Morphological and molecular variability in some Iranian almond
genotypes and related *Prunus* species and their potentials for rootstock
breeding

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ABSTRACT

In this study, in order to know the variability for a rootstock breeding program genetic diversity and relationships among 55 Iranian almond genotypes and seven related *Prunus* species were investigated. Morphological and molecular analyses were used. Principal component analysis showed that three components explained 67.6% of the total morphological variation for the first year and 68.06% for the second year of the study. Leaf traits were predominant in the first component and contributed most of the total variation. Leaf length and width, as well as, leaf area were highly correlated with each other and correlated to vigor. Also a negative correlation was found among leaf length/width ratio and vigor. Ward's method was used to construct cluster from morphological data which allocated individuals into their respective species. Out of 100 pre-screened RAPD primers, 16 with reproducible bands and maximum polymorphism were selected. Two-hundred and sixty bands were scored which 250 of them were polymorphic. Average value of polymorphism per primer was 95.81% and maximum value for polymorphism (100%) was obtained from TIBMBA-14, TIBMBA-17, TIBMBB-05, TIBMBB-08, TIBMBD-09, TIBMBD-10. On the other hand, the minimum value was obtained from TIBMBB-16 (86%). Primer TIBMBB-5 gave the maximum number of bands (25 fragments) and the minimum obtained from TIBMBE-18 (11 fragments). Genetic similarity based on Jaccard's coefficient ranged from 0.28 to 0.79 with an average of 0.53. Molecular analysis revealed a high degree of separation among samples regarding their geographical origin. Correlation between two approaches was low ($R = -0.38$). High molecular and morphological variability indicated that this collection includes rich and valuable plant materials for almond rootstock breeding.

Keywords: Almond - Rootstock - RAPD - Vegetative traits - Related *Prunus* spp.

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1. Introduction

Almonds are deciduous trees and/or shrubs adapted to arid and semi-arid environments. They also encompass a wide range of values from nutritional to ecological applications (Martínez-Gómez et al., 2007; Zeinalabedini et al., 2008). The cultivated almond, which is one of the oldest nut crops [*Prunus dulcis* (Mill.) D.A. Webb; syn. *P. amygdalus* Batsch], is thought to have originated in the arid mountainous regions of Central Asia (Grasselly, 1976b) and is grown commercially worldwide. Iran, due to a diverse variability in geographical regions such as mountain ranges and deserts spreading throughout the country and hence diverse kinds of climates, is one of the origin of almond (Grasselly, 1976a,b; Kester and Gradziel, 1996; Ladizinsky, 1999; Martínez-Gómez et al., 2007). Based on the latest statistics (FAO STAT Data sources, 2008) world production of almond was approximately 2,420,000 Tons, of which Iran produced 110,000 Tons and stands in the fifth place. Almond has been cultivated in Iran for millennia and its production is mostly based on orchards with traditional managements. These clones, local cultivars and seedlings as well as related wild species constitute a valuable source of genetic diversity and an excellent potential for improvement. Owing to the responsibility of rootstock for a wide range of fruit tree properties (Cummins and Aldwinckle, 1983; Layne, 1987; Zarrouk et al., 2005; Jiménez et al., 2007), they have an important role in modern horticulture and commercial orchards. Therefore, a suitable rootstock should have a range of characters from compatibility with cultivars to adaptation to biotic and abiotic stresses. Evaluation of genetic diversity and relationships among cultivated almond and its related wild species is of great importance in determining gene pools and developing better strategies in conservation and identification of genetic resources (Gradziel et al., 2001; Hend et al., 2009; Tahan et al., 2009)

and owing to the absence of crossing barriers, the possibility of interspecific hybridization supplies almond with a rich germplasm and a valuable source for improvement programs (Browicz and Zohary, 1996; Gradziel et al., 2001; Martínez Gómez et al., 2003b).

Although traditional approaches for germplasm characterization are based on morphological observations, they are time consuming, affected by environmental conditions, particularly due to the long generation time and large tree size. Also there is low level of variability in morphological traits (Casas et al., 1999; Sorkheh et al., 2007; Zeinalabedini et al., 2008; Bouhadida et al., 2009; Sorkheh et al., 2009b). In this concept, in order to supplement the morphology-based results, several molecular techniques including isoenzyme (Vezvaei, 2003), and DNA-based markers such as, ISSRs (Martins et al., 2003), SSRs (Cipriani et al., 1999; Martínez-Gómez et al., 2003a,b; Mnejja et al., 2004; Xu et al., 2004; Xie et al., 2006; Zeinalabedini et al., 2008; Bouhadida et al., 2009) and AFLPs (Sorkheh et al., 2007), which are not affected by environmental changes have been used for describing diversity and genetic characterization of *Prunus* germplasm throughout the world. Random amplified polymorphic DNA (RAPD) technique has been used to study the genetic diversity of *Prunus*, including almonds, in several studies (Gogorcena et al., 1993; Casas et al., 1999; Mir Ali and Nabulsi, 2003; Gouta et al., 2008; Shiran et al., 2007) and is considered as a quick, inexpensive and less laborious approach for studying genetic diversity. Previous studies showed that results of RAPD analysis are reliable and comparable to markers like SSR (Baránek et al., 2006; Shiran et al., 2007; Bouhadida et al., 2009; Gouta et al., 2010) and AFLP (Sorkheh et al., 2009a).

