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Evaluation of genetic relationships of some Iranian asian pears (*Pyrus pyrifolia*) using SSR markers

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Abstract

New several genotypes are found in the forests of the eastern Guilan province that are morphologically more like the Asian pears and have also some agronomical important traits such as very large, high quality of fresh fruits, and relative resistant to fire blight disease. In the present study, eleven microsatellite markers were used to assess the genetic relationships between these Iranian genotypes Asian pears. The UPGMA cluster analysis located the 20 genotypes into two main groups. The first main group had two subgroups including seven native genotypes and commercial Asian pears. The second group also had two subgroups involving the interspecific hybrids of Asian and European pears, and Bartlett pear. We were found a major population of the Asian pears that were grown in the northern forests of Iran and it seems that these forest are secondary centre of origin of Asian pear.

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Introduction

Pears are the third most important fruit produced in the temperate regions after grapes and apples. The genus *Pyrus* is believed to be originated from the central Asian (Asia Minor to India) and spreads around east and west (Wünsch and Hormaza, 2007). This genus commonly contains at least 22 widely recognized primary species involving all indigenous to Asia, Europe and the mountainous areas of North America as well as nine natural or artificial interspecific hybrids classified as different species (Bell, 1990; Bell *et al*., 1996). All species of *Pyrus* are diploid (2n= 34, x= 17) and the occasional tetraploid or the triploid cultivars are also found (Janick and Paull, 2008).

Commercial pear production is mainly represented by two species *P. communis* L. (the European pear) and *P. pyrifolia* (the Asian pear) (Bell *et al.*, 1996).The European fruit pears have a buttery, juicy texture with rich flavour and aroma, whereas the Asian pears have important features such as sweet, crisp, juicy texture and longer storage time and shelf life than the European pears.

The Asian pears are also considered to be originated from the East Asia, and many indigenous varieties are cultivated in China, Japan, Korea, etc. (Yamamoto *et al.*, 2002b). Among these countries, China is the largest producer country in the world with an average production of over 15 million t in 2010, almost exclusively of the Asian species; the world pear production is equal to 22731087 t in 2010. The production and the cultivation area of pear are estimated about 160000 tonnes and 13300 ha in Iran in 2010, respectively (FAO-STAT 2012).

The Asian pears are susceptible to the fire blight disease that is caused by *Erwinia amylovora*. This disease is known as a destructive disease of apple and pear for over 200 years. The bacteria can infect to host tissues by blossoms, fruits, vegetative shoots, woody tissues, and rootstock crowns and this makes a difficult control over this disease (Norelli *et al.*, 2003). This problem may be solved by the use of the pear genetic resources in the breeding programs for achieving resistant cultivars to fire blight.

With more than 10 species, Iran is one of the important genetic resources of the *Pyrus* in the world. For example, it has been reported that 12 species of *Pyrus* are grown in the Iranian plateau. This genus is an important element of Irano-Turanian region (species such as *P. syriaca, P. glabra*, and *P.oxyprion*) (Assadi *et al.*, 1989).The distribution zone of the Pyrus in Iran is Alborz and Zagros Mountains, south and some elevations in the east of Iran (Zamani *et al.*, 2008). In addition, native cultivars and introduced European pears are presently cultivated in the orchards of Iran. However, the fruit industry of Iran does not experience the culture and the production of the Asian [Japanese] pears (Arzani, 2004).The current cultivars that are presently cultivated in Iran are shown in a later work (Erfani *et al*., 2012).

In this regard, DNA markers are used for the genetic fingerprinting and studying the genetic diversity, and relatedness in different plant species. These markers are more important in the woody perennials due to some particularities of these species such as long generation time, large individual size, and vegetative propagation. Microsatellites or SSRs are one of the most applications of the DNA markers that are recognized as a useful tool for the molecular studies in the fruit tree species due to high reproducibility, co-dominant inheritance, high degree of polymorphism, abundance in genomes, and their transferability to other relative genera and species (Ercisli *et al.*, 2001).

The pear genetic resources are not fully identified because of low morphological diversity, lack of differentiating characters among the species, and widespread cross ability. Hence, the estimation of the genetic diversity among *Pyrus* spp. is often very difficult (Erfani *et al*., 2012; Yamamoto *et al*., 2002b). However, the genetic studies are not more about pear.

