Evaluation of genetic variations and phylogeny of the most popular pear *(Pyrus communis* L.*)* cultivars in Duhok city using AFLP markers

Evaluación de variantes genéticas y filogenia de la pera común *(Pyrus communis L.)* cultivada en la ciudad Duhok empleando AFLP como marcadores moleculares

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ABSTRACT

Introduction: Genotyping and evaluation of genetic variation and polymorphic information content of the locally cultivated pear (*Pyrus communis* L.) might play an important role in building the genetic bank. These are also immensely important for present and future pear breeding program in the region. **Methods:** In the current study, AFLP markers have been employed to estimate the level of genetic diversity and to assess the phylogeny among the seven most popular pear cultivars in Duhok city. **Results:** Eight selective primer combinations generated a total of 653 AFLP fragments from which 445 (68.2%) fragments were polymorphic. The number of visible amplified products per primer combination were varied and ranged from 66 to 96 bands. The highest percentage of polymorphism (78.4%) was observed by the primer pair P174/M182, while the lowest percentage of polymorphism (58.6%) was observed by the primer pair P174/M100. The highest PIC (0.85) was obtained with the primer combination P174/M182, while, the lowest PIC (0.49) was obtained by the primer combination P174/M307. The genetic distance was ranged from 0.1348 (between Danimarki and Amreki cultivars) to 0.3131 (between Italy and Zaafaran2 cultivars). Based on the AFLP data, all the seven pear genotypes were successfully clustered into two separate clusters (C1 and C2) with an out-group of Itali cultivar. **Conclusions:** Overall, it can be concluded that there was high polymorphism among the studied genotypes. Also, it can be stated that the AFLP was a reliable and a powerful technique in genotyping and discriminating of respective pear cultivars.

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INTRODUCCTION

Pear (*Pyrus spp.*) is one of the economically and nutritionally valuable and popular rosaceous fruit crop being cultivated in temperate climates, it originated in the mountain regions of south-western China and have spread all over the world [1, 2]. It considered as the third most significant fruit crop after grapes and apples in temperate areas [3]. Pear, which has a smooth and delicate pleasant taste, is nutritionally valuable fruit due to its low caloric value and considerable concentration of vitamins including; A, B1, B2, B3, and C. Besides, Pear also contains high amount of minerals namelycalcium, magnesium, sodium, phosphorus, potassium, and iron. As well as fibers which give significant results in treatment of, intestine inflammation, constipation and kidney stones [4].

The genus *Pyrus* (2n = 34 chromosomes), which belong to the tribe Pyreae, subfamily *Pomoideae* in the *Rosaceae* family, is widely distributed on six continents particularly in Asia, Europe, and Africa [3, 5, 1, 6]. Classically, based on domestication and the geographical distribution, *Pyrus* species are divided into two major native groups. The European pears (*P. communis),* which is also called the Occidental group that, is the most cultivated species in Europe and in the United State. While, the Asian pears (Oriental pears), which include; *P. ussuriensis*, *P. bretschneideri*, *P. sinkiangensis*, and *P. pyrifolia*, are the most grown pear species in East Asian countries including China and Japan [7, 8].

The genus *Pyrus* is variously described as a polymorphic fruit species which consist of 22 primary species with at least six naturally interspecific hybrids, and at least three artificial hybrids [6]. However, the easily cross-pollinations and the ambiguous taxonomic status of the obtained crosses make the determination of the exact number of *Pyrus* species relatively difficult. The presence of such huge number of species, sub-species, cultivars, hybrids, and clones make the application of reliable molecular tools crucial for their accurate identification, genetic characterization, and verification [9].

