Genetic Diversity of Albanian Pea (*Pisum sativum* L.) Landraces Assessed by Morphological Traits and Molecular Markers

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Abstract

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In order to investigate the genetic diversity present in the pea germplasm stored in the Albanian genebank, we analyzed 28 local pea genotypes of Albanian origins for 23 quantitative morphological traits, as well as 14 retrotransposon-based insertion polymorphism (RBIP) molecular markers. The study of morphological characters carried out during three growing seasons (2010, 2011 and 2012) had the objective of characterization of traits useful in breeding programs. RBIP marker analysis revealed the genetic similarity in range from 0.06 to 0.45. ANOVA, principal component analysis (PCA) and cluster analysis was used to visualize the association among different traits. Most of the quantitative morphological traits showed significant differences. PCA and cluster analysis (Ward's method) carried out for morphological traits divided the local pea genotypes into three clusters. Finally, the study identified the agronomicaly important traits which will facilitate the maintenance and agronomic evaluation of the collections.

Keywords: clusters analysis; genetic similarity; landraces; morphological traits; pea, retrotransposon

Pea is an important food grain legume of the temperate and elevated sub-tropical cropping zones, grown as dry grain, green immature fresh seed or pod for vegetable use and for canning, as well as fodder crop (Muehlbauer *et al*. 1988). The total world grain production fluctuates 10–12 million tons, with Canada as the leading producer, followed by USA, India, Russia, France and China (Smýkal *et al*. 2012). Up to half of the area sown by pea is used for production of vegetables, green snap pea pods, green seed for fresh vegetables (fresh, frozen or canned), green leaves and for direct livestock grazing.

The genus *Pisum* contains the wild species *P. fulvum* Sibth. & Sm. found in Jordan, Syria, Lebanon and Israel; the cultivated species *P. abyssinicum* A. Braun from Yemen and Ethiopia, possibly independently domesticated of *P. sativum*; and a large aggregate of both wild (*P. sativum* L. subsp. *elatius* (Steven ex M. Bieb.) Asch. & Graebn.) and cultivated forms of *P. sativum* subsp. *sativum* L. (Smýkal *et al*. 2011, 2013; Ellis 2011). Four centers of origin based on genetic diversity were proposed by Vavilov (1926), namely Central Asia, the near East, Abyssinia (Ethiopia) and the Mediterranean. Pea (*Pisum sativum* L.) is one of the world's oldest domesticated crops. Archaeological evidence dates the existence of pea back to 10 000 B.C. in Near East and Central Asia (Zohary & Hopf 1973). Pea among other grain legumes accompanied cereals and formed important dietary components of early civilizations in Middle East and Mediterranean. In

Europe, it has been cultivated since the Stone and Bronze Ages and in India from 200 B.C. (Smýkal *et al*. 2014).

Although there are around 98 thousand pea accessions preserved worldwide, the total germplasm collection is much smaller owing to substantial overlap. There is large bias (17%) towards Western and Central European accessions, as these regions represent modern pea breeding activities. Moreover, a high level of duplication exists between the collections, giving a misleading impression of the true level of diversity (Smýkal *et al*. 2013). Substantially less well are represented Mediterranean (2.5%), Balkan (2%) regions and Caucasus (0.8%), and Central Asia (2%) centres of pea crop domestication and diversity where higher variation can be anticipated (Smýkal *et al*. 2013).

Important gaps remain in the collections, particularly those of wild and locally adapted materials, that need to be collected before these genetic resources are lost forever (Maxted *et al*. 2010). The demand for productivity and homogeneity, as in other crops, has resulted in a limited number of standard, high-yielding varieties, at the price of the loss of heterogeneous traditional local varieties (landraces), known as genetic erosion. Landraces preserve much of this lost diversity and comprise the genetic resources for breeding new crop varieties to help cope with environmental and demographic changes (Esquinas-Alcazar 2005). There are significant gaps of the wild and landrace peas collected and held *ex situ* or reserved *in situ*. Zong *et al*. (2009) reported a recent example of significant gaps in Chinese pea landrace collection.