The cultivars belonged to two species of *P. communis* and *P. pyrifolia* were clearly separated according to their geographical origins by RAPD markers (Kim *et al.*, 2005). Also, more than 100 SSR markers have been isolated from the genome sequence of the Asian and European pears by RAHM, 5' anchored PCR, enriched genomic library, and pear GenBank sequences (Bassil *et al.*, 2004; Inoue *et al.*, 2007; Kimura *et al.*, 2002; Sawamura *et al.*, 2004; Yamamoto *et al.*, 2002a; Yamamoto *et al.*, 2002b; Yamamoto *et al.*, 2002c). These markers are used for the construction of the linkage maps, the assessment of the relationships, and the estimation of the genetic diversity in different pear species (Bao *et al.*, 2007; Erfani *et al.*, 2012; Ghosh *et al.*, 2006; Kimura *et al.*, 2002; Yamamoto *et al.*, 2002a; Yamamoto *et al.*, 2002b).

According to our research team, it seems that the genotypes of the north of Iran are somewhat resistant to the fire blight disease because this result is observed in the laboratorial and field studies and this research is likewise continued [unpublished data].These genotypes have good quality fruits in comparison with other wild genotypes. In the present study, the relationships among the Iranian genotypes and the commercial Asian pears are revealed by the microsatellite markers. Therefore, the present results may help utilize the native genotypes of the north of Iran for breeding programs in the feature.

Materials and methods

Plant material

Twenty pear genotypes 20 genotypes including seven native genotypes, nine registered cultivars of Asian pear, three genotypes of hybrid pear and Bartlett pear (Table 1). Afterward, leaf samples of the native genotypes were collected from the forests of the Eastern Guilan province (Chaboksar).Nine Asian pear cultivars named 'KS'6, 'KS'7, 'KS'8, 'KS'9, 'KS'10, 'KS'11, 'KS'12, 'KS'13, and 'KS'14 were introduced to Iran by Belgium and also used for the research purposes in the Iranian climatic conditions (Arzani, 2004).

DNA extraction and amplification

The genomic DNA was extracted from the fresh leaves based on the combination of the modified CTAB and the modified SDS procedures (Dellaporta *et al.*, 1983; Yamamoto *et al.*, 2001).

Eleven SSR markers including eight SSRs derived from the Japanese pear Housui, one SSR from the European pear Bartlett, and two SSRs from the apple Florina were used for PCR amplification (Table 2). The PCR amplification was performed in a 25 µl solution containing 1x PCR buffer, 1.5mM $MgCl_2$, 0.2 mM each of the dNTPs, 10 pmoles of each primer , 1 unit of Tag polymerase (CinnaGen Inc., Tehran, Iran), and 25 ng the genomic DNA. This study was performed by the markers that were previously used for the parentage analysis of different pear cultivars (Kimura *et al.*, 2003), identification of quince varieties (Yamamoto *et al.*, 2004), the estimation of the genetic diversity, and the identification of different pear species. The PCR amplification of the SSR loci of NH001c, NH002b, NH004a, NH011b, NH012a, NH014a, NH017a, CH03c02, and CH04g04 was performed according to the protocol Yamamoto *et al.*, (2002b). The touchdown program was also used for the other two SSR loci [KA16 and BGT23b] (Kimura *et al.*, 2002). After completion of the reaction 7 microliters of the PCR products were mixed with 1.5 µl of the formamide loading buffer (95% formamide, 10 mM EDTA, 0.5% bromophenol blue, and 0.5% xylene cyanol) and subsequently heat-denatured at 940C for 5 minutes. Afterward, 7 µl of each mixture and a molecular weight marker (Gene Ruler, #SM0371, Fermentas Inc., Rockford, IL, USA) were loaded onto a 6% (0.4mm- thick) polyacrylamide gel in a 1x TBE buffer. The gel was run at a constant 75 W, 50 0C for 1.5 h in a sequencing gel electrophoresis apparatus (Bio-Rad, USA). Besides, the gels were silver stained according to the method previously described (Creste *et al.*, 2001). Finally, the gels were scanned by using a Bio-Rad GS-800 Scanning densitometer.

