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Full Length Research Paper

Agronomic and molecular evaluation of recombinant inbred lines (RILs) of lentil

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Sequence-related amplified polymorphism (SRAP) and morphological markers were studied to compare the efficiency of both marker systems in the evaluation of twenty five lentil (*Lens culinaris* Medik.) recombinant inbred lines (RILs) and four testers. Data on 13 morphological traits were collected and analyzed. A total of 240 polymorphic SRAP's bands (76.7%) were scored using four combinations of primers. Cluster analysis and both principal component and principal coordinate analysis were carried out. The entries were grouped in five clusters through procrustes generalized analysis. Relationships among lines revealed by molecular markers were significantly correlated with those based on the agronomic traits (r = 0.75), suggesting that the two systems give similar estimates of genetic relations among the RILs. In future breeding programs parent selections could be based on these traits information in order to broaden the genetic.

Key words: Genetic distance, molecular markers, morphological markers, recombinant inbred lines, SRAP marker.

INTRODUCTION

The genus *Lens* is a member of the legume tribe *Vicieae* which includes the major legume crops of the classical Mediterranean civilizations, faba bean, pea and lentil. *Lens* is a small Mediterranean genus that comprises the cultivated lentil (*Lens culinaris* Medikus subsp. *culinaris*) and 6 related taxa (Ferguson et al., 2000). Lentil (*L. culinaris* Medik.) is a diploid (2n = 2x = 14), autogamous species which is one of the oldest crops in the world, originated in the Near East (Zohary, 1972). The cultivated species, *L. culinaris* has been divided into two sub-species (Barulina, 1930) namely macrosperma (seed diameter, 6 to 9 mm) and microsperma (seed diameter, 2 to 6 mm) . The macrosperma type has yellow cotyledons and very light or no pigmentation in their flowers and other plant parts, whereas the microsperma type has red, orange or yellow cotyledons with pigmented flowers and other plant parts. The small size of the seed has some advantages, for example, small seeded varieties in comparison with large seeded varieties were found to be better adapted to dry environments (Erskine, 1996). Genetic variation also exists in lentil for low temperature tolerance under drought condition. Photothermally sensitive genotypes are more tolerant to low temperatures (Keatinge et al., 1996). Large seeded varieties in comparison with small seeded varieties have greater cold tolerance (Erskine, 1996). For culinary use the small seeded varieties are preferred due to less cooking time they demand. However, ssp macrosperma has significantly higher seed saponin content than ssp microsperma (Ruiz et al., 1997). Saponins are a class of bioactive compounds with diverse good properties such as the inhibition of growth and sporulation of a wide range of fungi (Gestetner et al., 1971), the reduction of plasma cholesterol levels in humans (Sidhu and Oakenfull, 1986) and the exhibition of anticancer activity (Konoshima et al., 1992).

One of the major problems that face Argentinian lentil breeders is the narrow genetic base of the current cultivated germplasm (derived from four varieties). This must be broadened from other sources and isolate superior recombinant inbred lines (RILs). While the parental phenotypes can be defined in terms of seed diameter, cotyledon colour and the presence or absence of pigmentation in the flowers, the RILs obtained present mixed phenotypes due to recombination process and these traits lose their properties as agronomic markers.

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On the other hand, the seed diameter might be affected by the environmental conditions in which the crop is grown, and the cultural practices used for production as well (i.e. soil conditions, nutrient deficiency, water stress, extreme temperature, pest infestation, handling operations) (Bishaw et al., 2007). In this sense, varieties with intermediate values for seed diameter are difficult to classify or assign to either ssp. To avoid this problem, when dealing with genetically diverse lentil lines, others morpho-agronomic traits and molecular markers should be considered to provide a relatively unbiased method for their classification and the evaluation of the genetic diversity. Several molecular markers systems were used to determine allelic diversity in lentil collections (Datta et al., 2007). In this work, we propose the use of SRAP (Sequence-Related Amplified Polymorphism) (Li and Quirós, 2001), because they are simpler than AFLP and more reliable than RAPD markers. The objective of the present study was to evaluate morphological traits associated with macrosperma-microsperma seeded type and in combination with SRAP markers, access the efficiency of their use in the classification of recombinant inbreed lines derived from a current lentil breeding programmed.