A few studies have conducted on the assessment of genetic diversity and relationships among Iranian cultivated and wild almond genotypes with regard to molecular and

morphological analyses (Zeinalabedini et al., 2008; Sorkheh et al., 2007; 2009a). In other words, ongoing researches on Iranian Almond germplasm are increasing our knowledge about the morphological/molecular duality in these valuable and closely related plant materials.

Hence, the aim of present study was to investigate the genetic diversity and relationships among Iranian almond genotypes and relative wild *Prunus* species, from the breeding program at the University of Tehran, for their conservation, management, utilization and future selection of rootstocks, by comparing morphological and RAPD analyses.

2. Materials and methods

2.1. Plant Material

Samples in this study were classified into two groups: first, *Prunus dulcis* genotypes (cultivated almond) and second, wild species being relatives of almond (Fig. 1). In main production regions of the country an individual open pollinated almond tree was selected due to characters such as drought tolerance and vigor and considered as mother plant. Then approximately 30 seeds were collected from them, stratified and sown in the experimental orchard of the University of Tehran (Longitude: 50.56 and latitude: 35.47). All seedlings in each row were considered as a family and evaluated based on length and diameter of main trunk (two important vegetative traits for rootstock). At that point, the most vigorous seedlings (ranging from five to ten in each family) were selected for further morphological study. The samples were named with initials of cities at collection sites and/or local names. This material is growing in the experimental field of the Department of Horticulture, College of Agriculture, University of Tehran in Karaj. Second group, consisted of wild almonds species (*P. scoparia*, *P. arabica*, *P. eburnea*, *P. erioclada*, *P. lycioides*, *P. orientalis* and *P. communis*) characterized by high

tolerance to harsh environmental conditions (Kester et al., 1991; Gradziel et al., 2001) collected from natural habitats. Both groups together, included 62 genotypes (Table 1, Fig. 2).

2.2. Morphological Evaluation

During two growing seasons (2008 and 2009), fifteen morphological traits (eight quantitative and seven qualitative), were evaluated based on almond descriptors developed by the International Plant Genetic Resources Institute (IPGRI) (Gulcan, 1985), in the experimental orchard as well as in natural ecosystems. Leaf and branch traits were measured on samples from open-grown canopy, around the tree and in case of leaves, from middle part of normal current season's shoots. Diameter of main trunk was measured in the height of 15 cm above the ground which is the common height for grafting in nurseries.

2.3. Molecular Evaluation

2.3.1. DNA Isolation

Young leaves were freeze-dried immediately after sampling by liquid nitrogen. For total DNA extraction approximately 50 mg of young leaves were ground in a 1.5 ml Eppendorf tube with 750 μ l of CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO₃) (Doyle and Doyle, 1987). Samples were incubated at 65 °C for 20 min, mixed with an equal volume of 24:1 chloroform:isoamyl-alcohol, and centrifuged at 6000 $\times$ g (20 min). The upper phase was recovered and mixed with an equal volume of isopropanol at -20 °C. The nucleic acid pellet was washed in 400 μ l of 10mM ammonium acetate in 76% ethanol, dried, dissolved in 50 μ l of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and incubated with 0.5 mg of RNase-A at 37 °C for 30 min, to digest RNA. DNA quality and quantity was determined by comparing with a standard λ DNA set (Fermentase™ Life Sciences) on 1.2% agarose gel.

2.3.2. DNA Amplification

Amplifications were carried out in a 25 μ l reaction volume containing 1X PCR buffer, 1.75 mM MgCl₂, 200 μ M dNTPs, 0.4 μ M of each decamer primer, 1 U Taq DNA polymerase (CinaGene, Iran) and 10 ng of genomic DNA. Reactions were performed in a 96 well-block thermocycler (iCycler, Bio Rad Co., USA), programmed for a step of 94°C for 4 min, followed by 35 cycles of 92°C for 1 min, 37°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Primers with weak or ambiguous products were repeated to confirm the consistency. The amplified products were resolved in 1.5% agarose (Roche Co., Germany) gel electrophoresis in 1X TBE buffer under 120V for 2 h and stained with 5 μ g.ml⁻¹ ethidium bromide. Products were analyzed by a Gel Doc system (UVP, Bio Doc Co., USA).

2.4. Data Analysis

Morphological data analysis was performed using SPSS 16.0.0 (SPSS, 2007). The data set was converted to z-scores and a distance matrix was prepared based on squared Euclidean distance. For the establishment of the overall relationships among genotypes, morphological clustering was constructed based on the mean values of two years of evaluation using Ward's method. Also principal component analyses (PCA) were performed for each year of morphological evaluation and loading values greater than 0.55 were considered as significant and a 2D PCA plot was designed using combined data for two years of the study . Finally to show the relationships among the traits, a correlation analysis was performed on the complete set of data (62 samples and 15 variables) using the mean values of two years of evaluation. The parametric Pearson correlation and the non-parametric Spearman correlation were used for quantitative traits and for qualitative traits respectively.

