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RELATIONSHIPS AMONG CULTIVATED PEAS AND THEIR WILD RELATIVES: MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION

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Abstract

This study was conducted to determine relationship between some wild pea accessions (*Pisum fulvum* L., *P. abyssinicum* L., *P. sativum* var. *elatius*), local varieties (*P. sativum* var. *sativum* L. and *P. sativum* var. *arvense* L.) and commercial varieties "Boogie" and "Rondo". The genetic diversity was evaluated with 14 simple sequence repeat markers and 50 morphological characters. The results of morphology indicated that, genotypes showed a clustering pattern based on the taxonomic groups when considering only flower characters and all morphological characters. During the molecular study, a total of 48 alleles were obtained. Used all primers showed polymorphism in accessions. The number of alleles varied between 2 - 6 among 14 SSR loci revealing the polymorphism level of markers. Similarity coefficient (Dice's) ranged from 0.100 to 0.800 with an average of 0.378. A dendrogram grouped the 15 genotypes into two main clusters. This information can be utilized for genetic analysis, genotype identification from different sources and development of improved germplasm.

Introduction

The taxonomic classification of *Pisum* based on karyology and morphology has varied over time. Despite the wide phenotypic and genetic variability, available classifications are still confusing (Korstein and Bogdanova 2008). Davis (1970), Kupicha (1981) recognized species as *P. fulvum* and *P. sativum. P. abyssinicum* was not considered as third presumed species by these authors. In addition to these assumptions, Vershinin *et al.* (2003) reported strong relationships among *P. humile, P. elatius* and *P. sativum.* These scientists also grouped the genus into three major classes: *P. abyssinicum, P. fulvum* and *P. sativum- P. humile - P. elatius* complex. Three species (*P. abyssinicum, P. fulvum* and *P. sativum)* were recognized with two subspecies ssp. *sativum* and ssp. *elatius* (Bieb.) by Maxted and Ambrose (2001) in a phylogenetic organization of taxa. According to the actual integrated taxonomic information system database, the *Pisum* genus has only two species: *P. fulvum* and *P. sativum* L. According to same data, the *P. sativum* L. species has five varieties: *P. sativum* var. *arvense* (L.) Poir. (Austrian winter pea), *P. sativum* var. *elatius* (Steven ex M. Bieb.) Alef., *P. sativum* var. *macrocarpon* Ser., *P. sativum* var. *pumilio* Meikle, *P. sativum* var. *sativum* L. (garden pea) (ITIS 2018).

This study focused on examining the molecular and morphological features based on plant organs of the 15 *Pisum* accessions (wild peas, local varieties and commercial varieties). Results of the present study may ensure some beneficial information for the conservation and the use of these *Pisum* accessions in future breeding programs.

Materials and Methods

The genotypes of *Pisum sativum* var. *sativum, P. sativum* var. *arvense, P. sativum* var. *elatius, P. abyssinicum* and *P. fulvum* were used as plant material (Table 1).

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Fifteen *Pisum* accessions were planted on the 16th March 2016, and 23th March 2017. The experiment was designed according to the randomized block design with three replications. Data on different agronomic characters were recorded on individual plant basis from 20 plants randomly selected in each plot appropriately to the International Union for the Protection of New Varieties of Plants (Table 3).

Table 1. List of genotypes used in study and their origins.

	Accession name	Geographical origin	Taxonomic name	Resource
1	65PA099	Ethiophia	*P. abyssinicum*	ARS-USDA, USA
2	66PA099	Yemen	*P. abyssinicum*	ARS-USDA, USA
3	67PF099	Antalya, Turkey	*P. fulvum*	ARS-USDA, USA
4	68PF099	South Anatolia, Turkey	*P. fulvum*	ARS-USDA, USA
5	69PSE099	Denizli, Turkey	*P. sativum* var. *elatius*	ARS-USDA, USA
6	70PSE099	Mardin, Turkey	*P. sativum* var. *elatius*	ARS-USDA, USA
7	BOOGIE	Com. Variety	*P. sativum* var. *sativum*	Commercial var.
8	75PSE098	Mersin, Turkey	*P. sativum* var. *elatius*	JIC, UK
9	105PSC098	Aydın, Turkey	*P. sativum* var. *arvense*	JIC, UK
10	115PSA097	Mardin, Turkey	*P. sativum* var. *arvense*	AARI, TR
11	116PSA097	Kastamonu, Turkey	*P. sativum* var. *arvense*	AARI, TR
12	117PS097	Çorum, Turkey	*P. sativum* var. *sativum*	AARI, TR
13	122PSA097	Bayburt, Turkey	*P. sativum* var. *arvense*	AARI, TR
14	141PS097	Çanakkale, Turkey	*P. sativum* var. *sativum*	AARI, TR
15	RONDO	Com. Variety	*P. sativum* var. *sativum*	Commercial var.