Traditionally, numerous methodologies have been used for identification and characterization of pear cultivars depending on the morphological and physiological characteristics. However, most of these phenotypic traits are influenced by environmental factors, plant age, phenology, and are time consuming as well as not always available for analysis [10, 11]. After the advent of PCR technology, the molecular methods have become very popular in molecular identification, characterization, genotyping, and to determine the genetic diversity of many fruit species including pear [12, 13]. Among the most popular molecular approaches are; random amplified polymorphic DNA (RAPD), microsatellite markers (SSR), restriction fragment length polymorphism (RFLP), and the amplified fragment length polymorphism (AFLP) markers, which leaded to discover new relationships between cultivated and wild species [10, 6]. These DNA based techniques allowed researchers to distinguish species and cultivars at the molecular level through access to the stable information carried by DNA regardless the environmental, tissue, and growth stage of the plant [13].

A DNA barcoding technology AFLP, which combines DNA restriction with PCR amplification, is being considered as a powerful and suitable technique for characterization and fingerprinting plant species at the molecular level [6]. This technique has many advantages over the other molecular methodologies such as RAPD and SSR, due to its power to generate high number of polymorphic and informative bands per reaction with high reproducibility of banding patterns. This is mainly because of its higher specificity in the primer annealing to their corresponding complementary adapters [10, 1]. Due to the lack of comprehensive molecular characterization and diversity analysis of pear in the region, the AFLP technique was implemented in this study to molecular characterize and to assess the genetic diversity and phylogenetic relationships between pear cultivars in Duhok city.

MATERIALS AND METHOD

Plant materials

Fresh pear leave samples were collected from Malta Nursery for Agricultural Research in Duhok. Plant

materials were sampled during May 2017. The cultivars of pear (*Pyrus communis* L.) included in the current study were; Harmijali, Itali, Licont, Zaafaran1, Zaafaran2, Danimarki, and Amreki.

DNA Extraction

Genomic DNA for each sample was extracted from three gm of young fresh leaves according to a protocol described previously [14], with few modifications. In brief, leaves were socked in liquid nitrogen and then powdered using sterilized pre-chilled mortar and pestle, Crushed leaves, were mixed with 10ml of 2X CTAB buffer (CTAB 2gm, NaCl 5M, Tris-HCl 1M, and EDTA 0.5M). The composition was incubated for 30 minutes at 60°C in water bath with continuous shaking. The extraction was performed by adding an equal volume of chloroform/isoamyl alcohol (24:1, v/v) mixed well and centrifuged at 4000rpm for 30minutes. The top aqueous phase was transferred into a new sterile tube and re-extracted by an equal volume of chloroform/isoamyl alcohol, then centrifuged at 4000rpm for 30minutes. The aqueous phase was transferred into a new sterile tube and 2/3 volume of cold isopropanol was added to precipitate the genomic DNA. The precipitated DNA was then hooked out and dissolved in 500-1000µl of sterile TE buffer (10mM).

The purification of extracted genomic DNA from the previous step was performed by adding an equal volume of phenol/chloroform isoamyl (25:24:1, v/v), they gently mixed and centrifuged for 15mins at 12000rpm. After transferring the top aqueous phase to a new sterile Eppendorf tube, 2 volumes of absolute ethanol and 0.1 volume of sodium acetate was applied to the tube. After gentle mixing, the mixture was centrifuged at 12000rpm for 5 mins. The supernatant was discarded by gentle pipetting. The tube was left upside down on filter paper for 5 minutes to dry the pellet prior re-suspending it in 350-500µl of TE buffer and finally stored at -20°C for downstream processing.