For RAPD analysis, a presence/absence (1/0) matrix was prepared and a similarity matrix was generated using NTSYS-pc software ver. 2.02 (Roholf, 1998) based on Jaccard's similarity coefficient. Also a principal coordinate analysis (PCoA) was performed and neighbor-joining (NJoin) clustering module was used for constructing a molecular-based dendrogram as well. Bootstrap analysis was performed to estimate nodal support on the basis of 1,000 replications using DARwin5 software (Perrier and Jacquemoud-Collet, 2006).

3. Results

3.1. Morphological Analysis

In our study, correlation was observed among most of the traits (Table 2). Leaf length and width and leaf area were highly correlated with each other and correlated with traits related to vigor such as length and diameter of main trunk, foliage density, tree vigor and thickness of one year old shoot as well. Ramification was negatively correlated with length and diameter of main trunk, tree vigor, length of main branches, thickness of one year old shoot, leaf length and width and leaf area. There were also negative correlations among leaf length/width ratio and traits related to vigor such as length and diameter of main trunk, tree vigor, foliage density, length of main branches, thickness of one year old shoot, leaf length, leaf width and leaf area. Descriptive statistics of morphological traits for two years of the study are shown in table 3. Accessions exhibited great variability for most of the traits as indicated by their high value of coefficient of variation (CV). In each year, among all the traits, leaf area had the maximum values of CV which were 57.17 and 62.53 for the first and second year, respectively.

Principal component analysis showed that three components explained 67.6% of the total variation contributed by all traits for the first year of the study (Table 4). Leaf traits were predominant in the first component and contributed most of the total variation. The first component presented 34.77% of the variation in which leaf width, ramification, leaf area, leaf length, thickness of one year old shoot, length of main branches and Leaf length/width ratio had the highest loadings. The second component explained 22.55% of the total variation and featured foliage density, diameter and length of main trunk and tree vigor. Third component accounted for only 10.28% of the variation in which Tree habit and coloration of shoot tip and leaf were dominant (Table 4).

Results of the second year of evaluation also showed three components which explained 68.06% of the total variation contributed by all traits. The first component accounted for 31.53% of the variation and featured leaf area, leaf width and length, thickness of one year old shoot and ramification. The second component presented 25.5% of the total variation and featured length and diameter of main trunk, length of main branches, foliage density and tree vigor. Finally the third component explained only 11.03% of the variation in which tree habit and coloration of shoot tip and leaf were dominant (Table 4). Based on two dimensional PCA plot and with regard to the first two components, wild almonds except *P. communis* were separated from cultivated (Fig. 3).

Morphological cluster analysis showed two distinct groups as well (Fig. 4). The first group included related *Prunus* species (except *P. communis*). All the cultivated genotypes and *P. communis* were placed in the second group. Also GF677-based genotypes (GF677OP1 and GF677OP2) were placed in one cluster with each other.

3.2.Molecular analysis

3.2.1. Primer screening:

100 Primers (TIBMOBIO Co., Germany) were used for prescreening using two morphologically distinct genotypes (*P. scoparia*-B1 and *P. communis*-T) in which 16 decamer primers with reproducible bands and maximum polymorphism were selected (Table 5, Fig. 5). From 260 scored DNA fragments, 250 (96.15%) were polymorphic and the size of bands ranged

from 300 to 3000bp. Average value of polymorphism per primer was 95.81%. Maximum value for polymorphism (100%) was obtained from TIBMBA-14, TIBMBA-17, TIBMBB-05, TIBMBB-08, TIBMBD-09, TIBMBD-10 and the minimum from TIBMBB-16 (86%). TIBMBB-5 gave the maximum number of bands (25 fragments) and the minimum number of bands was obtained from TIBMBE-18 (11 fragments). The number of generated bands per primer depends on the primer sequence and also the extent of molecular variation in each genotype (Shiran et al., 2007).

Genetic similarity coefficient ranged from 0.28 (between E-5 and O-4T) to 0.79 (between *P. scoparia*- ES and *P. scoparia*- N1) with an average of 0.53. The molecular neighbor joining dendrogram based on Jaccard's similarity matrix indicated four main groups among the samples, mostly according to their geographic origins and relatively to their botanical classifications. The first group included three samples (SH, SHSH-3773 and SAH). The second group included one of the GF677-based samples (GF677OP2). *P. communis* was also placed independently from other wild genotypes in this group. Group III completely showed Section *Spartioides* Spach and the maximum value of similarity (0.79) was seen in this group. In forth group, *P. erioclada*-M, *P. lycioides*-T and *P. eburnea*-T (members of Section *Lycioides* Spach) were grouped in one cluster with the average similarity of 0.62. On the other hand *P. orientalis*-G was grouped more closely to the cultivated almonds. Another GF677-based sample (GF677OP1) was grouped in this cluster with two cultivated genotypes.

The results of principle coordinate analysis showed that first three coordinates accounted for 63.33% of the molecular variation. The first coordinate accounted for 55.17% of the variation while the second and third coordinates featured 5.53% and 2.63% of the variation respectively. The separation of the samples with regard to the first three coordinate axes as 3D PCoA plot,

were lead to similar groupings as were seen in neighbor-joining molecular dendrogram (data not shown).