Table 2. Details of the SSR primers used in the study.

SSR marker	Temp.(°C)	Linkage group	Allel size (bp)	No. of alleles	PIC
AA122	61	IV	175-225	4	0.833
AA205	51	II	175-225	2	0.154
AA446	51	VII	450-465	5	0.892
AA5	61	III	225-250	3	0.311
AB141	61	III	175-225	3	0.592
AB23	61	V	200-225	3	0.681
AC58	61	V	200-225	3	0.585
AD146	51	VII	375-425	6	0.658
AD147	61	I	300-325	3	0.753
AA67	51	I	330-390	4	0.820
AB72	55	II	450-500	4	0.523
AA175	61	III	225-250	3	0.716
AA285	51	IV	250-275	3	0.574
AB64	61	III	350-400	2	0.611

Table 3. Morphological traits regarding leaf-stem, flower and pod-seed characters.

Leaf-stem (LS)	Flower (F)	Seed-pod (SP)
Length of leaflet (LS1)	Time of flowering (F1)	Shape of seed (SP1)
Width of leaflet (LS2)	Maximum number of flowers	Color of cotyledon of seed (SP2)
Size of leaflet (LS3)	per node (F2)	Marbling of testa (SP3)
Length of stipule (LS4)	Color of wing (F3)	Violet or pink spots on testa
Width of stipule (LS5)	Intensity of color of wings (F4)	(SP4)
Color of leaflet (LS6)	Intensity of color of standard	Hilum color on seed (SP5)
Intensity of color of leaflet (LS7)	(F5)	Color of testa (SP6)
Leaflets (absent or present) (LS8)	Color of standard (F6)	Wrinkling of seed cotyledon
Waxiness of upper leaflet (LS9)	Width of standard (F7)	(SP7)
Dentation of leaflet (LS10)	Shape of base of standard (F8)	Type of starch grains (SP8)
Degree of dentation of leaflet (LS11)	Undulation of standard (F9)	Width of seed (SP9)
Size of stipule (LS12)	Width of upper sepal (F10)	Curvature on pod (SP10)
Shape of stipule (LS13)	Shape of apex of upper sepal	Type of curvature of pod (SP11)
Flecking of stipule (LS14)	(F11)	Shape of distal part of
Density of flecking of stipule (LS15)	Length of peduncle	pod (SP12)
Anthocyanin coloration of stem (LS16)	(up to first flower) (F12)	Color of pod (SP13)
Length of plant (LS17)		Intensity of green color of pod
Fasciation of stem (LS18)		(SP14)
Intensity of color of foliage (LS19)		Anthocyanin coloration
Stem length (LS20)		of parchment (SP15)
Number of nodes up to first fertile		Anthocyanin coloration of
Node (LS21)		pod (SP16)
Length from axil to first leaflet or		
tendr (LS22)		

Cluster analysis conducted on the matrix of Euclidean distances generates a dendrogram using the Ward method for each observation group (Ghixari *et al.* 2014). SAS Institute Inc. JMP® and IBM SPSS® Statistics Ver. 221 were used for all the statistical procedures during morphological characterization studies.