AFLP analysis

The digestion reaction (30µl total volume per sample) was composed of sterilized deionized distilled water (18µl), 10X one-phor-all buffer (3µl), bovine serum albumin 10µg/µl (2µl), purified genomic DNA (250ng per sample), *Pst*1 10U/µl (0.5µl), and *Mse*1 10U/µl (0.5µl). The mixture was gently shaken by fingers, spin downed and incubated for three hours at 37°C. The ligation master mixture (25µl total volume per sample) was consisted of sterilized nuclease free water (4.4µl), 10X one-phor-all buffer (0.7µl) (Promega), ATP 10mM (0.5µl), *Pst*1 adapter 5pmol/µl (0.5µl), *Mse*1 adapter 5pmol/µl (0.5µl), digested DNA (18µl), and T4 DNA ligase 3U/µl (0.4µl). The ligation mixture was gently shaken by fingers, briefly spin downed and incubated overnight at 37°C. The diluted digested and ligated DNA were tested using 1% agarose gel. The pre-amplification reaction (20µl total volume per sample) was comprised of sterilized nuclease free water (9.3µl), 10X PCR buffer (2µl), dNTPs 2mM/µl (2.5µl), sevenfold diluted (1:7) ligated DNA (4µl), *Pst*1 and *Mse*1 pre-amplification primers 50ng/µl (1µl each), and*Taq* DNA polymerase 5U/µl (0.2µl) (Promega). The master mixture was briefly spin downed and was put into PCR machine. The PCR for pre-amplification reactions were carried out with the following cycling conditions; 30 cycles of 94°C for 30 seconds; 56°C for 30 seconds and 72°C for 30 seconds. A tenfold (1:10) dilutions have made from the pre-amplified products. The selective master mixture (20µl final volume per sample) was composed of sterilized nuclease free water (9.7µl), 10X PCR buffer (2µl), dNTPs 2mM/µl (2µl), 1µl of each of *Pst*I and *Mse*1 selective primer combinations 50ng/µl each (Table 1), and *Taq* DNA polymerase 5U/µl (0.3µl). The mixture was briefly spin downed and was put into PCR machine. The PCR for selective-amplification reactions were performed according to Vos *et al*., (1995)[15] using the following cycling conditions; 12 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minutes, the annealing temperature was decreased by 0.7°C per each cycle and followed by 23 cycles of 94°C for 30°C, 56°C for 30°C and 72°C for 1 minutes. The separation of amplified DNA fragments was done on polyacrylamide gels 8% (v/v). Gels were stained and visual-

ized by using silver staining kit as described by the supplier (Promega, Madison, Wis), and captured by digital camera after air drying.

Data analysis:

Data obtained from clearly amplified and reproducible bands were analyzed by the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), Version 1.8 (Applied Biostatistics) software program [16]. The unambiguously scored AFLP band positions were converted into a matrix of binary characters (1= presence and 0 = absence of a band at each position). Genetic similarities obtained between any two variants were evaluated by using SIMQUAL (Similarity for Qualitative Data) similarity index method [17]. The phylogenetic tree was constructed by UPGMA cluster analysis method [18, 19]. The TreeView program was implemented to obtain a better graphic representation.

Table 1. AFLP primers and adapters and amplicons sizes used in this study

Name	Sequence (5' - 3')	Amplicon size (bp)	Reference
Adapter P	F: CT CGT AGA CTG CGT ACA TGCA R: TGTACGCAGTCTAC	Multilocus (100-21000 bp)	Encyclo Lab Tech East
Adapter M	F: GACGATGAGTCCTGAG R: TACTCAGGACTCAT		
P00	GACTGCGTACATGCAG		
P100	GACTGCGTACATGCA G AACC		
P104	GACTGCGTACATGCA G		
P107	GACTGCGTACATGCA G AATA		
P109	GACTGCGTACATGCAG		
P114	GACTGCGTACATGCA G ACAC		
P174	GACTGCGTACATGCA G CATG		
P294	GACTGCGTACATGCA G TACC		
M43	GATGAGTCCTGAGTAAATA		
M95	GATGAGTCCTGAGTAAAAA		
M100	GATGAGTCCTGAGTA A AACC		
M181	GATGAGTCCTGAGTA A CCCC		
M182	GATGAGTCCTGAGTA A CCCG		
M237	GATGAGTCCTGAGTA A GATA		
M289	GATGAGTCCTGAGTA A TAAA		
M291	GATGAGTCCTGAGTA A TAAG		
M293	GATGAGTCCTGAGTA A TACA		
M301	GATGAGTCCTGAGTA A TATA		
M307	GATGAGTCCTGAGTA A TCAG		