Data analysis and determination of SSR marker

polymorphism

The SSR fragments' sizes were obtained by the DNA ladder and then the presence (1) or absence (0) of the bands in the genotypes were noted. Some of the markers were amplified by the multi loci that separately scored each locus. In the present study, the genetic relationships among the genotypes and the cultivars were constructed based on the Nei's genetic identity by the unweighted pair-group method using the arithmetic averages (UPGMA) algorithm (Nei, 1972). The programme of NTSYS-PC (version 2.02) was used to construct the dendrogram (Rohlf, 1992).

Results and discussion

SSR analysis

The eleven SSR markers produced 65 alleles in the 20 pear genotypes with an average of 5.9 alleles per locus (Table 2). The genetic relationships of the genotypes with 65 alleles were correctly revealed. All primers used in this study showed polymorphism. The number of the observed alleles ranged from 3 for NH002b and NH012a to 11 for NH014a. In five SSRs (NH004a, NH002b, BGT23b, NH017a, and NH012a), only one or two bands were amplified in each genotype suggesting a single locus existed for these markers in the genotypes and the used cultivars genome. In this respect, five other SSRs (NH011b, NH014a, KA16, CH03c02, and CH04g04) produced 1, 2, and 3 discrete reproducible bands in some Iranian genotypes indicating the amplification of at least two different loci for these markers. The SSR markers including CH04g04 and KA16 produced three discrete fragments in Bartlett pear that was a diploid cultivar. In the SSR loci of NH001c, except 1or 2 bands, produced 3 and 4 discrete fragments in some native genotypes. This primer also produced four discrete fragments in Bartlett. In the present study, all the SSR markers only produced 1 or 2 bands in the commercial cultivars (KS cultivars) but some markers produced more than two discrete fragments in the Iranian genotypes and Bartlett. The identical allele sizes were observed in each of the two populations (Table 3). The size of the observed alleles ranged from 77bp for NH004a and NH014a to 264bp for BGT23b. Some primers produced no amplification in some genotypes such as NH004a in Ch111, Hm, Ha, also NH012a in Sa, Ri, Ab, and NH017a and CH03c02 in Ab.

The UPGMA dendrogram of 20 pear genotypes was also constructed based on the SSR data (Figure 1). The cophenetic correlation coefficient between the cophenetic matrix derived from the dendrogram and the similarity matrix was high (r= 0.82). The UPGMA cluster analysis also clearly separated the genotypes and the cultivars according to their pedigree and the geographical origin. The genetic similarity coefficient ranged from 0.44 (Bartlett and KS11) to 1 (Gh, Hs and Ch24) among the studied genotypes. Moreover, the UPGMA dendrogram separated the genotypes and the cultivars into two main groups consist of 16 Asian pears and 3 interspecific hybrids. The first main group (I) consisting of two subgroups included (1 sub-group) seven Iranian genotypes and (2 sub-group) ten commercial Asian cultivars. In the first subgroup, Gh, Hs, and Ch24 were closely clustered together. The similarity coefficient was 1 for them that presumably revealed these genotypes originated from one genotype or their plant materials were transferred by grafting and then were shown by different names. Among the studied genotypes, Sa, Ab, Ri, and Bartlett were also clustered together in the second main group (II). In this respect, the first three genotypes were interspecific hybrids between the Asian pears and the European pears; they were obtained from the Agricultural Ghaboksar office. These genotypes showed identical fragments' size in some of the markers. Some markers were produced the identical alleles in each of three hybrids. The examples include 264bp at BGT23b; 136bp, 145bp, 162bp, and 175bp at NH001c; 104bp at NH004a; 195bp and 200bp at NH011b; 125bp and 133bp at NH014a; 119bp, 133bp, and 136bp at CH03c02; 185bp at CH04g04, and 176bp at KA16 locus. However, these alleles were not observed in other local genotypes.

Primers used in this study produced 65 alleles in the pear genotypes with an average of 5.9 alleles per

locus. It was previously reported that 174 alleles in 47 pear genotypes using 28 SSR markers (6.21 alleles per locus) (Erfani *et al.*, 2012). It was also identified 54 apricot landrace cultivars with 103 alleles using 26 SSR markers (four alleles per loci) (Krichen *et al.*, 2006). Zhang *et al.*, (2011) reported 100 alleles at 19 SSR loci with an average of 5.3 alleles per locus in 29 apple accessions as well (Zhang *et al.*, 2012).