4. Discussion

The existing gene pool in cultivated almond restricts its introduction to new regions and also limits its cultivation due to always changing environmental conditions. These problems have led to an inevitable need for genetic solutions and utilization of genetic diversity (Gradziel et al., 2001). In the studies of genetic diversity, the combination of the necessary and also less laborious morphological evaluation with molecular markers leads to more reliable conclusions in the assessment of genetic diversity. (Sorkheh et al., 2007; Khadivi-khub et al., 2008; Hend et al., 2009; Sorkheh et al., 2009a).

Close relationships among traits could have positive or negative role in the transmission of traits through gene introgression, since strong selection for a desirable trait, could support the presence of other trait(s) (Dicenta and García, 1992). The existence of close correlations among leaf length, leaf width, leaf area and also traits related to vigor such as length and diameter of main trunk and thickness of one year old shoot indicate that more leaf expansion leads to stronger aerial growth. This correlation could be considered as a suitable relationship for improving vigorous rootstocks suitable for dry environments where a fast and strong growth is needed in the beginning of the seasonal life cycle to induce and maintain appropriate vigor in

scion and also for reaching to an appropriate size for budding and/or grafting as soon as possible in nurseries.

Negative correlation between leaf length/width ratio and traits related to vigor is an important relationship for improving drought tolerant rootstocks in a different point of view. As in water deficiency conditions plants reduce leaf expansion and aerial growth by some strategies like reducing leaf area, leaf abscission and rolling of leaves (Scott, 2008; Hopkins and Hüner, 2009), having thinner leaves (high leaf length/width ratio) accompanied by less aerial growth, may lead to more root penetration. This could be considered as a possible potential for drought tolerance, particularly for the purpose of establishing orchards on slopes and also under non irrigated conditions where a good and quick root penetration is necessary. In a similar study Lansari et al., (1994) concluded that Moroccan almond clones have smaller leaves than European and American cultivars (a probable property in Moroccan almond germplasm to flourish in dry conditions). In this concept, leaves of *P. scoparia* and its close relative *P. arabica* drop in natural ecosystems after a few weeks of growth and the green shoots continue to photosynthesis (as a mechanisms for drought tolerance).

As an undesirable trait interfering with an upright and strong trunk, also a negative correlation was observed among ramification, length and diameter of main trunk. This relationship indicates that more ramifications by the induction of lateral branches hamper an upright trunk growth and possibly could affect the time needed in reaching to a proper thickness of budding and/or grafting in nurseries.

In principle component analysis, in comparison with other vegetative traits in our study, Leaf traits such as leaf width and leaf area were predominant in the first component in both years of the study, indicating that they are not only useful for the assessment of genetic diversity but

also for almond germplasm characterization. Our results are in correspondence with the results of previous studies. Lansari et al., (1997) showed that leaf traits have a more significant role in characterizing the almond germplasm when plants are under stress. Zeinalabedini et al., (2008) studied nine fruit, kernel and leaf traits in four wild *Prunus* species including *Prunus eleagnifolia*, *Prunus hauskenchtii*, *Prunus scoparia* and *Prunus lycioides*. Their results showed that leaf width showed significant differences among these species and indicated the possibility of the use of leaf traits in distinguishing *Prunus* germplasm during the vegetative growth period.

Results of morphological cluster analysis showed a distinction with regard to similarity in appearance among genotypes. The separation of all the wild species was in good agreement with their botanical classification. As an example *P. eburnean-T*, *P. eburnean-N*, *P. lycioides-T* and *P. erioclada-M* which belong to the section *Lycioides* Spach were gathered together in one cluster. These species are characterized by thorny branches and dwarf growing habits (Mozaffarian, 2005). The other expected grouping is among *P. scoparia* genotypes. Although they have collected from different provinces throughout the country, their morphology is so similar that except one sample (*P. scoparia-T*) they have grouped together. Unlike *P. scoparia*, its close relative, *P. arabica*, is very dwarf and also has larger leaves (Mozaffarian, 2005). Possibly close resemblance of leaf traits and growing habits among *P. scoparia-T* which is unexpectedly dwarf, *P. orientalis-G* which also has larger leaves than other wild species and *P. arabica-G* have placed them in one cluster. *P. communis* were placed in one cluster with genotypes from Fars, Khorasan and East Azarbayjan provinces indicating a good similarity of appearance between *P. communis* and Cultivated almond (*Prunus dulcis*). Two dimensional morphological PCA plot based on the first two components, also discriminated the samples into two different groups as shown in figure 3 and the ordination of the genotypes confirmed the

morphological clustering results (Fig. 3 and Fig. 4). In the first group among cultivated almonds, GF677OP1 and GF677OP2 were placed together which show their close relationships in appearance. Wild and cultivated almonds were separated into two distinct groups and as seen in morphological dendrogram, *P. communis* was placed among the cultivated almonds (Fig. 3 and Fig. 4).

RAPD approach has been used to study the genetic diversity of almond germplasm in several studies (Casas et al., 1999; Mir Ali and Nabulsi, 2003; Shiran et al., 2007) and also has been shown to be a reliable marker with a series of advantages such as performing quick, inexpensive and less laborious assays with a high level of band-sharing and the possibility of the use of unlimited number of arbitrary primers (Baránek et al., 2006; Shiran et al., 2007; Sorkheh et al., 2009a). Our results also showed that RAPD marker could properly detected the similarity in the studied plant materials.