Several studies of pea germplasm using morphological descriptors and agronomical traits and lately DNA markers have been published (Baranger *et al*. 2004; Jing *et al*. 2005; Loridon *et al*. 2005; Smýkal *et al*. 2008, 2011; Zong *et al*. 2009; Kwon *et al*. 2012). Traditionally, germplasm diversity is assessed by morphological descriptors, which remain the only legitimate marker type accepted by the International Union for the Protection of New Varieties of Plants (UPOV 2009). Morphological characterization is the first step in the description and classification of the germplasm (Smith & Smith 1989). An understanding of morphological characters facilitate the identification, selection of desirable traits, designing new populations, in transferring their desirable genes into widely grown food legumes through biotechnological means, resistance to biotic and a biotic stresses that are known to individual accessions increase the importance of the germplasm (Santalla *et al*. 2001). Since many morphological characters (especially quantitative or polygenic characters) are influenced by environmental factors (Simioniuc *et al*. 2002; Smýkal *et al*. 2008), the analysis of genetic diversity among pea local populations in this study is realized on combination of morphological characters and molecular markers.

The legume collection in Albania genebank (AGB) contains more than 200 accessions (landraces) with known or unknown origin. The aim of the study was to characterize and to assess the level of genetic diversity among and within pea (*P. sativum* L.) landraces of Albanian or Balkan origins, using morphological traits and molecular markers to aid in the selection and more efficient use of this germplasm in breeding programs.

MATERIAL AND METHODS

Plant materials. Twenty-eight pea accessions from different origin were used to assess genetic diversity among pea landraces stored in Albanian genebank. Six pea accessions (BGJR2, BGJR5, BGJR7, BGJR10, BGJR11, BGJR12) were from Albania; seven pea accessions (BGJ137, BGJ138, BGJ139, BGJ140, BGJ141, BGJ142, BGJ143) were repatriated from Germany, five pea accessions (BGJ2507, BGJ2508, BGJ2509, BGJ2510, BGJ2511) were from Sweden, two accessions (BGJ1589 and BGJ1590) from Russia and eight accessions (BGJ1582, BGJ1583, BGJ1584, BGJ1585, BGJ1586, BGJ1587, BGJ1588, BGJ1591) were signed with unknown origin. This study was carried out at the experimental field of Agriculture University of Tirana (latitude: 40°24'05''N; longitude: 01°94'108''E; elevation 40 m) during three growing seasons (2010, 2011 and 2012). The experimental scheme was randomized block design with four replications. All observations and measurement were realized on 20 plants per plot (80 plants per accessions) situated under the same field and soil conditions.

Morphological traits. The twenty-three morphological quantitative traits were assessed to characterize and estimate genetic diversity among Albanian pea landraces, using the International Union for the Protection of New Varieties of Plants (UPOV 2009) methodology. The quantitative traits measured were stem length (STL), number of nodes including first fertile node (NNod), maximum number of leaflets (MxNLL), leaflet size (LLS), leaflet length (LLL) and width (LLW), leaflet position of broadest part (LLP), stipule length (StL) and width (StW), stipule size (StS), stipule length from axil to tip (StLax-t), stipule length of lobe below axil (StLlob-ax), petiole length from axil to first tendril (PtLax-firstT), petiole length from axil to last tendril (PtLax-lastT), peduncle length of spur (PedLsp), peduncle length from stem to first pod (PedL-1P), peduncle length between first and second pods (PedL1P-2P), pod length (PL) and

width (PW), number of seed per pod (NSP), weight of seeds per plant (WSpPL), weight of 1000 seeds (W100-S) and yield per genotype (YpG).

DNA isolation. All plants chosen for DNA extraction were first described morphologically. Fresh young leaves collected from ten randomly chosen plants per accession were bulked together (Smýkal *et al*. 2008) and stored at –80°C until DNA isolation. Genomic DNA was manually isolated using the Invisorb Plant Genomic DNA Isolation Kit (INVITEK, Berlin, Germany). DNA obtained from approximately 100 mg fresh weight leaf material per accession resuspended in 300 µl of the kit's elution buffer at concentration of 50–100 ng/µl and were stored at –20°C until use. The DNA quality was checked electrophoretically and spectrophotometrically.