MATERIALS AND METHODS

The present investigation was conducted at the research station of the Faculty of Agriculture of Rosario University, Argentina (33° 1 S and 60° 53 W), in 2008. The experimental material consisted of 25 F_5 recombinant inbred lines (RILs) (obtained from crosses between lines developed by ICARDA) and four tester (two microsperma type, T_1 and T_2 and two macrosperma type, T_3 and T_4). For each material, 30 seeds were sowed in a randomized block design using 5 dm^3 pots (Kgsoil/pot) filled with a mixture of sterile soil, peat and perlite (1:1:1) as substrate. Plants were grown in greenhouse with natural light. Observations were recorded on number of branch (NB), length (LF) and width (W) of folioles, length (LP) and width (WP) of pods, number of pods per plant (NP), number of folioles (NF), plant height (PH), height of the first pod (HFP), number of nodes at the first pod (NNFP), days to flowering (DF), days to maturity (DM), number of total nodes (NTN) and seed diameter (SD). With the collected data Euclidean distances between RILs were calculated and a cluster analysis was carried out. A dendrogram was generated using ‘‘Ward’s minimum variance’’ through the InfoGen software (Balzarini and Di Renzo, 2003).

For DNA extraction and SRAP procedure about 100 mg of fresh leaf was ground in liquid nitrogen and the total genomic DNA was extracted using the Murray and Thompson (1980) protocol based on CTAB. The amplifications were carried out in a thermo-cycler MyCyclerTM (BIO-RAD). At the beginning of the PCR reaction, the annealing temperature was set at 35˚C and run for five cycles. Annealing temperature was raised to 50˚C for another 35 cycles. Denaturing was done at 94˚C for 1 min, while extension was carried out at 72˚C for 1 min in all cycles. Four primers forward and two primer reverse were used originating eight primers combinations.

Primers “Forward”

me1, 5’-TGAGTCCAAACCGGATA-3’
me2, 5’-TGAGTCCAAACCGGAGC-3’
me4, 5’-TGAGTCCAAACCGGACC-3’
me5, 5’-TGAGTCCAAACCGGAAG-3’

Primer “Reverse”

em1, 5’-GACTGCGTACGAATTAAT-3’
em2, 5´-GACTGCGTACGAATTTGC-3´

The amplified fragments were separated in denaturing acrylamide sequencing gels and revealed with silver (Li and Quirós, 2001). SRAP fragments were scored for presence or absence as 0 and 1, respectively. Genetic distances were calculated with SRAP data according to Dice’s similarity index and a dendrogram was performed. A comparison between morphological and molecular data was carried out through the procrustes generalized analysis using the InfoGen program (Balzarini and Di Renzo, 2003).

RESULTS AND DISCUSSION

Morphological analysis

The variable seed diameter (SD) showed mean values that ranged between 0.4 and 0.62 cm, so, lines could not be classified clearly as belonging to microsperma or macrosperma type. The tester lines showed an average value of 0.4 cm (T1 and T2) and 0.7 cm (T3 and T4). In the cluster analysis this variable was excluded to avoid interference. Relationships among the 25 (RILs) and the four testers revealed by cluster analyses are presented in Figure 1. Three main clusters or groups were formed. Except for LF, HFP and NNFP, significant differences were obtained between clusters for the rest of the evaluated traits. The traits DF, DM and NP showed the highest discriminating values ($F = 47.7$; $p < 0.001$; $F = 45.6$; $p < 0.001$ and $F = 36.4$ $p < 0.001$, respectively).

The RILs included in clusters 1 presented the lowest values for number of branch, number of pods per plant, days to flowering, plant height and number of total nodes; and the highest values for the traits related to the size of leaflets and pods. The testers T3 and T4 were also included in this cluster. This fact implies that the RILs of cluster 1 could be considered of the macrosperma type. The RILs included in groups 2 and 3 differed only by the number of pods and the days to flowering. Group 3 had the highest values for these variables (Table 1); T1 and T2 were included in this group, suggesting that the RILs associated with this group would be microsperma type. The lines associated with cluster 2 correspond to recombinant lines with intermediate characteristics of both subspecies.