Based on the molecular dendrogram, grouping of many samples into their respective regions (collection sites) was evident. This could be due to the DNA sharing by plants from similar regions through the intra-location hybridization among them. Woolley et al., (2000), Martínez-Gómez et al., (2003b) and Shiran et al., (2007) reported groupings of different almond cultivars according to the geographic origin, using RAPD or SSR markers. The presence of GF677-based genotypes (GF677OP1 and GF677OP2) in groups with samples from East Azarbayjan province, possibly could be due to the existence of parental relationships between this region's plant materials and plants used in breeding programs of la Grande Ferrade research station in France for introducing GF677 (Fig. 6).

On the other hand, accessions did not completely cluster according to their geographical origins. Almond is an ancient nut crop in Iran and has a short juvenile period (Kester and Gradziel, 1996). Therefore, its traditional propagation and accordingly its ancient distribution were by seeds. Thus during millennia, because of communication throughout the country, almond germplasm may have been under exchange among different regions of Iran. This could explain the overlapping groupings of some genotypes from different sites (as an example SHA-12, GA-21AG and AV-151).

Average Jaccard's similarity coefficient was 0.53 (with the maximum of 0.79 and the minimum of 0.28) and the mean value of polymorphism per primer was 95.81%. In the study conducted by Mir Ali and Nabulsi, (2003) on 19 almond cultivars from Syria, America and Europe, the reported average similarity value was 0.78, which is lower than our result. Also in a previous study, Gouta et al., (2008) used 12 polymorphic RAPD primers to assess the genetic diversity and phylogenetic relationships among 58 Tunisian almond and one peach cultivars. Our results showed much more variation than Tunisian almond germplasm as well. Species differences at molecular level could be masked by random mating and gene exchange through natural interspecific hybridization, owing to the lack of crossing barriers in almond and its related species (Browicz and Zohary, 1996). This probable interaction between wild and cultivated genotypes have resulted in the observed high level of variation.

A notable divergence was observed between the morphological and molecular analysis. In general, groupings obtained from RAPD dendrogram were not congruent with those obtained by morphological dendrogram. The morphological analysis differentiated individuals into their respective species while molecular analysis reveals a high degree of separation among genotypes and species regarding to geographical regions. In addition, both morphological and molecular

analyses did not show completely expected groupings. The correlation between matrices of these two data sets was not significant ($R = -0.38$). RAPDs investigate the whole genome including both coding and non-coding regions (Williams et al., 1990; Kumar, 1999) but morphological traits are related to coding regions. In addition, most of the genome size consists of non-coding regions, therefore, this low correlation could be interpreted by differences in the nature of these two approaches. It is possible that the evaluation of more morphological traits could lead to a higher correlation between morphological and molecular analyses. Several studies have compared the use of morphological and molecular techniques in different species and examined their relationships and most of these studies showed low level of correlation between them (Kjar et al., 2004; Kelleher et al., 2005; Vollmann et al., 2005; Sorkheh et al., 2007; Zamani et al., 2007).

However, in our study there are agreements between morphological and molecular groupings. As an examples, based on molecular NJoin cluster, grouping III was in correspondence with the morphological dendrogram. Another agreement between morphological and molecular dendrograms was observed in the case of *P. communis* (Fig. 4 and Fig. 6). This species which is considered as one of the closest ancestor of cultivated almond was placed among cultivated almonds both in morphological and molecular clusters. No matter cultivated almond is derived from natural hybridization (Evreinoff, 1958) or by selection within *P. communis* populations (Vavilov, 1930; Kovaleff and Kostina, 1935; Denisov, 1988), our results confirm that this species has close relationships with cultivated almonds both at morphological and DNA levels. Our results are in the same line with a recent study of Sorkheh et al., (2007) who used cultivated and wild almond genotypes to determine genetic relationships among them using 19 AFLP marker combinations. They showed that *P. communis* was highly similar to

cultivated almonds at DNA level. Also Shiran et al., (2007) showed that *P. communis* is highly related to *P. dulcis* using RAPD and SSR markers which is in agreement with our results.

Previous authors have determined genetic relationships and diversity in almond using different kinds of markers such as isoenzyme (Vezvaei, 2003), SSRs (Shiran et al., 2007; Zeinalabedini et al., 2008), AFLPs (Sorkheh et al., 2007, 2009a) and morphological traits (Sorkheh, 2009b). This is possibly the first study conducted on the Iranian almond germplasm with regard to rootstock breeding point of view using both morphological and molecular techniques.

Zeinalabedini et al., (2008) and Sorkheh et al., (2009) showed that presence of undesirable and possibly worthless fruit and kernel traits such as self-incompatibility, bitter kernel taste and small fruit size limits the use of wild almond species for transferring useful properties to cultivated almond and these authors concluded that wild *Prunus* species are less useable in almond cultivar improvement. In contrast, our results showed that the studied plant materials are valuable sources for root stock improvement programs which is in the same direction of our long-term objective for introducing suitable rootstocks for arid and semi arid regions.

Our results showed high level of variability at both morphological and molecular levels. Almond is predominantly an outbreeder crop in which natural (Serafinov, 1971; Denisov, 1988) and also controlled hybridization (Grasselly, 1976a; Kester et al., 1991; Kester and Gradziel, 1996; Gradziel et al., 2001; Martínez-Gómez et. al., 2005) occurs among its species.

