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Characterization of genetic variation between local and exotic Iranian strawberry (*Fragaria×ananassa* Duch.) cultivars using amplified fragment length polymorphism (AFLP) markers

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Abstract

The goal of this study was to determine the level of genetic diversity and relatedness among 17 local strawberry cultivars collected from 'Kurdistan' province (north-west of Iran) with 13 exotic cultivars, using ten AFLP markers. The 10 AFLP primer pairs produced 228 polymorphic bands. The number of polymorphic bands per primers ranged from 11 to 33, whereas the maximum was observed in *E36/M35* primer pairs. The genetic diversity (GD) and markers PIC values ranged from 0.28 to 0.77 and 0.33 to 0.48, with an average 0.54 and 0.41, respectively. Cluster analysis grouped cultivars in four distinct clusters and all local and exotic cultivars separated in referred clusters. We obtained narrow range of diversity in the group of exotic cultivars (from 0.27 to 0.44) while higher diversity values (from 0.33 to 0.77) were observed in local cultivars. The results clearly demonstrate that AFLP markers can be used in a genetic diversity study as well as in genotypic identification of strawberries.

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Introduction

The cultivated strawberry (*Fragaria×ananassa* Duch.), an octoploid (2n=56) hybrid between the Scarlet or Virginia strawberry (*F. virginiana* Duch.) and the pistillate South American *F. chiloensis* (L.) Duch., is a dicotyledonous, perennial low-growing herb grown in most arable regions of the World. The commercially important strawberries (*Fragaria* × *ananassa* Duch.) belong to the family *Rosaceae* and the genus *Fragaria*, which comprises 23 species (Rousseau-Gueutin et al., 2009). All of these strawberries have seven basic types of chromosomes. However, they exhibit a series of ploidy levels, ranging from diploid species such as Fragaria vesca (2n = 2x = 14), to decaploid species, such as some accessions of *Fragaria iturupensis* (2n = 10x = 70). The cultivated strawberry, *F.* × *ananassa*, is an octoploid (2n = 8x = 56) (Nathewet et al., 2010). Because of this, it is difficult to breed it successfully to develop new varieties with differing genetic characteristics. Marker-assisted selection (MAS) will be of benefit with the help of polymorphic markers. However, for MAS, a genome scan of the progeny is currently not possible because of a lack of evenly distributed markers that are polymorphic in the respective breeding population (Shulaev et al., 2008). Crop development efforts with strawberries began in the mid-18th century (Hancock et al., 1996) and significant progress has been made in the past 50 yr. Today, more than 500 commercial cultivars are grown worldwide (Hancock et al., 1999). However, the commercial strawberry has a narrow germplasm base, even though its progenitor species have an extensive geographical range (Sjulin and Dale, 1987; Hancock et al., 2002). Strawberry was imported into Iran about 100 years ago from France. Its cultivation area in Iran is about 3000 ha and the total production averages about 21 Kt yearly. Strawberry can be grown successfully in different parts of Iran. Strawberry cultivation and production in Iran has doubled in the last two decades. At present the two main regions of the country producing strawberries are Kurdistan (North-west) and Golestan (North) provinces (Eshghi et al., 2007). Kurdistan province grows about 80% of the total strawberry production in the Iran. Currently, morphological traits are used to certify the identity of strawberry cultivars, but this information often does not yield clear answers concerning discrimination of a plant variety, due to ambiguous differences or phenotypic modifications caused by environmental factors (Garcia et al., 2002). Molecular markers are increasingly used in breeding programs of many horticultural crops. The introduction of molecular biology techniques, such as DNA-based markers, allows direct comparison of different genetic material independent of environmental influences (Weising et al., 1995). The degree of similarity between the banding patterns can provide information about genetic similarity, and relationships between the samples studied. AFLP has already been used successfully with a number of crops such as rice (Mackill et al., 1996), tea (Paul et al., 1997), almond (Sorkheh et al., 2007), Coffee (Steiger et al., 2002), chickpea (Talebi et al., 2008), wheat (Talebi et al., 2012) and has been shown to reveal significant levels of DNA polymorphism in plants (Vos et al., 1995). The advantages of this technique include the large number of loci analyzed, high polymorphism levels, high reproducibility without prior sequence knowledge, and genome-wide marker distribution (Powell et al., 1996). So far, the genetic diversity of Iranian local strawberry accessions is not well understood. This study was designed to assess genetic diversity of some local and exotic strawberry genotypes at the molecular level using AFLP markers.