Table 1. Pear genotypes and cultivars used in this study.

No.	Genotype name	Species	Origin
1	Ch111	Unknown	Iran
2	Gn	Unknown	Iran
3	Hm	Unknown	Iran
4	Hs	Unknown	Iran
5	Ch24	Unknown	Iran
6	Ha	Unknown	Iran
7	Sa	*Pyrus hybrid (P.pyrifolia × P.communis)*	Iran
8	Ab	*Pyrus hybrid (P.pyrifolia × P.communis)*	Iran
9	Hb	Unknown	Iran
10	Ri	*Pyrus hybrid (P.pyrifolia × P.communis)*	Iran
11	KS6	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
12	KS7	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
13	KS8	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
14	KS9	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
15	KS10	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
16	KS11	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
17	KS12	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
18	KS13	*Pyrus pyrifolia* Nakai.	Europe (Belgium)
19	KS14	*Pyrus pyrifolia* Nakai.	Europe (Belgium)

In the present study, the genetic relationships of the genotypes with 65 alleles were correctly revealed. The observed larger number of the alleles is because of the use of the genotypes with different geographical origin and high level of the polymorphism of 11 used SSRs; this suggestion was also previously described by Bao *et al.*, (2007).

In our study the number of the observed alleles ranged from 3 for NH002b and NH012a to 11 for NH014a. In five SSRs only one or two bands were amplified in each genotype and it seems that a single locus existed for these markers in the genotypes. However, Yamamoto *et al.*, (2004) reported that NH004a and NH017a markers produced more than two amplified bands that were presumably derived from the multi loci. Kimura *et al.*, (2002) and Erfani *et al.*, (2012) also described that the SSRs produced one or two bands that were presumably derived from a single locus.

In this respect, five other SSRs (NH011b, NH014a, KA16, CH03c02, and CH04g04) produced 1, 2, and 3 discrete reproducible bands in some Iranian genotypes indicating the amplification of at least two different loci for these markers. Besides, it has been reported that CH03c02 and CH04g04 markers derived from apple amplified only a single locus (Liebhard *et al.*, 2002). However, this finding was not supported by the present study.

The SSR markers including CH04g04 and KA16

produced three discrete fragments in Bartlett pear that was a diploid cultivar (2n= 34, x= 17). Liebhard *et al.*, (2002) also revealed that all the tested SSR makers derived from the apple amplified the fragments in other genera of sub-family Maloideae (*Amelanchier*, *Cotoneaster*, *Pyrus*, *Cydonia*, etc.), whereas only CH04g04 marker produced the fragments in the sub-family Amygdaloideae that was also one of the used SSRs in the present study. Moreover, it was previously reported that the SSR loci of the CH01f02 produced three discrete fragments in some genotypes suggesting that at least two different loci were amplified by this marker (Wünsch and Hormaza, 2007). Conversely, Liebhard *et al.*, (2002) described CH01f02 as a single locus.

Table 2. Primer sets and characteristics of SSR loci used in this study.

primer	Annealing temperature	Alleles	Allele Size (bp)	source	Reference
NH001c	52	9	114- 175	Housui	Yamamoto *et al.* 2002b
NH002b	59	3	193-211	Housui	Yamamoto *et al.* 2002b
NH004a	60	6	77-124	Housui	Yamamoto *et al.* 2002b
NH011b	58	6	192- 223	Housui	Yamamoto *et al.* 2002b
NH012a	59	3	118- 136	Housui	Yamamoto *et al.* 2002b
NH014a	55	11	77- 133	Housui	Yamamoto *et al.* 2002b
NH017a	57	4	104- 128	Housui	Yamamoto *et al.* 2002b
KA16	59	8	143- 202	Housui	Yamamoto *et al.* 2002a
BGT23b	55	5	222- 264	Bartlett	Yamamoto *et al.* 2002a
CH03c02	63	4	111- 136	Florina	Liebhard et. al. 2002
CH04g04	63	6	174- 207	Florina	Liebhard et. al. 2002

In the present study the SSR loci of NH001c, except 1or 2 bands, produced 3 and 4 discrete fragments in some native genotypes. This primer also produced four discrete fragments in Bartlett. The presence of several amplification fragments in most of the studied loci can be due to the allopolyploid origin of Maloideae subfamily from two primitive forms of the Rosaceae such as Prunoideae (x=8) and Spiraeoideae (x=9) (Janick and Paull, 2008; Layne and Qamme, 1975). Genus Maloideae has a basic chromosome number of 17, whereas other subfamilies of the Rosaceae have basic chromosome numbers of 7, 8 or 9 (Brini *et al.*, 2008).