RESULTS AND DISCUSSIONS

AFLP fingerprinting was carried out for all pear samples used in the current study. Results from AFLP amplification (Figure 1), implementing eight selective primer pairs, generated a total of 653 AFLP fragments from which 445 (68.2%) fragments were polymorphic. The number of visible amplified products per primer combination were varied and ranged from 66 to 96 bands. The polymorphism detection ability of individual primer combination in the analyzed genotypes is presented in Table 2. The highest number of polymorphic fragments (70 bands, 72.9%) was generated by the P174/M237 primer combination; however, the highest percentage of polymorphism (78.4%) was produced by the primer pair P174/M182. The lowest number of variable fragments (47 bands, 64.4%) was observed by the primer combination P174/M307. The average percentage of polymorphic fragments obtained in this study (68.2 %) was lower than that recorded by Wolf *et al.*, [6] who reported 89.5% of the average percentage of polymorphism within pear cultivars. The real reason of this variation is not quite clear. It might reflect the genuine differences between the two pear populations (pear resources).However, the differences in thenumber of cultivars and/or rootstocks belonging to each studied population as well as the variation in the implemented primer combinations in each study should also be taken into consideration.

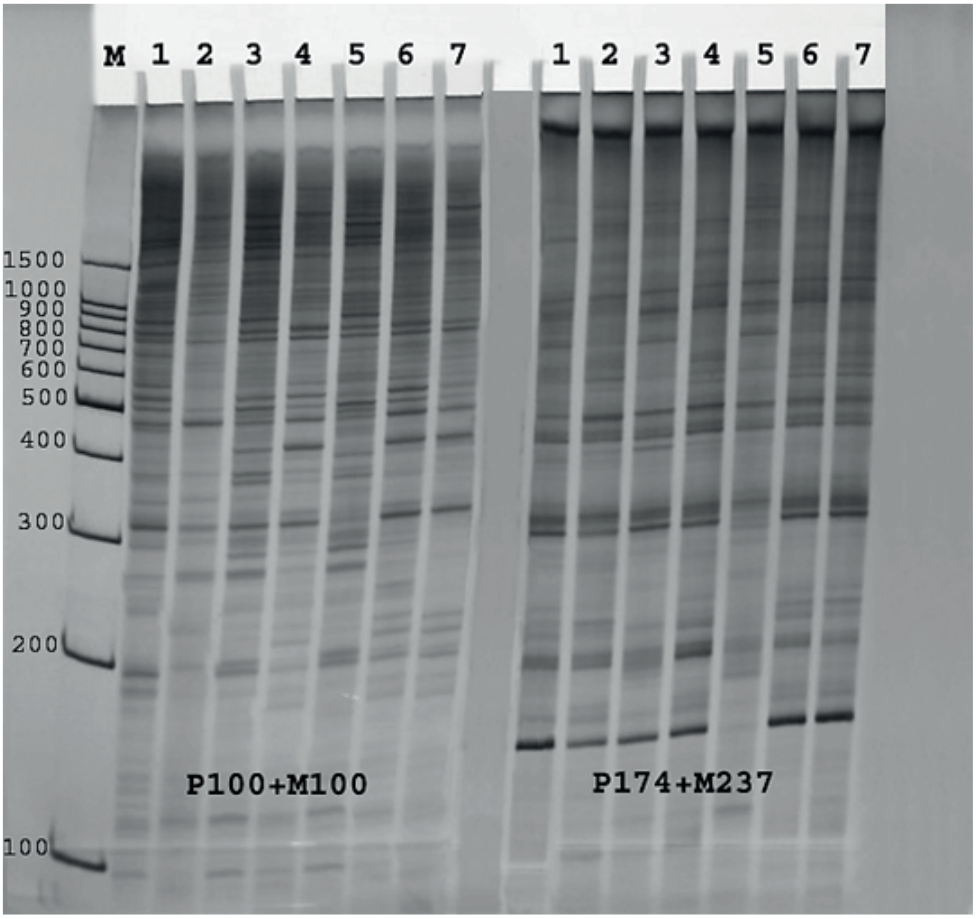

Figure 1. Examples of AFLP amplification implementing primer pairs P100+M100 and P174+M237. M= Ladder, numbers 1-7 represent pear samples used in this study (Harmijali, Itali, Licont, Zaafaran1, Zaafaran2, Danimarki, and Amreki, respectively).