Materials and methods

Thirty genotypes of strawberry, which 17 of them collected from different villages of Kurdistan province, north-west of Iran. All of these genotypes named as 'Kurdistan' variety, while huge morphological differences existed between them. Remained thirteen genotypes are famous exotic varieties that grown in most strawberry production zones of Iran (Table 1). Genomic DNAs were extracted from approximately 2 g of young leaves

tissue from all genotypes. Leaf tissue was ground to a fine powder in liquid nitrogen and stored at -70 °C until total genomic DNA was isolated. DNA was extracted using the CTAB method according to Lassner et al. (1989) with the modification described by Torres et al. (1993). The DNA final concentration was determined by agarose-gel electrophoresis using a known concentration of uncut λ DNA as a standard. A 200 ng sample genomic DNA of each accession was digested simultaneously with 10 units each of *MseI* and *EcoRI* (Vos et al. 1995) (Table 2) at 37 °C for 4 h. Following digestion, *EcoRI* and *MseI* adapters (Cinagene, Iran) were ligated to restricted fragments at 20 °C for 2 h and the digested fragments were preamplified using 20 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min according to the manufacturer's instructions. Selective amplification was then performed according to the manufacturer's instructions (Cinagene, Iran). Diluted preselective PCR product samples (3.75 µL) were used as DNA templates for selective amplifications. The PCR selective amplification was carried out in the same manner as PCR preamplification. For selective amplification, the following cycle profile was used with 12 primer combinations: 2 min at 94 °C; 10 cycles: 30 s at 94 °C, 30 s at 63 °C (touchdown 1 °C per cycle to 54 °C), 2 min at 72 °C and 23 cycles: 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C. All amplifications were performed in a BioRad thermocycler (BioRad Laboratories Inc., Hercules, CA, USA). Amplification reaction products were separated on a 6% denaturing polyacrylamide gel in a 50 cm Sequi-Gen GT Sequencing Cell gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). The amplified fragments were detected by silver staining. The resulting gels were scored as present (1) or absent (0) for each marker and a binary data matrix was created. DARwin version 5.0 was used for calculating pair-wise genetic distances and for constructing the dissimilarity matrix (Perrier et al., 2003). The dissimilarity matrix thus obtained was subjected to cluster analysis using the un-weighted neighbour-joining (UNJ) method (Gascuel, 1997), followed by bootstrap analysis with 1,000 permutations to obtain a dendrogram for all the 30 accessions (Perrier et al., 2003). Polymorphic information content (PIC) values were calculated for each AFLP primer combination according to the formula: PIC= 1- $\Sigma(Pij)^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (Botstein et al. 1980).

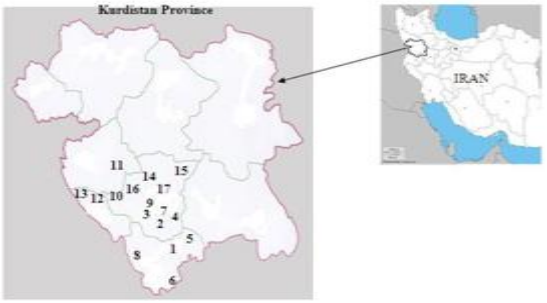

Fig. 1. Map of Iran showing the approximate areas of collection site of the 17 local strawberry varieties used in this study.

Results

The polymorphism rates of AFLP primers were evaluated using thirty strawberry genotypes: 17 local and 13 exotic cultivars. Among the 30 sets of *Eco*RI/*Mse*I primers with the three nucleotides extension surveyed, the ten most polymorphic sets were selected for genotyping 30 strawberry genotypes. Figure 2 shows a representative amplification pattern obtained using *E36/M33* primer combinations. The summary of AFLP markers produced by 10 primer-pairs across all genotypes is given in Table 3. The 10 primer-pairs generated a total of 525 bands of which 228 (43%) were polymorphic over all the genotypes. The capability of different primer-pairs to generate polymorphic AFLP markers varied significantly, ranging from 11 to 33 polymorphic bands per primer-pair over all the genotypes. The percentage of polymorphism ranged from 33% (in the combination *E45/M65*) to 54% (in combination *E36/M35*) (Table 3). PIC values ranged between 0.34 and 0.48, with a mean of 0.41. PIC provides an estimate of the discriminatory power of a marker by taking into account not only the number of alleles,

but also their relative frequencies. The distribution of PIC scores was nearly uniform (random) for all 525 polymorphic AFLP markers. Results show that most of the markers have a high discrimination power. The average genetic distance (GD), based on F_{st} values among 30 genotypes was 0.54, ranging from 0.28 to 0.77.