Barulina (1930) classified lentil landraces from diverse world areas into macrosperma and microsperma subspecies according to seeds, pods and leaflets characters. The RILs included in Clusters 2 and 3 should be classified like microsperma types. However, the RILs included in Cluster 2 have a lower number of pods per plant. Solh and Erskine (1984) found that seed size and other morphological characters form a continuum between macrosperma and microsperma types. Biçer

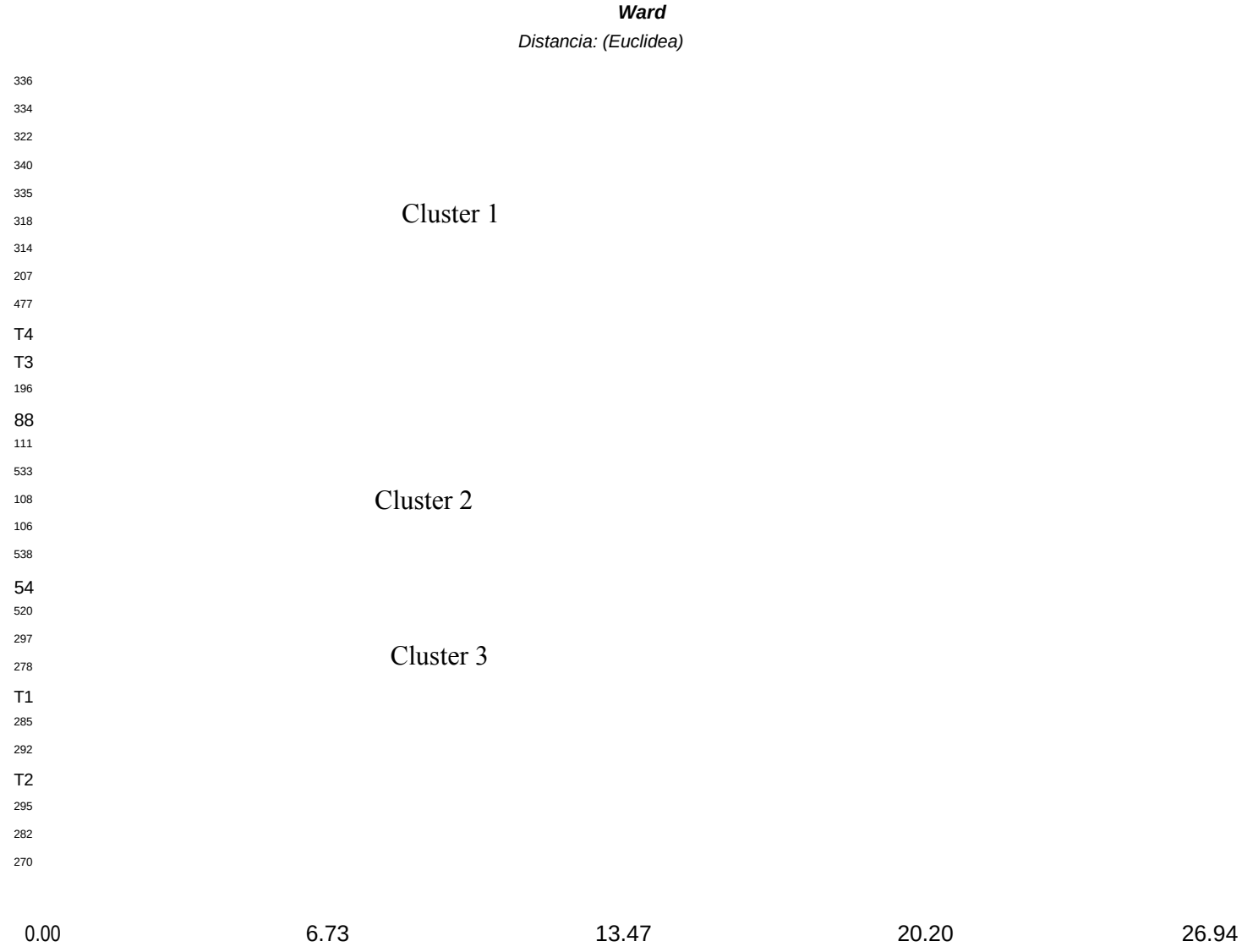

Figure 1. Dendrogram compiled by Ward's method showing the grouping of 25 lentils RILs and four testers based on morphologic traits.

and Sakar (2008) found that the correlation between 1000 seed weight and grain yield was positive, but low and insignificant. Thus, recombinant could have been generated with the morphological characteristics of type microsperma but with less number of seeds per plant.

Molecular data

A total of 8 primers combinations were assayed on the 25 accessions and the four testers. Primer banding patterns that were difficult to score and those that failed to amplify consistently in all genotypes was excluded. Consequently, only four combinations were selected. One RIL was excluded from the trial because the DNA obtained had low quality and it did not allow good amplification patterns. Pejic et al. (1998) reported that with 150 polymorphic bands it is possible for a researcher to reliably estimate genetic similarities among genotypes within a species. From a total of 317 bands, we found 240 polymorphic fragments (76.7%) with an average of 60 polymorphic bands per combination. This percentage of polymorphism is consistent with those obtained with SRAP markers by Ferriol et al. (2004) in squash, Ahmad et al. (2005) in pistachio, Smutkupt et al. (2006) in highland legumes, and Esposito et al. (2007) in a pea collection.