In our research, all the SSR markers only produced 1 or 2 bands in KS cultivars but some markers produced more than 2 discrete fragments in the Iranian genotypes and Bartlett. The Iranian genotypes were recognized as a wild and cross-pollination populations. Hence, these genotypes presumably have more SSR loci. A total number of 33 and 47 alleles amplified in commercial Asian cultivars and other genotypes, respectively.

The identical allele sizes were observed in each of the two populations. The size of the observed alleles ranged from 77bp for NH004a and NH014a to 264bp for BGT23b. The smallest fragment in the markers NH004a and NH014a were also produced in the previous studies (Bao *et al.*, 2007; Kimura *et al.*, 2002). Using these markers, the size of the observed alleles in the present study was very near to the previous performed studies. The number of the observed 65 alleles was also due to different geographical origins of the genotypes and high level of the polymorphism of the SSR markers.

Some primers produced no amplification in some genotypes such as NH004a in Ch111, Hm, Ha, also NH012a in Sa, Ri, Ab, and NH017a and CH03c02 in Ab. The loci for these primers may presumably not exist in these genotypes or that the flanking regions of

the SSR loci may mutate during the evolution of the Iranian Asian pears. In addition, Yamamoto *et al.*, (2002b) reported some of the SSR loci that were not used in the European pears designed from the sequences of Housui. This suggests that these primer sequences may not completely be conserved between *P. pyrifolia* and *P. communis* or that the flanking regions around repeats may sometimes have mutated..

Table 3. Amplified fragments profile in the genotypes analyzed.

primer	Local genotypes and Bartlett	Commercial cultivars	Other works	Refrence
NH001c	114/119/136/141/145/150/162/175	141/ 145/ 156	103-403	Ghosh, A.K.,*et al.*(2006)
NH002b	193/200/211	200/ 211	174-185	Kimura, T., *et al.*(2003)
NH004a	77/102/104	102/ 112/ 119/ 124	78-130	Jiang,Z., *et al.*(2009)
NH011b	192/195/200/223	209/ 217/ 223	156-241	Kimura, T., *et al.*(2002)
NH012a	118	122/ 136	105-108	Kimura, T., *et al.*(2003)
NH014a	77/88/98/107/114/118/125/133	86/ 98/ 111/ 114/ 123	60-130	Jiang,Z., *et al.*(2009)
NH017a	104/108	104/ 112/ 128	88-120	Jiang,Z., *et al.*(2009)
KA16	143/146/153/158/164/176/202	158/ 171	120-160	Yamamoto, T., *et al.*(2002a)
BGT23b	222/264	222/ 233/ 241/ 250	184-236	Yamamoto, T., *et al.*(2002a)
CH03c02	119/133/136	111/ 119	116-136	Leibhard, R., *et al.*(2002)
CH04g04	174/181/185/191/197/207	185/ 191/ 207	170-186	Leibhard, R., *et al.*(2002)

The UPGMA cluster analysis also clearly separated the genotypes according to their pedigree and the geographical origin. Furthermore, Brini *et al.*, (2008) examined the genetic diversity among the local Tunisian pears with the SSR markers; the cophenetic correlation coefficient was high (r=0.91). They described that the tree branching was considered as a good representation of the genetic similarity among the studied genotypes. In this regard, Wuncsh and Hormaza (2007) reported that the cophenetic correlation coefficient was low (r= 0.65) in the identification of 63 European pear cultivars. They also described that the cluster analysis was not considered as the genetic similarity among varieties. However, the UPGMA cluster analysis separated the varieties according to their pedigree and the geographic origin.