DNA marker analysis. Retrotransposon-based insertion polymorphism (RBIP) analysis was performed according to Flavell *et al*. (2003), with the exception that DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, USA) was used. The following 14 RBIP primers pairs selected from Jing *et al*. (2005) were applied: Birte-B1, Birte-x5, Birte-x16, RBIP3, RBIP4, RBIP7, 1006-x19, 45x31, 399-14-9, 399-80-46, 281x40, 281x44, 95x2, 2055nr1. PCR products were resolved by electrophoresis as described in Smýkal *et al*. (2008) and Jing *et al*. (2005).

Genetic similarity, cluster and data analysis. RBIP scores were converted into binary data by presence (1) or absence (0) of the selected fragment. In the case of RBIP analysis, a fourth state, namely complete absence of any PCR product corresponding to primer site mutation (Jing *et al*. 2005) was added. Genetic similarity coefficients were calculated using the Jaccard index of similarity (Nei 1973, 1978; Reif *et al*. 2005) using SPSS 12 software (SPSS 2003).

Morphological descriptors were analysed using principal component analysis (PCA). The number of principal components to retain in the analysis was determined using the minimum eigenvalue criterion proposed by Kaiser (1960). Genetic similarity/distances carried out on the matrix of Euclidean distances were assessed using cluster analysis (Ward) method. The statistical treatment of morphological traits were performed using SAS JMP Statistical Discovery (SAS 2012).

RESULTS AND DISCUSSION

Analysis of morphological quantitative characters. ANOVA analysis showed the presence of significant differences between pea accessions for most number of the morphological traits analyzed with probability $F < P_{0.05}$ (Table 2). High degree of variation was observed for all the morphological characters. There were significant differences between pea genotypes related to STL, NNod, leaflets characters (LLL and LLW), PtLax-firstT, PtLax-lastT, PedLST-1P and PedL1P-2P, pod characters (PL, PW and NSP), W100-S, StW and YpG. All these quantitative traits were significant at the probability $0.0001 < P_{0.05}$. There were also significant differences between pea genotypes related to LLS, LLP, and StW traits (significant at the respectively probabilities 0.0016, 0.0264, 0.0180, $< P_{0.05}$).

Principal components analysis on correlations of quantitative traits identified the variances of the principal components (PC) and the proportion of the total variance accounted for by each factor. Comparing the eigenvalues for each factor (Table 1) using the minimum eigenvalue criterion (Kaiser 1960), only three principal components were retained for further analysis. All 23 quantitative variables contribute in the total source of variation 100% of variance. The percentage of variation accounted for by three PC was 86.91%. The percentages of total variation accounted for by each of the three principal components were 72.76, 7.71 and 6.44% respectively (Table 1). The proportion of total variation more than 75% is acceptable (Cadima & Jolliffe 2001; Jolliffe 2002) for characterization and evaluation of accessions in this genebank collection.

Relationships among the morphological characters and pea genotypes. In the present study where the first two PCs explain 80.4% > 75% of the original variation, the maximum information from morphological quantitative data was received using ordination methods (PCA and principal coordinates analysis) in

Table 1. Matrix of eigenvalues of three principal components (PC) for 28 peas and 23 morphological quantitative traits

Principal components/factor analysis						
PC No.	eigenvalue	percent variance	cumulative percent	χ^2	*DF*	prob > χ^2
1	16.7353	72.762	72.762	1186.99	239.613	< 0.0001*
2	1.7738	7.712	80.475	679.025	248.548	< 0.0001*
3	1.4806	6.437	86.912	578.971	228.959	< 0.0001*
4	0.7567	3.290	90.202	468.660	209.120	< 0.0001*

DF – degree of freedom; prob – probability; *significance level equal to the 0.05 of probability

combination with cluster analyses (Messmer *et al*. 1993; Jolliffe 2002).

Two-dimensional scaling for relationships among pea genotypes and quantitative morphological traits that accounts for the larger proportion of the total variance in PC1, PC2 and PC3 revealed by PCA indicate that the contribution of each pea genotype and of each quantitative morphological trait on the total of variation is not equal. There were 17 pea genotypes included in PC1 that account for 72.76% of total variation, and seven pea genotypes in PC2 which contribute with 7.71% on the total variation. Four pea genotypes included in PC3 account for 6.44% on the total variation (Table 2, Figure 1).