The relationships between the 24 RILs and the testers revealed by cluster analyses based on Dice distance are shown in Figure 2. Two main clusters can be observed with 5 and 23 accessions, respectively. One of the clusters (cluster 1) included only RILs with a high number

Table 1. Mean values (MV) and standard error (SE) for the four clusters performed with all RILs considering morphologic data.

	Cluster 1	Cluster 2	Cluster 3
	MV±S.E	MV±S.E.	MV±S.E.
Number of branch	3.1 ± 0.25[b]	4.0 ± 0.45[a]	4.1 ± 0.28[a]
Width of folioles (cm)	0.3 ± 0.01[a]	0.2 ± 0.02[b]	0.2 ± 0.01[b]
Length of foliole (cm)	1.1 ± 0.06[a]	0.9 ± 0.13[a]	0.9 ± 0.05[a]
Number of folioles	11.4 ± 0.36[a]	9.6 ± 0.40[b]	10.2 ± 0.20[b]
Width of pods (cm)	0.6 ± 0.01[a]	0.5 ± 0.04[b]	0.5 ± 0.02[b]
Length of pods (cm)	1.3 ± 0.03[a]	0.9 ± 0.06[b]	0.9 ± 0.03[b]
Number of pods per plant	20.9 ± 0.94[c]	38.6 ± 7.10[b]	55.1 ± 3.79[a]
Days to flowering	87.4 ± 1.18[b]	83.4 ± 2.80[b]	101.3 ± 0.21[a]
Days to maturity	99.4 ± 1.09[b]	90.2 ± 2.37[c]	110.0 ± 0.93[a]
Plant height (cm)	27.2 ± 1.11[b]	37.0 ± 3.27[a]	34.9 ± 1.12[a]
Number of total nodes	16.6 ± 0.67[b]	22.4 ± 1.75[a]	21.1 ± 0.81[a]
Height of the first pod (cm)	15.8 ± 0.88[a]	13.8 ± 1.24[a]	12.2 ± 1.40[a]
Number of nodes at the first pod	8.7 ± 0.32[a]	8.8 ± 0.97[a]	7.9 ± 0.74[a]

The values followed the same letter are not different at the 5% level.