Results of the present investigation were further confirmed the already established fact that AFLP technique can be applied virtually on any DNA regardless its source. Additionally, the repeated AFLP experiments (replicated 10% of the samples) produced identical and reliable fingerprint patterns (data not shown). Thus, the AFLP technique was confirmed to be a highly valuable method in fingerprinting of the corresponding pear samples due to its sensitivity, reliability, as well as its reproducibility in production of high amount of polymorphic bands among the studied individuals. Furthermore, the polymorphic information content (PIC), which is a good index for evaluation of genetic diversity, was also calculated to evaluate the level of genetic variation and polymorphism between the studied pear samples (Table 2). Results revealed thatthe highest PIC (0.85) was obtained with the primer combination P174/M182. This indicated that this primer combination were highly informative to discriminate among the studied pear cultivars and to assess the genetic diversity among this local population. In contrast, the lowest PIC (0.49) was demonstrated by P174/M307 primer combination, which indicated that the amplified loci by this primer combination are of low variability among pear samples. The relative amount of the marker's PIC is ranging from 0 to 1. The bigger the PIC is (PIC>0.5), the higher the variability of the loci is. In contrast, the smaller the PIC is (PIC<0.5), the lower the diversity of the loci is [20].

Table 2. The polymorphism characteristics of AFLP primer combinations used in this study.

Primer combination	No. of amplified bands	No. of polymorphic bands	polymorphic %	PIC
P174/M307	73	47	64.4	0.49
P174/M301	92	63	68.5	0.60
P100/M100	84	54	64.3	0.52
P174/M291	66	50	75.7	0.55
P174/M293	81	52	64.2	0.57
P174/M100	87	51	58.6	0.56
P174/M237	96	70	72.9	0.63
P174/M182	74	58	78.4	0.85
Total	653	445	68.2	0.59
P174/M307	73	47	64.4	0.49

To estimate the genetic similarity among the studied pear cultivars, SIMQUAL similarity index method was implemented, based on AFLP data, to generate the genetic distance matrix, which is the degree of genetic variations between species or within a species of populations [21, 22]. Results showed that the highest genetic distance (0.3131) was observed between Italy and Zaafaran2 cultivars, while the lowest genetic distance (0.1348) was detected between Danimarki and Amreki cultivars (Table3). This confirms that the Danimarki and Amreki cultivars are closely related, as they share a more recent common ancestor (Figure2).

Table 3. Depicts the genetic distance values between respective pear samples.

	1	2	3	4	5	6	7
1	0.0000						
2	0.2501	0.0000					
3	0.1829	0.2719	0.0000				
4	0.2439	0.2728	0.2230	0.0000			
5	0.2362	**0.3131**	0.1679	0.2441	0.0000		
6	0.2281	0.2812	0.2271	0.1639	0.2153	0.0000	
7	0.2448	0.2559	0.2420	0.2394	0.2400	**0.1348**	0.0000

1= Harmijali, 2= Itali, 3= Licont, 4= Zaafaran1, 5= Zaafaran2, 6= Danimarki, and 7= Amreki.

Based on the genetic similarity values recorded in the current study, which was ranged from 0.6869 to 0.8652, it can be stated that the investigated genotypes can be considered as a different cultivars. This is based on what reported by Cervera*et al.*, [23], if the similarity coefficient is equal or higher than 0.9 between two individuals, one of the individuals can be considered as a clone of the same cultivar [6].

Genetic similarities, obtained through AFLP data, were further analyzed and employed to construct a phylogenetic relationships among respective *Pyrus communis* cultivars using UPGMA cluster analysis. The result show that the AFLP primer combinations were successfully clustered all the seven pear genotypesinto two separate clusters (C1 and C2) with an out group of Italy cultivar (Figure 2).