Thirteen quantitative traits show higher contribution on the PC1 variance. In total contribution of quantitative traits includes in PC1 account for 58.1% of PC1 variance. For PC1 the morphological quantitative traits as StS, PtLax-firstT, LLS, and LLW traits (with eigenvectors > 0.23) followed by LLP, StLax-t, LLL, PtLax-lastT, and Nnod (with eigenvectors > 0.22) were the quantitative traits (variables) with larger values and more significant weighting on the PC1 variance. These traits can be used successfully as morphological quantitative marker traits for characterization and classification of the pea germplasm (Smith & Smith 1989) stored in genebank, and in plant breeding programs (Javaid *et al.* 2002; Zahir *et al*. 2007).

Good understanding of the most important morphological characters can facilitate identification of any individual accession and selection of desirable traits (genes), increasing the information and the representativeness of the *Pisum* germplasm (Santalla *et al*. 2001) in genebank. The other quantitative traits as StL, StW, NSP, WSpPL, W100-S, and PW with eigenvectors < 0.21 show less contribution on the PC1 variance.

Variation in PC2 (7.71% of total variance) was mainly the result of differences between quantitative traits as PW, PL, PedL1P-2P, Nnod, PedLsp, and and StLlob-ax with eigenvectors > 0.2150. In total their contribution account for 33.3% of PC2 variance (Figure 2). In addition, Nnod and StLlob-axil traits are important to the PC1, but at the same time, these characters also account for PC2 the part of variance that was not account for in PC1. In PC3, there are W100-S, PW and LLL characters that account for 6.44% of the total

Table 2. Matrix of eigenvectors of three principal components (PC) for 28 peas and 23 morphological quantitative traits

Morphological quantitative traits	Prob > *F*	PC1	PC2	PC3
Stem length (STL)	0.0001*	**0.21979**	0.11505	0.09309
Number of nodes (Nnod)	0.0003*	**0.22289**	**0.23986**	0.01140
Maximum number of leaflets (MxNLL)	0.5663	0.21235	0.02827	–0.13626
Leaflet size (LLS)	0.0016*	**0.23092**	–0.03047	0.07202
Leaflet length (LLL)	< 0.0001*	**0.22374**	0.18085	0.22990
Leaflet width (LLW)	< 0.0001*	**0.23046**	–0.20560	0.03683
Leaflet position of broadest part (LLP)	0.0264*	**0.22569**	–0.07327	–0.06414
Stipule length (StL)	0.6784	0.20343	–0.04966	–0.06755
Stipule width (StW)	0.0180*	0.20416	–0.10765	0.04051
Stipule size (StS)	< 0.0001*	**0.23374**	–0.11126	–0.03160
Stipule length from axil to tip (StLax-t)	0.0002*	**0.22494**	–0.15252	–0.13685
Stipule length of lobe below axil (StLlob-ax)	< 0.0001*	**0.21595**	**0.21791**	–0.12802
Petiole length from axil – first tendril (PtLax-firstT)	< 0.0001*	**0.23233**	0.08809	–0.13816
Petiole length from axil – last tendril (PtLax-lastT)	0.0002*	**0.22316**	–0.19615	–0.06733
Peduncle length of spur (PedLsp)	< 0.0001*	0.21360	**0.23914**	0.14522
Peduncle length from stem-first pod (PedLST-1P)	0.0004*	0.21367	–0.01593	**–0.29086**
Peduncle length first- second pods (PedL1P-2P)	< 0.0001*	0.19986	**0.28905**	0.20142
Pod length (PL)	< 0.0001*	0.20328	**0.29102**	0.12940
Pod width (PW)	< 0.0001*	–0.12555	**0.31067**	**0.52058**
Number of seed per pod (NSP)	< 0.0001*	**0.21900**	–0.06296	0.03992
Weight of seeds per plant (WSpPL)	< 0.0001*	**0.21861**	–0.08726	0.21016
Weight of 1000 seeds (W100-S)	< 0.0001*	0.01641	**–0.42076**	**0.57770**
Yield per genotype (YpG)	0.0001*	0.16267	**–0.43924**	0.17226

F – *F*-ratio; *significance level equal to the 0.05 of probability; all eigenvectors > 0.2150 are in bold