The bulked representative of six individual plants was used for DNA extraction using a MACHEREY-NAGEL NucleoSpin® Plant II kit (MACHEREY-NAGEL GmbH & Co. KG., Düren, Germany). SSR primer pairs were preferred for high power of discrimination, considering the previous report (Loridon *et al*. 2005) (Table 2). The PCR was performed in a 25 µl volume of a master mixture containing 2 mM $MgCl_2$, 1 U Taq DNA polymerase (Fermentas, Pittsburgh, PA, USA), 20 - 25 ng genomic DNA, 200 µM deoxyribonucleotide triphosphates, 1X Taq buffer, and 0.6 mM reverse and forward primers (Kumari *et al.* 2011).

Amplification protocol was 1 cycle of denaturation for 3 min at 94°C (preamplification); 30 sec at 94°C for denaturation, 30 sec at 5°C for annealing, followed by 1 min at 72°C for extension, for a total of 40 cycles; and the final extension at 72°C for 10 min. PCR products were analyzed on 3% agarose gel stained with ethidium bromide (EtBr) in Tris-borate EDTA (TBE) buffer and

visualized under UV light (Kumari *et al*. 2011). Microsatellite bands were scored as either present (1) and absent (0). The values of polymorphic information content (PIC) were obtained using this formula (Hildebrand *et al.* 1992):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2 \quad (1)$$

The p_i and p_j represent the population frequency of the i_{th} and j_{th} allele, respectively.

Dice's coefficients were used to obtain similarity matrices. To obtain the matrix which was computed with the UPGMA algorithm (unweighted pair group method with arithmetic mean) the XLSTAT program was used (Garcia-Valle *et al.* 1999).

Results and Discussion

Allelic variation was clearly observed among the accessions with the used primers. A total of 48 bands were detected by 14 SSR primers, thus amplifying an average of 3,43 bands for each primer. Among them, 47 bands were found polymorphic. The SSR primer AA122 (175 bp) was monomorphic among accessions, therefore, it was not used for further evaluations. Genetic distance between *Pisum* accessions varied in the range of Dice's similarity coefficient from 0.100 to 0.800 and average distance has been identified as 0.379. Among the accessions, genetic similarity coefficient found 0.800 as the highest value between genotypes 075PSE098 (from Mersin province) and 070PSE098 (from Mardin province). The lowest value was determined between 75PSE098 and "Rondo" (a commercial variety). The dendrogram obtained from the analysis of *Pisum* accessions is divided into two groups (G1-2) at 30% genetic distance (Fig. 1).

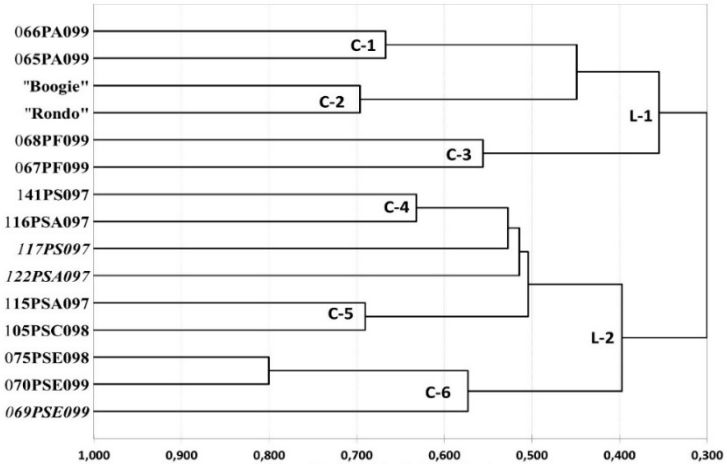

Fig. 1. Cluster analysis of *Pisum* genotypes based on molecular data.

Group-I is comprised of six accessions, having two commercial varieties. This group includes also two *P. fulvum* L. and two *P. abyssinicum* L. accessions. The combination of cultured peas and wild genotypes was remarkable. Group-II is comprised of nine genotypes, having three P. *sativum* var. *elatius* landraces; two local varieties (*P. sativum* var. *sativum* L.), four *P. sativum* var. *arvense* L. accessions. Polymerase chain reaction-based assays have been used to study the genetic

polymorphism in various plant species. In *Pisum* species, these SSR primers have made possible the characterization of different accessions, the understanding of phylogenetic relationships, and genetic mapping.