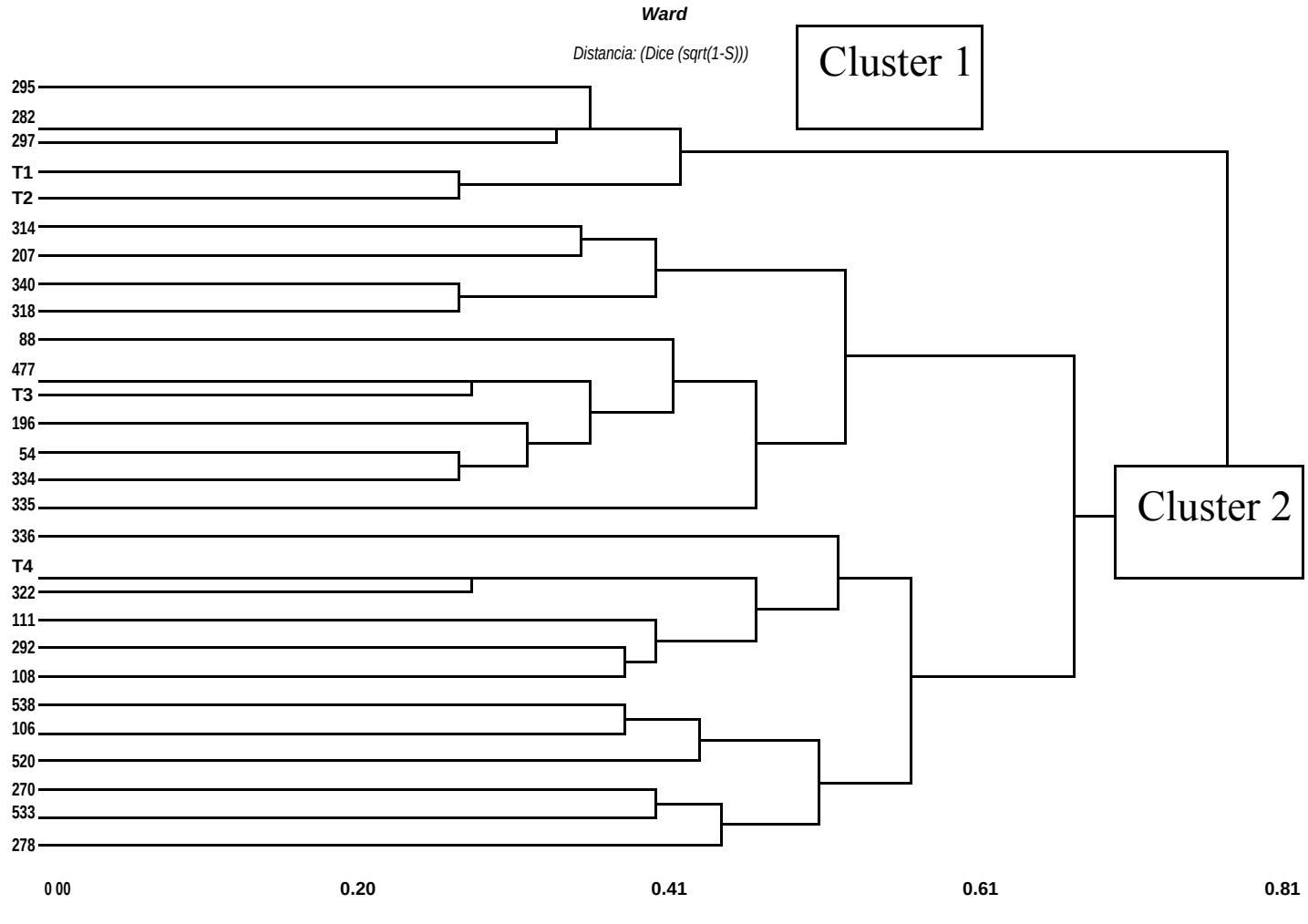

Figure 2. Dendrogram compiled by Ward's method showing the grouping of 24 lentil RILs and four testers based on Dice's distances.