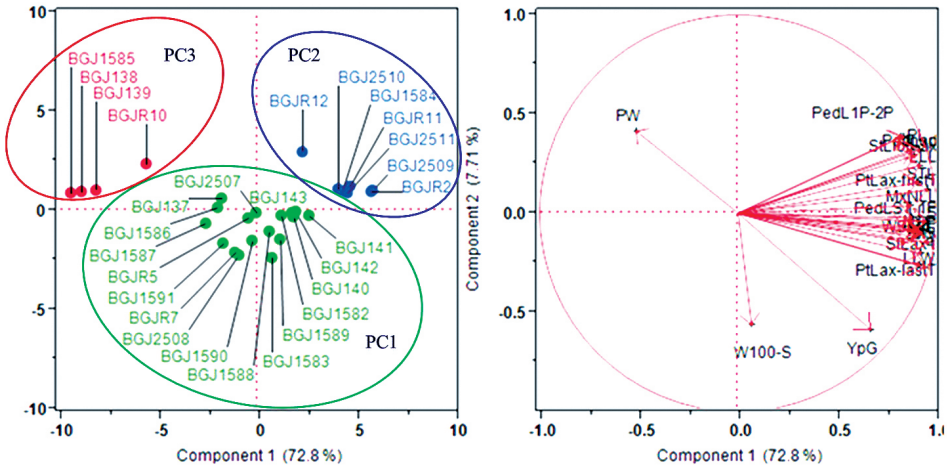

Figure 1. Two-dimensional relationships among the 28 pea genotypes based on morphological quantitative traits revealed by principal component analyses

of variation. PW trait accounts for PC3 the variance that was not account for in PC2 (Table 2, Figure 2).

Genetic similarity/distances assessed by morphological data. Genetic similarity/dissimilarity evaluated by combination of quantitative morphological traits using cluster analysis (Ward) method (Figure 3) show the presence of similarity and distances between Albanian local pea landraces. Comparisons of data and cluster analysis generate a dendrogram where 28 pea genotypes were grouped into three main clusters (Figure 3). In the dendrogram, 17 pea genotypes of cluster I were grouped into three main subclusters consisting of six, four and seven genotypes, respectively. Cluster II consists of seven pea genotypes and cluster III consists of four genotypes (Figure 3).

Study results show presence of similarity between three pea accessions with unknown origin (BGj1582, BGJ1585, BGj1587) and pea accessions repatriated from Germany (BGj137, BGJ138, BGJ140, BGJ141, BGJ142 and BGj143). There were also four other pea genotypes with unknown origin (BGJ1583, BGJ1584, BGJ1588, BGj1591) that show similarity with pea accessions repatriated from repatriated from Sweden (BGj2507, BGJ2508, BGj2509, BGJ2510, BGJ2511). The latest accession (BGj1586) with unknown origin shows similarity with BGjR7 from Albania. The results show that there was a large similarity between pea landraces with unknown origin and pea landraces repatriated from Sweden. Maximal distance (17.544669) was found among BGJ138 accession (from Germany) leader and BGJ1586 (unknown origin) joiner, and minimal distance (0.8154849) among BGJ1584 (unknown origin) leader and BGJ2511 (from Sweden) joiner. The higher estimated genetic distance could be ascribed to differences between pea accessions of different origin. The coefficient of genetic similarity obtained in the present study quantitative morphological data ranged from 0.45 to 0.94, indicating that a high level of genetic diversity existed among the 28 pea genotypes.

In the present study, the cluster results were similar to those of PC analysis. The genotypes of PC1 formed cluster I of the dendrogram, and genotypes of PC2 formed cluster II of the dendrogram (Figure 1, 3). Similarities between some of the genotypes could be explained by common parent origin in their pedigree.