Allele sizes were found similar to expected values (Loridon *et al.* 2005, Cupic *et al.* 2009, Kumari *et al.* 2011). Nisar *et al.* (2017) reported that an average of 4.69 alleles per SSR locus was obtained in newly developed Pakistani pea lines. Similarly, 2 to 4 alleles per locus were reported in Spanish pea accessions (Martin-Sanz *et al.* 2011). Using SSR markers, Hagenblad *et al.* (2014) reported 5 to 10 alleles in the Swedish garden pea. Similarly, allele number per locus averaged 3.1 in the work of Teshome *et al.* (2015).

PIC values of all studied primers ranged from 0.154 to 0.892 with a mean of 0.622. PIC values obtained in the present research were higher than the previous studies of the various researchers. Nisar *et al.* (2017) obtained the maximum PIC value of 0.630 in 23 pea accessions while Kumari *et al.* (2011) obtained the maximum PIC value of 0.657 in 28 genotypes. This value varied from 0.055 to 0.660 with a mean of 0.460 in the work of Ahmad *et al.* (2012). In the present study, the high polymorphism values (average PIC, 0.622; maximum PIC, 0.892) is through the efficiency of the preferred SSR primer pairs. Clusters of studied accessions revealed based on molecular data. Only *P. sativum* var. *arvense* and *P. sativum* var. *sativum* L. accessions grouped in different clusters (Fig. 1). According to the results of the cluster analysis, it was interesting that the commercial varieties "Rondo" and "Boogie" were distinguished from other *P. sativum* var. *sativum* accessions (Fig. 1). The high level of genetic diversity obtained among the 15 *Pisum* genotypes based on Dice's similarity coefficients (ranged from 0.100 to 0.800). Samec and Našinec (1996) have reported a narrow diversity (0.69 - 0.88) between cultivars of *P. sativum* ssp. *sativum* and *P. sativum* ssp. *arvense*, whereas a much higher range (0.49 - 0.98) was obtained between the wild species *P. sativum* ssp. *elatius* and *P. sativum* ssp. *humile*. Ford *et al.* (2002) reported the largest distance among *P. sativum* and *P. fulvum* accessions.

According to the results of molecular marker application, used microsatellite markers confirmed that the present experiment *Pisum* accessions have big genetic variability. All 14 primer pairs cross-amplified in specimens of the widespread sister-subspecies *P. sativum* var. *arvense*, *P. sativum* var. *elatius*, *P. abyssinicum*, and *P. fulvum*. These molecular markers will be useful for studying genetic diversity and structure as well as for better assessing the conservation status of populations of *Pisum*. In the future these results can help breeders for interspecific crossing attempts. Also, the application of different marker systems such as ISSR, RAPD etc., may make a significant contribution to the findings obtained in this study.

The cluster analysis was made separately for each agronomic character's group (leaf and stem, flower, seed and pod) for detailed evaluations. In addition, each dendrogram was prepared for both individual *Pisum* accessions (Figs 2 - 5). The members of groups did not correspond in terms of taxonomic classification based on leaf and stem characters (Fig. 2). Two main clusters were obtained using flower characters for all individual accession (Fig. 3). Members of groups did not correspond in terms of taxonomic classification when considering the flower characters. For example, two *P. sativum* var. *elatius* accessions were located in two separate groups. When considering the seed-pod characters, the largest distance coefficient was 44.09 between 65PA099 and 67PF099, while the least was 0.01 (between 67PF099 and 68PF099; 65PA099 and 66PA099). Average distance coefficient among all investigated accessions was 13.18 (Fig. 4).