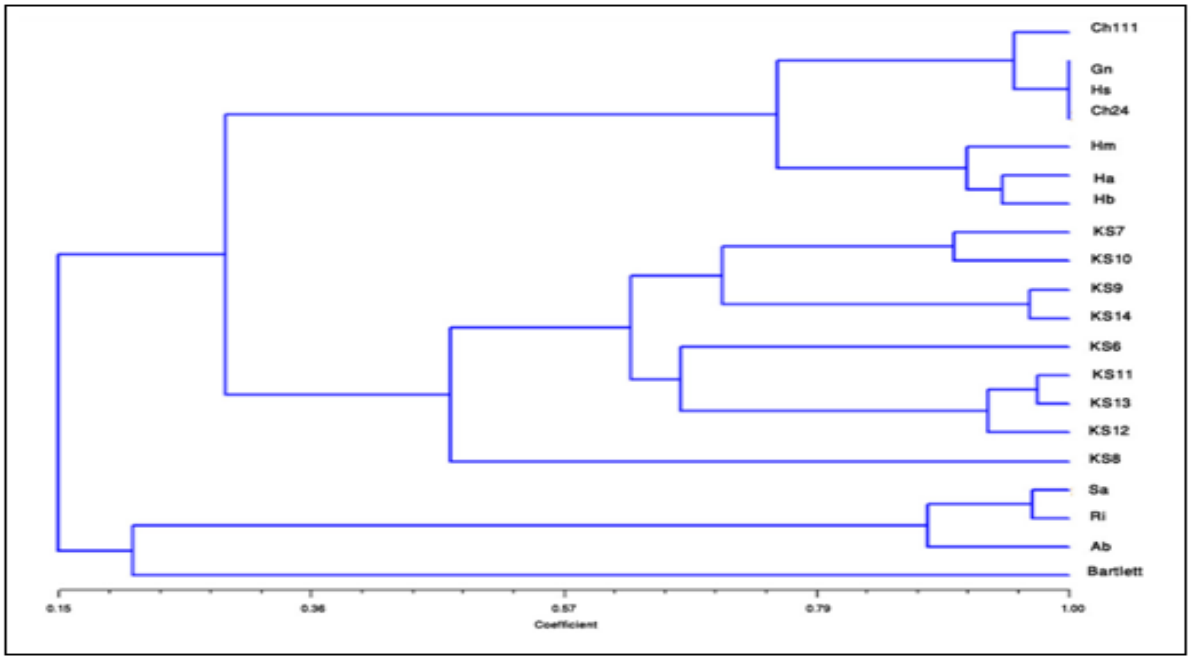

Fig. 1. A phenogram for 20 pear genotypes constructed by the UPGMA method based on Nei's genetic identity.

European pear is one of the parents all the hybrids and Bartlett pear also belong to this species. Afterward, some identical alleles were presumably transferred from P. communis to each of the four genotypes. Kimura *et al.*, (2002) also described the hybrids inherited the SSR alleles from their parents without any discrepancy.

In the present study, some markers were produced the identical alleles in each of three hybrids. It is worth noting that the native genotypes of the north of Iran differ very much in morphology from the European pears. They have also different Botanical and Molecular characteristics with the European pears that are not originated from *P. communis*. However, Erfani *et al.* (2012) reported the genotypes that grew in the forests of the north of Iran originated from "Khoj" (a local name for the pears in our study) genotype and these researchers believe that these genotypes belonging to P. communis. Although they did not describe the base of the taxonomy of Khoj genotypes but they reported that Khoj genotypes is native to Iran and both Khoj and P. salicifolia (that is also native to Iran) were grouped together in the introduced cultivars subgroup. Therefore, the UPGMA cluster analysis did not separate them according to their geographical origin (Erfani *et al.*, 2012). In our research obviously different results were obtained with this study which could be indicative of greater accuracy of markers used in the current study.

Conclusions

The purpose of the present study was to determine the genetic relationships between the Iranian genotypes and the commercial Asian pears based on the SSR data. The used markers are also appropriate to improve these valuable genotypes in the future because these markers inherited in a Mendelian fashion that permits to carry out the paternity analyses. The native genotypes of the north of Iran were botanically and morphologically very much similar to the species of *P. pyrifolia*. However, as far as we know the presence of them was not reported in Iran as Asian pears and it seems that Northern Iran is secondary centre of origin of Asian pear or at least these forests are the centers of diversity of Asian pear. Therefore, it is necessary to use the Iranian genotypes as the genetic resources for improving the fruit quality and improving biological conditions for the plant.

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