5.Conclusion

Although our plant materials does not represent the whole almond germplasm in Iran, but considerable genetic diversity was observed both at morphological and molecular levels indicating that there are rich and valuable plant materials for almond rootstock improvement. Lack of crossing barriers and therefore resultant inter specific hybridization and introgression among the species and genotypes could also have a significant role. With the continued use of molecular techniques for describing diversity and species characteristics, a potential source of markers is available for characterizing almond and for testing species differentiation at the molecular level. Seemingly we report for the first time, the potentials of almond germplasm in Iran for root stock improvement. In this study superior genotypes and families were selected which are going to be used as parents in further steps. Wild species are also potential sources for transferring valuable resistances regarding their ability to withstand unfavorable environments. These results emphasize the usefulness of closely related *Prunus* spp. in almond rootstock breeding programs.

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Fig. 1. Relative wild species of almond, *P. eburnean* (A), *P. scoparia* (B), *P. arabica* (C), *P. lycioides* (D), collected from different regions of IRAN.

Fig. 2. Distribution of collection sites of studied plant materials in Iran.

Fig. 3. Two dimensional PCA plot according to the combined morphological data of the two years of the evaluation in this study.

Fig. 4. Cluster analysis of 62 cultivated almond and wild *Prunus* genotypes based on morphological traits.

Fig. 5. RAPD profiles of cultivated almond and wild *Prunus* genotypes amplified by decamer primer TIBMBA06. The numbers represent different samples according to Table 1.

Phylogenetic tree showing the relationships between various *Pseudomonas* species based on 16S rDNA sequences. The tree is rooted at the bottom left and branches upwards. The tree is divided into four main groups labeled on the left: First Group (purple), Second Group (black), Third Group (red), and Fourth Group (black). Bootstrap values are indicated at various nodes. Species names are listed at the tips of the branches, with some highlighted in color: *P.orientalis-G* (black), *P.perioclada-M* (cyan), *P.lycioides-T* (cyan), *P.eburnea-T* (cyan), *P.scoparia-ES* (red), *P.scoparia-N1* (red), *P.scoparia-N2* (red), *P.scoparia-B1* (red), *P.scoparia-MJ* (red), *P.scoparia-T* (red), *Parabica-C* (red), *P.scoparia-B2* (red), *P.communis-T* (green), and others in black. A scale bar at the bottom left indicates 0.1 substitutions per site.

Table 1

Name/abbreviation and species of the studied plant materials with indication of their geographical origin in IRAN.

No.	Accession Name	Species	IRAN Province	Longitude	Latitude	No.	Accession Name	Species	IRAN Province	Longitude	Latitude
1	AV-14	<i>Prunus dulcis</i>	Khorasan (Site 1)	59.14	36.64	32	S-9	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
2	AV-151	<i>Prunus dulcis</i>	Khorasan (Site 1)	59.14	36.64	33	T-2BT	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
3	AV-169	<i>Prunus dulcis</i>	Khorasan (Site 1)	59.14	36.64	34	T-3	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46
4	AV-41	<i>Prunus dulcis</i>	Khorasan (Site 1)	59.14	36.64	35	TAS-2	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
5	AV-74	<i>Prunus dulcis</i>	Khorasan (Site 1)	59.14	36.64	36	JS-4T	<i>Prunus dulcis</i>	Kohgiluyeh Boyer Ahmad	50.42	31.2
6	GA-12	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.16	37.41	37	JS-7T	<i>Prunus dulcis</i>	Kohgiluyeh Boyer Ahmad	50.42	31.2
7	GA-16	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	38	AL-1	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
8	GA-21AG	<i>Prunus dulcis</i>	Fars (Site 1)	52.97	29.81	39	AL-2	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41
9	GF677(OP1)	<i>Prunus dulcis</i> × <i>Prunus persica</i>	East Azarbayjan (Site 1)	46.21	37.92	40	JS-1	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
10	GF677(OP2)	<i>Prunus dulcis</i> × <i>Prunus persica</i>	Fars (Site 2)	53.05	29.07	41	JS-11	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
11	SHA-12	<i>Prunus dulcis</i>	Fars (Site 2)	53.05	29.07	42	JS-12	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92
12	SHSH-3773	<i>Prunus dulcis</i>	Fars (Site 1)	52.97	29.81	43	JS-15	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46
13	SH	<i>Prunus dulcis</i>	Fars (Site 1)	52.97	29.81	44	JS-17	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46
14	SAH	<i>Prunus dulcis</i>	Fars (Site 1)	52.97	29.81	45	JS-18	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46
15	D-1	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46	46	JS-19	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41
16	D-3	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46	47	JS-20	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41
17	D-5	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	48	KA-1	<i>Prunus dulcis</i>	Tehran (Site 1)	51.62	35.72
18	D-10	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	49	<i>P. communis</i> -T	<i>P. communis</i>	Tehran (Site 1)	51.62	35.72
19	E-1	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46	50	<i>P. orientalis</i> -G	<i>P. orientalis</i>	Kohgiluyeh Boyer Ahmad	50.42	31.2
20	E-5	<i>Prunus dulcis</i>	East Azarbayjan (Site 3)	46.98	38.46	51	<i>P. erioclada</i> -M	<i>P. erioclada</i>	Fars (site 2)	53.05	29.07
21	E-6	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	52	<i>P. lycioides</i> -T	<i>P. lycioides</i>	Tehran(Site 2)	50.73	35.86
22	E-7	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41	53	<i>P. eburnea</i> -N	<i>P. eburnea</i>	East Azarbayjan (Site 2)	46.16	37.41
23	E-9	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41	54	<i>P. eburnea</i> -T	<i>P. eburnea</i>	Tehran(Site 2)	50.73	35.86
24	E-10	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41	55	<i>P. scoparia</i> -MJ	<i>P. scoparia</i>	Fars (site 2)	53.05	29.07
25	O-4T	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41	56	<i>P. scoparia</i> -B1	<i>P. scoparia</i>	Khorasan (Site 2)	59.21	32.83
26	O-5	<i>Prunus dulcis</i>	East Azarbayjan (site 3)	46.98	38.46	57	<i>P. scoparia</i> -N2	<i>P. scoparia</i>	Khorasan (Site 2)	59.21	32.83
27	O-8	<i>Prunus dulcis</i>	East Azarbayjan (site 3)	46.98	38.46	58	<i>P. scoparia</i> -B2	<i>P. scoparia</i>	Khorasan (Site 3)	59.28	32.86
28	O-9	<i>Prunus dulcis</i>	East Azarbayjan (site 3)	46.98	38.46	59	<i>P. scoparia</i> -N1	<i>P. scoparia</i>	Fars (Site 1)	52.97	29.81
29	S-3	<i>Prunus dulcis</i>	East Azarbayjan (Site 2)	46.16	37.41	60	<i>P. scoparia</i> -ES	<i>P. scoparia</i>	Fars (Site 1)	52.97	29.81
30	S-7	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	61	<i>P. scoparia</i> -T	<i>P. scoparia</i>	Tehran (Site 1)	51.62	35.72
31	S-8	<i>Prunus dulcis</i>	East Azarbayjan (Site 1)	46.21	37.92	62	<i>P. arabica</i> -G	<i>P. arabica</i>	Kohgiluyeh Boyer Ahmad	50.42	31.2