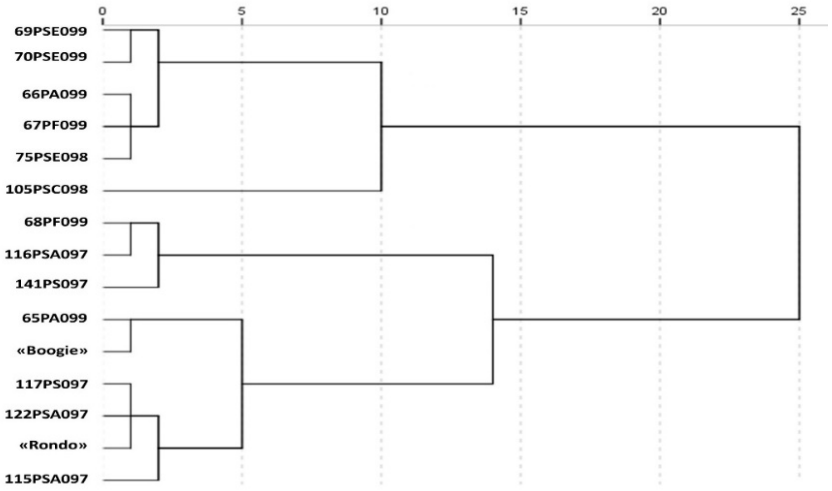

Fig. 2. Cluster analysis of individual genotypes based on leaf and stem morphology.

The 15 accessions formed two clusters at the average taxonomic distance of 142,86 considering whole morphology (Fig. 5). The largest distance was 505,06 between 65PA099 and 68PF099 while the least was 6,30 between 117PS097 and "Boogie". When considering the whole morphology, members of groups did not correspond in terms of taxonomic classification. For example, one of three *P. sativum* var. *arvense* took place in group II while the others were in group I. Similarly, *P. sativum* var. *sativum* accessions were divided into two separate groups.

The measurement of the phenotypical traits can provide a convenient technique for quantifying genetic similarity while at the same time defining genotype performance under relevant growing environments (Shuaib *et al.* 2007). The present research provided significant

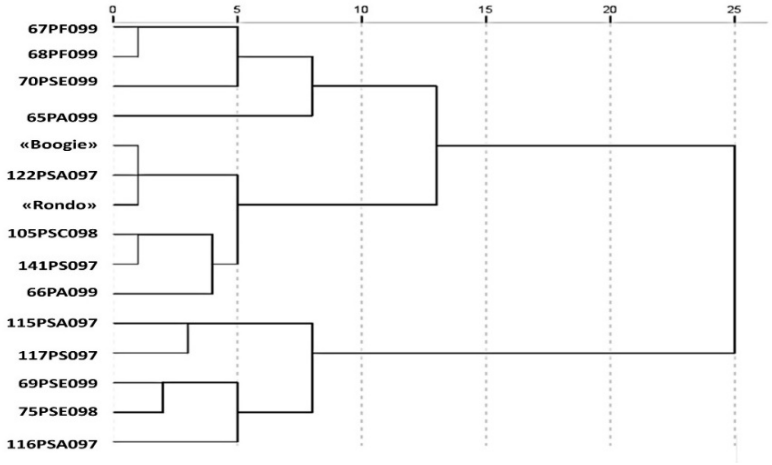

Fig. 3. Cluster analysis of individual genotypes based on flower morphology.

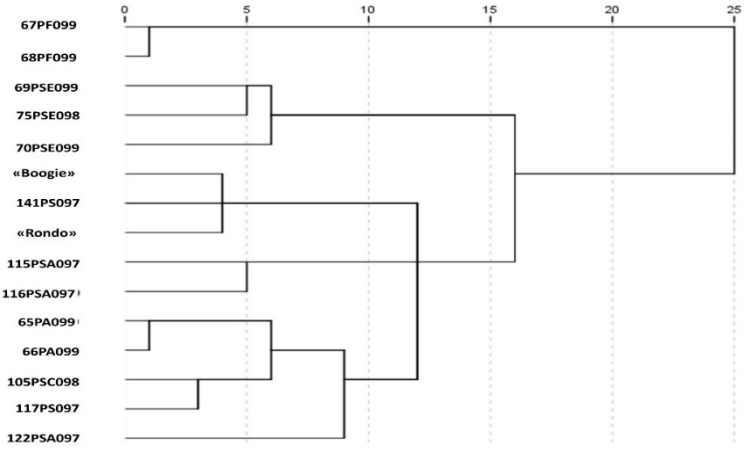

Fig. 4. Cluster analysis of individual genotypes based on seed and pod morphology.