Table 1. List of 30 strawberry (*Fragaria* x *ananassa* Duch.) varieties used for SSR fingerprinting.

No.	Genotype	Source	No.	Genotype	Source
1	NashurVasat	Local (Kurdistan province-Iran)	16	Sanandaj2	Local (Kurdistan province-Iran)
2	Barkulan	Local (Kurdistan province-Iran)	17	Sanandaj1	Local (Kurdistan province-Iran)
3	Yaminan	Local (Kurdistan province-Iran)	18	Missionary	Exotic (Europe)
4	NashurSafli	Local (Kurdistan province-Iran)	19	Ventana	Exotic (Europe)
5	Yaminan2	Local (Kurdistan province-Iran)	20	Fresho	Exotic (Europe)
6	Toriver1	Local (Kurdistan province-Iran)	21	Karcy	Exotic (Europe)
7	Karabad	Local (Kurdistan province-Iran)	22	Marak	Exotic (Europe)
8	Toriver2	Local (Kurdistan province-Iran)	23	Gaviota	Exotic (Europe)
9	Qalaji	Local (Kurdistan province-Iran)	24	Camarosa	Exotic (Europe)
10	Danikesh	Local (Kurdistan province-Iran)	25	Diamant	Exotic (Europe)
11	Sanandaj3	Local (Kurdistan province-Iran)	26	Paroos	Exotic (Europe)
12	Sanandaj4	Local (Kurdistan province-Iran)	27	Classica	Exotic (Europe)
13	Drileh	Local (Kurdistan province-Iran)	28	Pajaro	Exotic (Europe)
14	Dorood	Local (Kurdistan province-Iran)	29	Selva	Exotic (Europe)
15	Shian	Local (Kurdistan province-Iran)	30	Queen	Exotic (Europe)

Table 2. Oligonucleotide sequences used in the AFLP analysis.

		Sequence
Adapters		
	EcoRI adapter	5'-CTCGTAGACTGCGTACC
		CATCTGACGCATGGTTAA-5'
	MseI adapter	5'-GAC GAT GAG TCC TGA G / TAC TCA GGA CTC AT-5'/
Non-selective primers	E-000	5'-GACTGCGTACCAATTC-3'
	M-000	5'-GATGAGTCCTGAGTAA-3'
Selective primers		
	E-ATG (E45)	5'-GACTGCGTACCAATTCATG-3'
	E-ACT (E38)	5'-GACTGCGTACCAATTCACT-3'
	E-ACC (E36)	5'-GACTGCGTACCAATTCACC-3'
	M-AAG (M33)	5'-GATGAGTCCTGAGTAAAAG-3'
	M-AAT (M34)	5'-GATGAGTCCTGAGTAAAAT-3'
	M-ACA (M35)	5'-GATGAGTCCTGAGTAAACA-3'
	M-AGT (M42)	5'-GATGAGTCCTGAGTAAAGT-3'
	M-ATA (M43)	5'-GATGAGTCCTGAGTAAATA-3'
	M-ATC (M44)	5'-GATGAGTCCTGAGTAAATC-3'
	M-ATG (M45)	5'-GATGAGTCCTGAGTAAATG-3'
	M-CAC (M48)	5'-GATGAGTCCTGAGTAACAC-3'
	M-GAA (M63)	5'-GATGAGTCCTGAGTAAGAA-3'
	M-GAG (M65)	5'-GATGAGTCCTGAGTAAGAG-3'

Table 3. Characteristics of the amplification products obtained with 10 AFLP primer pairs used to analyze the genetic diversity of 30 strawberry cultivars.