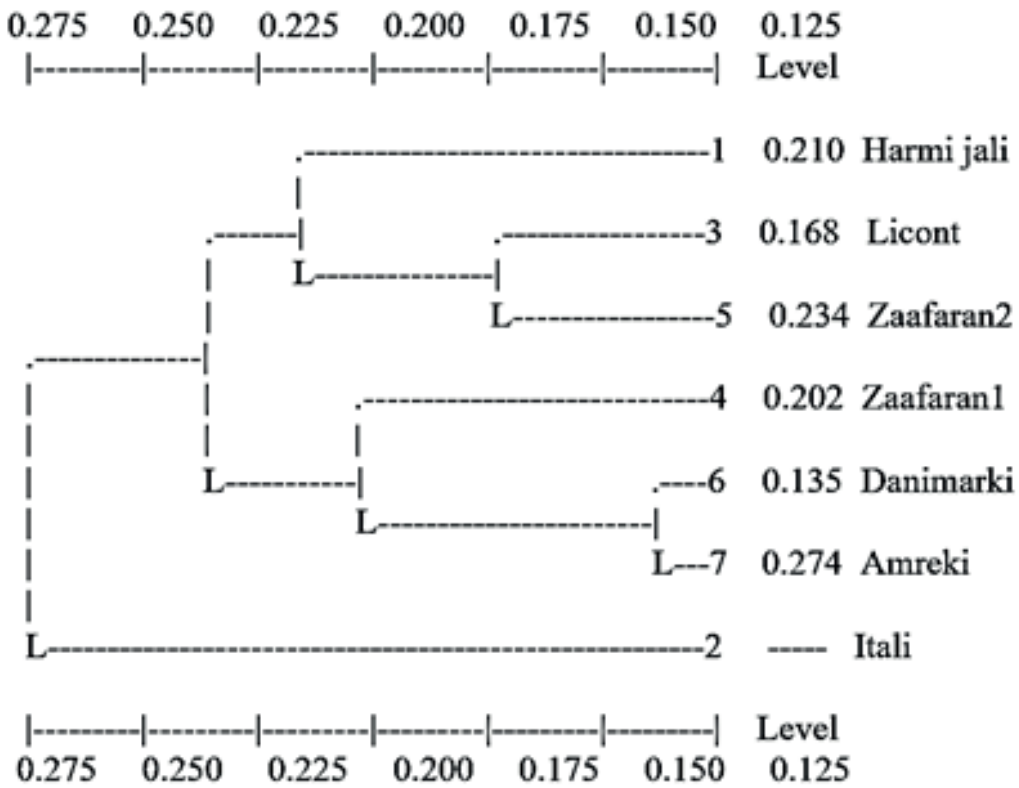

Figure 2. Phylogenetic relationships among the studied pear samples.

The findings of the present study proved that the locally cultivated pear, which to date has been less exploited, are rich in genetic variation. Therefore, the results of the estimation of genetic variation as well as investigation of the polymorphic information content in local pear cultivars might play an important role in building a genetic bank for these cultivars. Besides, these results might be immensely important for present and future pear breeding and genetic improvement program in this region.

Over all, the AFLP technique was confirmed to be an efficient tool for genotyping and estimation genetic variation in pear cultivars. In addition, the selected AFLP markers in the present study enabled the discrimination and characterization of these cultivars. The results obtained in the current investigation revealed high polymorphism between the studied pear cultivars. Also, the study observations detected a mixture of closely related and distantly related pear cultivars in the region. This observation will make the selection of genetically variable cultivars for breeding purposes much easier and more accurate.

CONCLUSIONS

The molecular technique used in the current study enabled the genotyping and discrimination of pear cultivars. AFLP analyses also confirmed the presence of high polymorphism among pear cultivars in Duhok city. Interesting results were found that the degree of genetic variations between studied genotypes confirmed that they are different cultivars and grouped them into two major genetic clusters, which might be considered as good gene pool for breeding program.The information obtained in the current investigation will be useful and helpful for pear breeders in order to carry out pear breeding within a broad gene pool and to work toward increasing the genetic diversity of pear cultivars in this region.

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