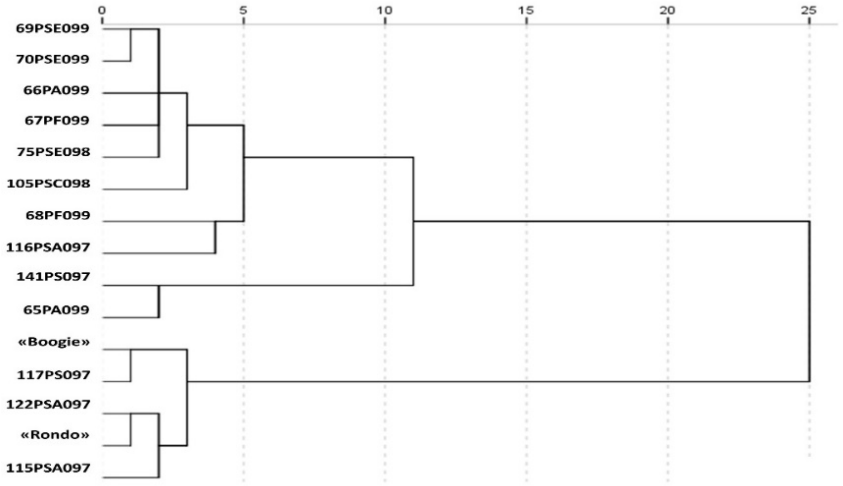

Fig. 5. Cluster analysis of individual genotypes based on whole morphology.

information in genetic variability of different *Pisum* accessions. In the present study, the results indicated that, when considering only flower characters and all morphological characters, *Pisum* accessions show clustering pattern based on the taxonomic groups. However, in clustering analysis according to the only leaf-stem characters or only seed-pod characters, accessions did not show any clustering pattern based on the taxonomic groups.

To examine the genetic relationships among different *Pisum* accessions, a dendrogram was performed using molecular and morphologic data together (Fig. 6). *Pisum* accessions were clustered into two main groups. The first group included two commercial varieties: 'Rondo' and 'Bogie'. This group includes all *P. sativum* var. a*rven*se (115PSA097, 116PSA097, 105PSC098, and 122PSA097) and all *P. sativum* var. *sativum* (117PS097 and 141PS097) accessions. The combination of commercial varieties and these accessions was an expected result. However,

according to only molecular markers results, commercial varieties were clustered together with wild forms (Fig. 1). The second group included accessions of *P. fulvum* L., *P. abyssinicum* L., *P. sativum* var. *elatius* L.

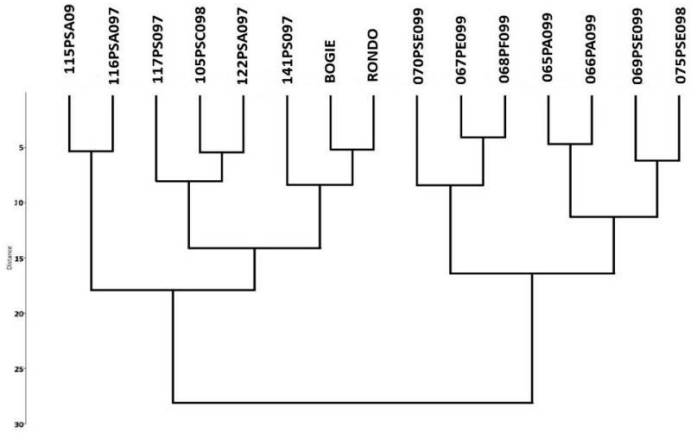

Fig. 6. Cluster analysis of *Pisum* genotypes based on molecular and morphological data.

In all, 15 accessions belonging to five different *Pisum* taxonomic groups originated from different geographic regions were analyzed using 14 SSR markers and 50 morphologic traits. Even though the number of accessions examined was restricted, the genetic variation of the samples investigated was very high as revealed by a large number of alleles, and polymorphism information content values, compared to previous studies. A broad range of diversity was also determined among morphological traits, primarily flower, seed/pod, leaf and stem.

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