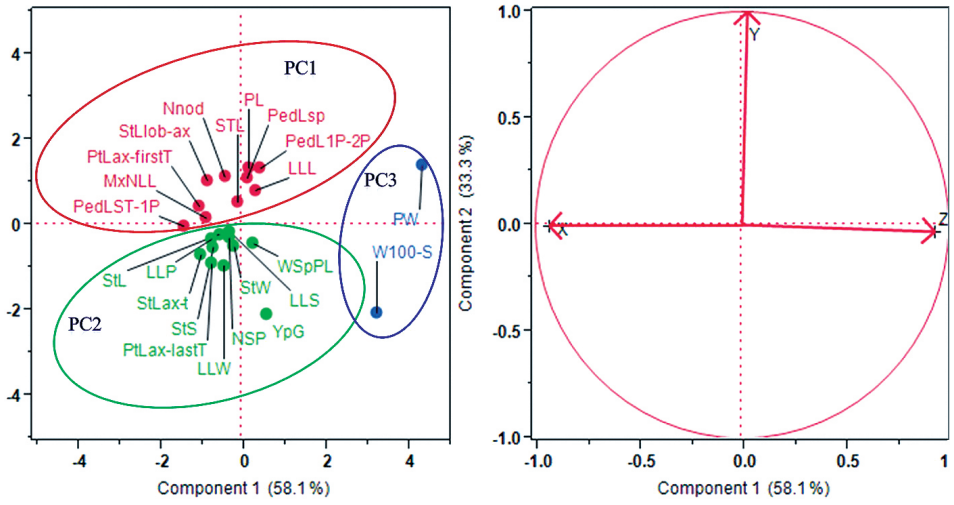

Figure 2. Two-dimensional relationships between the most important pea morphological quantitative traits revealed by principal component analyses

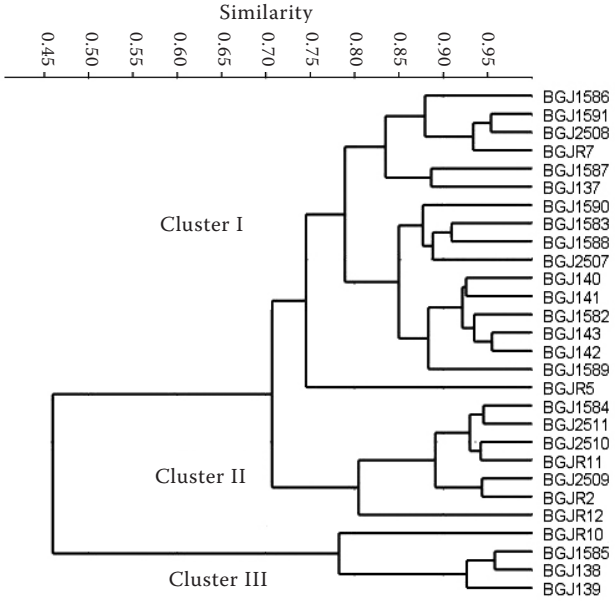

Figure 3. Dendrogram of relationships among pea accessions based on morphological data

Genetic similarity/distances assessed by RBIP marker data. Genetic similarity assessed by RBIP markers data, using the Jaccard index of similarity (Nei 1973, 1978; Reif *et al*. 2005) show the presence of similarity and differences between Albanian local pea landraces. Comparisons of data and cluster analysis range pea landraces into different clusters. In comparison with quantitative morphological traits analysis the cluster analysis based on molecular data generates a dendrogram with higher number of clusters (Figure 4).

Study results show presence of similarity between three pea accessions with unknown origin (BGj1585, BGJ1587, BGj1588) and pea two pea accessions repatriated from Germany (BGj138, BGJ139). There were also two pea genotypes with unknown origin (BGJ1583, BGJ1584) that show similarity with pea accessions repatriated from repatriated from Sweden (BGj2507, BGJ2508, BGJ2511). One pea accession (BGj1586) with unknown origin shows similarity with two pea accessions (BGJ1589, BGJ1590) repatriated from Russia. All pea accessions repatriated from Germany show high similarity among them.

Genetic similarity / distances estimated by RBIP markers data in comparison with similarity or distances estimated by conventional methods (Smith & Smith 1989; Burstin *et al*. 2001; Santalla *et al*. 2001; UPOV 2009) showed higher similarity with genetic distance estimated by morphological data. Molecular markers have coefficient of correlation 0.71 larger than coefficient of correlation of morphological traits 0.67 demonstrated the importance of molecular markers usage in this type of study. The relationship between morphological traits and molecular markers results is 68%. Differences between molecular and morphological data shown are probably due to difficulties in obtaining and inaccurate field morphological data. Results of this study were congruent with results of Baranger *et al*. (2004); Simioniuc *et al*. (2002); Hoey *et al*. (1996); Tar'an *et al*. (2005), who suggested low to medium correlations among molecular and morphological data.