Table 2

Correlations among 15 morphological traits in this study.

	Leng T	Dia T	Leng M	ThicY	Ll	Lw	L Area	Ll/Lw	Tree H	Tree V	Fol D	R	Col T	Leaf M	Col L
Leng T	1														
Dia T	0.748**	1													
Leng M	0.776**	0.858**	1												
ThicY	0.525**	0.607**	0.579**	1											
Ll	0.655**	0.825**	0.828**	0.778**	1										
Lw	0.628**	0.778**	0.785**	0.802**	0.944**	1									
L Area	0.597**	0.770**	0.761**	0.773**	0.954**	0.948**	1								
Ll/Lw	-0.677**	-0.636**	-0.640**	-0.620**	-0.671**	-0.794**	-0.673**	1							
Tree H	0.137	0.186	0.213	-0.142	-0.093	-0.224	-0.135	0.154	1						
Tree V	0.586**	0.737**	0.650**	0.344**	0.481**	0.384**	0.477**	-0.252*	0.066	1					
Fol D	0.452**	0.581**	0.413**	0.161	0.266*	0.278*	0.280*	-0.373**	0.045	0.422**	1				
R	-0.262*	-0.316*	-0.386**	-0.554**	-0.561**	-0.558**	-0.585**	0.365**	-0.035	-0.442**	0.061	1			
Col T	0.031	0.196	0.168	0.315*	0.228	0.184	0.327**	-0.046	0.287*	0.101	0.137	-0.177	1		
Leaf M	0.137	0.258*	0.317*	0.218	0.267*	0.341**	0.287*	-0.388**	0.250*	0.129	0.106	-0.267*	0.221	1	
Col L	0.147	-0.040	0.154	0.025	-0.107	0.015	-0.027	-0.086	-0.147	-0.011	0.087	0.007	-0.126	-0.084	1

Abbreviation of studied traits: Length of main trunk (Leng T), Diameter of trunk (Dia T), Length of main branches (Leng M), Thickness of one year old shoot (ThicY), Leaf length (Ll), Leaf width (Lw), Leaf area (L Area), Leaf length/width ratio (Ll/Lw), Tree habit (Tree H), Tree vigor (Tree V), Foliage density (Fol D), Ramification (R), Coloration of shoot tip (Col T), Leaf margin (Leaf M), Coloration of leaf (Col L).

Table 3

Descriptive statistics for fifteen morphological traits (abbreviation and units), among 62 almond and wild *Prunus* genotypes during two years of the study.