No	Primer Combination	Total number of amplification products	Number of polymorphic bands	Percent of polymorphism	PIC value
1	*E36/M33*	52	21	40	0.42
2	*E36/M34*	64	31	48	0.44
3	*E36/M35*	61	33	54	0.48
4	*E36/M42*	58	25	43	0.46
5	*E36/M43*	51	18	35	0.35
6	*E36/M44*	56	22	39	0.34
7	*E38/M45*	36	14	39	0.39
8	*E38/M48*	48	22	45	0.41
9	*E45/M63*	66	31	47	0.47
10	*E45/M65*	33	11	33	0.39

To evaluate the phylogenetic relationships between strawberry genotypes a dendrogram based in the 228 loci AFLPs was constructed by UNJ. Cluster analysis of 30 genotypes showed a clear separation of the strawberry accessions. Four clusters that consist of two or more accessions can be distinguished (Fig. 3). First cluster consist of eight genotypes, which all of them local cultivars that originated from south zones of 'Kurdistan' province. Second cluster consisted only two cultivars (Queen and Pajaro). Third cluster contained six genotypes, which all of them are exotic cultivars originated from Europe, same as two genotypes in second cluster. Fourth cluster consisted 14 genotypes which five of them (Marak, Karcy, Fresho, Missionary and Ventana) were exotic genotypes and remains were local cultivars that originated from central and north zones of 'Kurdistan' province. A further evaluation of the relationships among the strawberry cultivars taken into consideration has been obtained through the principal coordinate analysis (PCOA) based on the same dissimilarity matrix and cultivars were plotted in the coordinate system for the first two coordinates which accounted for 48% and 29% of the variation, respectively (Fig. 4). Most of the cultivars are separated by the first or second PCoA (Fig. 4) which demonstrated distinct groups of cultivars corresponding to cluster analysis (Fig. 3).

Discussion

Whereas phenotypic descriptors may vary with climate, geography or cultural practices, forensic tools based on genetic material allow cultivar authentication with great certainty. DNA markers provide a robust, rapid, and relatively inexpensive means to differentiate closely related plant materials. Strawberry plants are propagated vegetatively and can be easily misidentified based on phenotype (Bassil et al., 2006). Being a vegetative propagated species, collected strawberry accessions show considerable levels of genetic divergence. This is the first report on the use of AFLPs for fingerprinting and evaluating genetic relationships of Iranian local strawberry cultivars. The percentage of polymorphic AFLP fragments detected in this studied cultivars is 43%. The AFLP markers amplified from each pairs of primers are sufficient to distinguish among all 30 cultivars, some of which are belonged to closely geographic origins. Therefore, AFLPs prove very effective at fingerprinting strawberry cultivars. We obtained narrow range of diversity in the group of exotic cultivars (from 0.27 to 0.44) while higher diversity values (from 0.33 to 0.77) were observed in local cultivars. The results clearly demonstrate that AFLP markers can be used in a genetic diversity study as well as in genotypic identification of strawberries, as noted by Degani et al. (2001), Doulati Baneh et al. (2009).

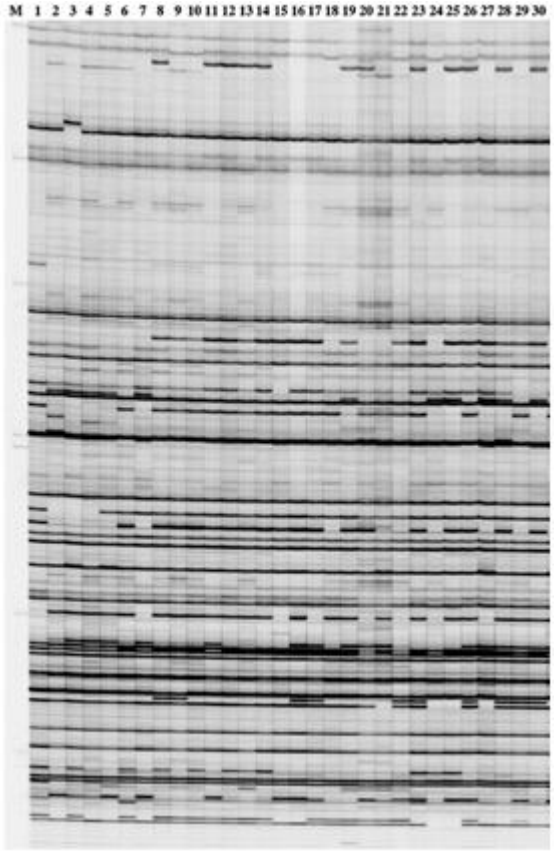

Fig. 2. Amplification profile obtained with AFLP primer pair E36/M33 detected in 30 strawberry cultivars.

