety/cultivars in numerous plant species i.e., apple (Goulao and Oliveira, 2001), strawberry (Arnau et al., 2003) and mulberry (Kar et al., 2008). High intra specific variation within the cultivar in the present study is in agreement with the Lopes et al. (2004) who reported intra-varietal polymorphism with 60% of allele differences in 130 olive samples corresponding to 67 different olive cultivars collected from same locations. Similarly, two microsatellite-based methodologies (SSR and ISSR) were used (Goulao and Oliveira, 2001) for potential use in fingerprinting and determination of the similarity in 41 commercial cultivars of apple and found that the SSR and ISSR markers were useful for cultivar identification and assessment of phenetic relationships. Several other studies have been performed on apple cultivars using different molecular markers. Genetic variability of several phenotypically differing apple trees, based on DNA polymorphism analysis using ISSR markers was determined (Smolik et al., 2004). Results demonstrated a high level of the inter-cultivar polymorphism in the apple trees. Therefore, for assessing genetic relationships between apple cultivars, ISSR-PCR can effectively be used in apple breeding programs for designing crosses and production of hybrids.

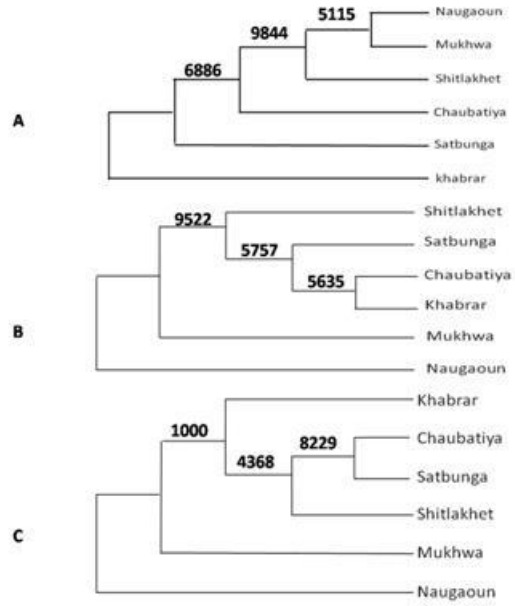

Fig 1: Dendrogram showing genetic relationship in selected apple cultivars collected from six different locations (A) Golden delicious (B) Red Delicious, and (C) Royal delicious

Table 4. Analysis of Molecular variance (AMOVA) based on 14 different ISSR markers from of Red delicious, Royal delicious and Golden delicious

	Degree of freedom	Sum of squared deviation	Mean square deviation	Estimated variance	%
Golden delicious					
Among Orchards	5	242.147	48.429	9.147	45 %
Within Orchard	19	208.333	10.965	10.965	55 %
Red delicious					
Among Orchard	5	155.567	31.113	4.719	27 %
Within Orchard	18	225.933	12.552	12.552	73 %
Royal delicious					
Among Orchard	5	208.442	41.688	8.488	47 %
Within Orchard	17	164.167	9.657	9.657	53 %

A study has been done using RAPD marker to check the authenticity of quality planting material from five different commercial orchards located in different counties of Transylvania, Romania, and emphasized on the fact that there should be available standard forms of the interested cultivars from which DNA could be analyzed and used as control (Mitre et al., 2009). The study found an obvious polymorphism at molecular level, illustrated by more or less large genetic distance within the same cultivar depending on location. In this study using ISSR primers low level of polymorphism was found among the cultivars. While comparing studied cultivars, comparatively higher polymorphism was recorded in Red Delicious (37%) and Golden Delicious (34%) as compared to Royal Delicious (28%). A similar report available from Transylvania, Romania showed low polymorphism in Jonathan and Starkrimson but high in Golden Delicious, Florina and Idared cultivars (Mitre et al., 2009). High polymorphism in some cultivars might be associated with management as mother plants belonged to locally grown clones of the cultivar multiplied than to the standard form of the respective cultivars. It has been seen that most of the orchards has their own mother plant. Although, a study on Grapes concluded that at molecular level, no variability exists depending on location (Pop et al., 2003) is not in agreement with the present study, which showed variation among the locations and cultivars type. In addition, microsatellites were also used for genetic diversity analysis (Hokanson et al., 2001) and variety discrimination (Hokanson et al., 1998). Using the 8 microsatellites markers, 66 accessions of Golden Delicious were characterized for genetic diversity, however, 7 pairs of accessions could not be differentiated due to sport mutations of closely related genotypes (Hokanson et al., 1998).

Relationships among 159 accessions of wild and domesticated apples including Iranian indigenous apple cultivars, selected wild species, and old apple scion and rootstock cultivars from different parts of the world was showed that there were more intra-orchard variation as compared to inter orchard in apple (Gharghani et al., 2009). Similarly, a total of 29 accessions including 12 Chinese wild species and 17 popular commercial cultivars were assessed for genetic diversity and their linkages with Xinjiang wild apple indicated that Chinese wild apple species have considerable higher genetic diversity and can be used in breeding programme (Zhang et al., 2011). The study conducted by Evans et al. (Evans et al., 2010) identified variations in the reported pedigrees of some of the apple cultivars and found that accessions of cultivars like Priscilla differed for 53% of their SSR loci. The study also found that accessions of Tydeman's Early Worcester were also represented by several different accessions. These results are in agreement with the present study where a 27 - 47% variation among locations and 53 to 73% in the accessions of different cultivars was reported.

Conclusion

The present study found that there is intraspecific variation in the selected apple cultivars inspite of the fact that these were propagated through grafting. This intraspecific variation might be originated from the source of material used for grafting. Therefore, in order to reduce variation, source of mother plants should be uniform. Higher genetic diversity in the Chaubatiya locations for golden Delicious and Khabrar for Red and Royal Delicious suggest that these locations are suitable for cultivation of these cultivars.

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