The genetic similarity coefficient among 28 Albanian local pea accessions evaluated by RBIP markers varied from 0.06 to 0.45 (Figure 4) indicating high level of genetic diversity existing among the 28 pea genotypes. Results of this study were congruent with results of other studies. Tar'an *et al*. (2005) reports genetic distances

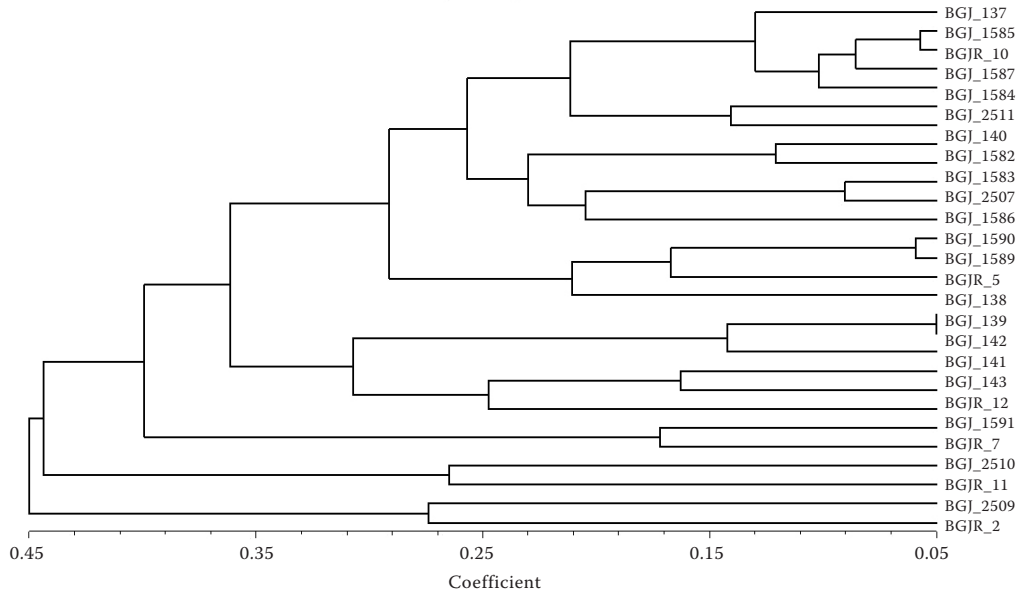

Figure 4. Dendrogram of relationships among pea accessions based on molecular data

based on SSR markers range from 0.0 to 0.66, Cupic *et al.* (2009) reports genetic diversity that range from 0.24 to 0.84 and Ford *et al.* (2002) reports a genetic distance range from 0.05 to 0.48. Simioniuc *et al.* (2002) reported a relatively higher similarity range (0.80–0.94) with RAPD markers compared with that obtained using AFLP markers in pea cultivars (0.85–0.94). Baranger *et al.* (2004) obtained a very wide range of similarity (0.0-1.0) in 148 *Pisum* genotypes using protein and PCR-based markers. The differences could be attributing to differences between pea accessions of different origin and software used.

In this study, pea accessions repatriated from Sweden had the largest level of genetic diversity, followed by Russian pea accessions and pea accessions signed with unknown origin in genebank. Pea accession with unknown origin show higher level of genetic diversity than pea accessions repatriated from Germany and peas originated from Albania, and these pea genotypes have interest as possible reserve of desirable traits (genes) for breeding schemes. In this study, the pea accessions repatriated from Germany were more uniform showing low level of genetic diversity. Uniformity of pea accession repatriated from Germany could be ascribed to possible their inclusion in modern breeding programs that usually result in low level of genetic diversity (Pasquet 2000; Baranger *et al.* 2004).

The Albanian pea gene pool was found to be narrow in genetic diversity and this suggests its enrichment through introgression of new traits (genes). The selection of genotypes from peas with higher genetic diversity level (pea genotypes from Sweden, Russia, and landraces with unknown origin) should be considered in pea breeding programs. The results of this study are beneficial to pea germplasm database and to breeding programs in pea. Moreover, Balkan origin pea accessions might often have specific alleles of resistance genes, as shown for *eIF4E* gene conferring resistance to potyviruses (Konečná *et al.* 2014).

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