Traits		Unit	Min		Max		Mean		SD		CV	
			First Year	Second Year	First Year	Second Year	First Year	Second Year	First Year	Second Year	First Year	Second Year
Quantitative	Length of main trunk (Leng T)	cm	34	73	293	390	171.65	249.08	56.78	70.17	33.08	28.17
	Diameter of trunk (Dia T)	mm	3.37	4.68	36.41	88.23	21.81	44.61	8.11	20.83	37.2	46.71
	Length of main branches (Leng M)	cm	27	40	230	293	134.06	169.58	54.45	66.32	40.61	39.11
	Thickness of one year old shoot (ThicY)	mm	1.08	1.8	4.9	5.52	3.03	3.74	0.8	0.79	26.33	21.08
	Leaf length (Ll)	mm	11.28	13.4	89.45	134.7	52.91	80.64	20.94	34.1	39.57	42.29
	Leaf width (Lw)	mm	1.49	1.9	25.03	37.04	15.57	22.3	7.34	10.87	47.15	48.76
	Leaf area (L Area)	mm ²	9.48	44.2	1597.4	3906.97	798.17	1717.82	456.31	1074.08	57.17	62.53
	Leaf length/width ratio (Ll/Lw)	-	2.1	1.82	11.73	10.55	4.25	4.28	2.27	1.89	53.4	44.13
Qualitative	Tree habit (Tree H)	code (1-9)	1	1	5	5	2.05	2.05	1.08	1.08	52.61	52.61
	Tree vigor (Tree V)	code (3-7)	3	3	7	7	4.94	4.94	1.54	1.54	31.1	31.1
	Foliage density (Fol D)	code (3-7)	3	3	7	7	5.02	5.02	1.62	1.62	32.39	32.39
	Ramification (R)	code (0-9)	2	2	9	9	5.92	5.92	2	2	33.83	33.83
	Coloration of shoot tip (Col T)	code (0-7)	0	0	7	7	4.11	4.11	2.25	2.25	54.65	54.65
	Leaf margin (Leaf M)	code (1-2)	1	1	2	2	1.48	1.48	0.5	0.5	33.95	33.95
	Coloration of leaf (Col L)	code (3-7)	3	3	7	7	6.52	6.52	0.94	0.94	14.37	14.37

Table 4

Eigenvectors of the 3 principal component axes from PCA analysis of the studied 62 almond and wild *Prunus* genotypes for first and second year of evaluation.

Traits		PC 1		PC 2		PC 3	
		First Year	Second Year	First Year	Second Year	First Year	Second Year
Quantitative	Leng T	0.371	0.361	0.728	0.794	-0.025	0.057
	Dia T	0.463	0.470	0.764	0.776	0.171	0.236
	Leng M	0.707	0.473	0.532	0.763	0.078	0.177
	ThicY	0.760	0.799	0.277	0.141	-0.152	-0.039
	Ll	0.787	0.860	0.450	0.400	0.119	0.158
	Lw	0.866	0.866	0.401	0.411	0.008	0.083
	L Area	0.813	0.882	0.426	0.322	0.052	0.128
	Ll/Lw	-0.695	-0.534	-0.459	-0.548	0.049	0.153
Qualitative	Tree H	-0.126	-0.303	0.180	0.262	0.743	0.710
	Tree V	0.446	0.321	0.590	0.668	0.038	0.080
	Fol D	0.019	-0.016	0.876	0.738	-0.027	-0.062
	R	-0.864	-0.758	0.035	-0.122	-0.080	-0.151
	Col T	0.278	0.332	-0.005	0.027	0.597	0.616
	Leaf M	0.471	0.292	0.025	0.264	0.451	0.477
	Col L	0.112	-0.006	0.081	0.336	-0.586	-0.589
Percentage		34.77	31.53	22.55	25.5	10.28	11.03
Cumulative		34.77	31.53	57.32	57.03	67.6	68.06

Length of main trunk (Leng T), Diameter of trunk (Dia T), Length of main branches (Leng M), Thickness of one year old shoot (ThicY), Leaf length (Ll), Leaf width (Lw), Leaf area (L Area), Leaf length/width ratio (Ll/Lw), Tree habit (Tree H), Tree vigor (Tree V), Foliage density (Fol D), Ramification (R), Coloration of shoot tip (Col T), Leaf margin (Leaf M), Coloration of leaf (Col L).

Table 5

16 selected RAPD primers and the degree of polymorphism obtained among all 62 genotypes in this study.

Number	Primer	Sequence 5 →3	No. of Bands	No. of Polymorphic bands	Polymorphism Percentage
1	TIBMBA-05	TGCGTTCCAC	15	14	93
2	TIBMBA-06	GGACGACCGT	16	15	93
3	TIBMBA-14	TCGGGAGTGG	15	15	100
4	TIBMBA-17	TGTACCCCTG	12	12	100
5	TIBMBB-05	GGGCCGAACA	25	25	100
6	TIBMBB-08	TCGTCTGAAGG	19	19	100
7	TIBMBB-09	AGGCCGGTCA	23	22	95
8	TIBMBB-12	TTCGGCCGAC	13	12	92
9	TIBMBB-16	TCGGCACCGT	15	13	86
10	TIBMBC-05	GAGGCGATTG	14	13	92
11	TIBMBD-04	TCGGGTGTTG	18	17	94
12	TIBMBD-06	AAGCTGGCGT	19	18	94
13	TIBMBD-09	CCACGGTCAG	12	12	100
14	TIBMBD-10	GACGCTATGG	16	16	100
15	TIBMBE-18	CCAAGCCGTC	11	11	100
16	TIBMBE-20	CAAAGGCGTG	17	16	94
17	total	-	260	250	96.15
18	mean	-	16.25	15.63	95.81