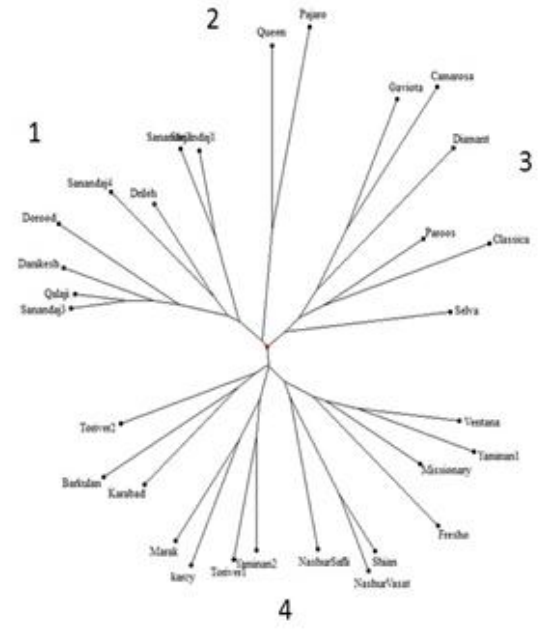

Fig. 3. Un-weighted pair grouping method of arithmetic averages dendrogram of 30 strawberry varieties based on genetic distances computed from AFLP markers.

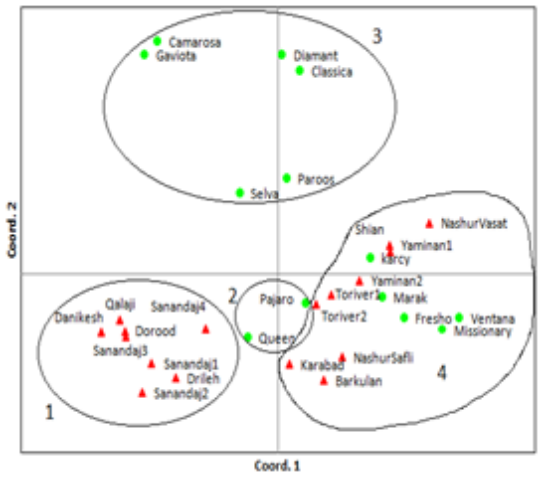

Fig. 4. Two-dimensional representation of genetic relations among 30 strawberry cultivars using AFLP data.

The exploitation of crosses between genetically distant parents and those from diverse local sources may produce higher heterosis, better genetic recombination and segregation in their progenies and result in varieties with broad genetic base (Chahal and Gosal, 2002). The selection of genotypes for this study is primarily based on different geographic origin and also the important role of north-west of Iran in strawberry production for poor farmers. Therefore, we believe that there needs a molecular markers studies as a complementary studies for the morphological traits in the field. It will reduce the amount of materials for study as well as the costs of experiments. It is expected that when such diverse genotypes are involved in breeding programs, as a result of reshuffling of the alleles due to recombination, there are better chances for the appearance of transgressive segregation with beneficial traits that can be selected to extract high yielding cultivars with desirable trait combination. Further, large amount of genetic variation which exists between strawberry genotypes can be used efficiently for gene tagging and genome mapping of crosses to introgress the favorable traits such as high yield potential, disease and insect resistance into the cultivated genotypes (Hallauer, 1999; Stuber, 1994). The existing genetic diversity observed in studied cultivars, indicated the efforts underway to widen

the genetic base of strawberry for various traits. There could be a possibility to exploit hybrid in strawberry. The previous crossing programs in most research centers of Iran were based on only phenotypic characters. The current study confirmed the importance of molecular studies beside the morphological data in detecting genetic variation among genotypes in selecting diverse parents to carry out a new crossing program successfully. Results of the present study indicate that extent of genetic variability in Iranian strawberry gene pool seems to have remained quite constant. This constancy should be considered of qualitative relevance, as it indicates that local and exotic genotypes germplasm was enriched by material different from the native and locally adapted germplasm, which resulted in a consistent broadening of the genetic background in different regions from north-west of Iran. Therefore, the classification obtained for these Iranian strawberry genotypes, based on morphological traits and molecular markers will be a useful tool to Iranian breeders to plan crosses for high yield and positive qualitative characters by choosing genotypes with appropriate diversity. Recommendations for conservation and breeding based solely on molecular markers should be made with caution until the quantitative diversity can be evaluated intensively. However, some general considerations can be outlined for the appropriate use of genetic diversity to build an efficient core germplasm collection and also to choose parental lines that would avoid the effect of inbreeding depression as has been suggested for *Fragaria* L. species (Shaw, 1995, 1997). In the future, it would be interesting to expand these analyses including accessions from other geographic areas of Iran such as North provinces (Mazandaran and Golestan) to strengthen the analyses of the pattern of variation present.

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