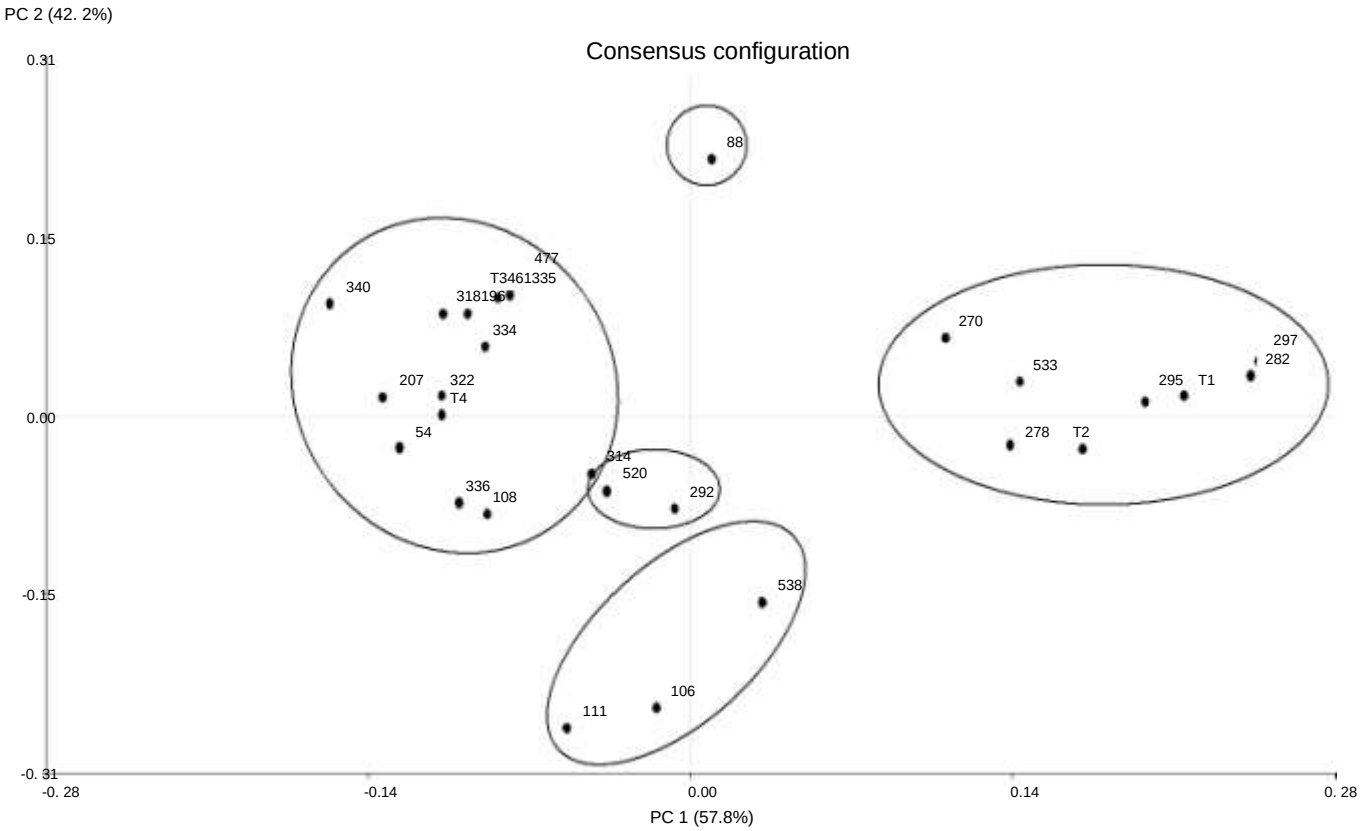

Figure 3. Scatter diagram of the first two principal components (PC_1 and PC_2) from procrustes generalized of 28 accessions based on 240 SRAP fragments and morphological characters.

of pods. The second cluster comprised RILs with a reduced number of pods. The largest DDI (Dice Distance Index) were observed between RILs included in cluster 1 (DDI = 0.77), meanwhile the lowest DDI were found between the pairs of RILs 54- 334 (DDI = 0.27), 54-196 (DDI = 0.29) and 54-461 (DDI = 0.29). Different types of molecular markers were used to determine diversity in lentil collections (Datta et al., 2007). Pioneering works of Abo-elwafa et al. (1995); Ford (1997) with RAPD´s gave important information for germplasm diversity, but also showed that nonspecific PCR based markers could not provide repeatable results in differentiating lentil genotypes. Babayeva et al. (2009) using SSR markers, found a high diversity in Azerbaijan lentil germplasm, as revealed by the low mean pairwise genetic similarities. In our case, the distance index ranged from 0.24 to 0.77 revealing a high genetic variability.

Comparisons between morphological traits and molecular data

Comparison between SRAP and morphological data was carry out using the procrustes generalized analysis. The correlation between Dice similarity index (SRAP data) and Euclidean distance (morphological data) matrices was 0.75, indicating good correspondence between both data set (Tatineni et al., 1996). RILs distribution for this analysis is showed in the Figure 3. Five big clusters of RILs can be observed and the tester's distribution could be associated with the seed size and the microsperma-macrosperma classification (Barulina, 1930). In several studies carried out in lentil with RAPD markers, the microsperma varieties clustered together whereas the macrosperma varieties conformed another cluster (Sharma et al., 1995, Alvarez et al., 1997, Duran and Pérez de la Vegal, 2004) however, Williams et al. (1974) study did not support the separation of the two types of lentil based on seed size. Our results confirm that this classification could be valid.

The first group included 8 RILs, all of them corresponding to the microsperma type. The second group included fourteen RILs of the macrosperma type. The other three clusters were conformed by RILs with intermediate values between macro and microsperma groups. Abo-elwafa et al. (1995) found identical patterns between accessions belonging to different types. This is an indication that, even when there are differences between the two types of lentils based on grain size and cotyledons colours, this classification is not so suitable, since there is a continuum between the two types,

which share the same genetic background.

In breeding programmes for self -fertilizing crops, a large number of crosses are made every year for the introgression of complementary useful genes from one type to other and to isolate transgressive segregants RILs. Therefore, in order to select parents to be involved in a cross for isolating transgressive segregants, greater emphasis needs to be given on the dispersion of genes in the parents. Chahota et al. (2007) founded that, the crosses displaying transgressive segregants having practical utility were observed in the macrosperma × microsperma crosses. Thus, the identification of both types of lentils is one of the key steps in the lentil breeding programmed. The information provided by our study allowed to establish a correlation between morphological and molecular data of r = 0.77. This value indicates an excellent consis-tency between both types of markers and suggests that both types of traits (morphological – molecular) would provide similar estimates on the variability between RILs. This implies that, SRAP markers are an efficient tool in the differentiation of genetic variability. In turn, both morphological and molecular markers are useful to differentiate itself homogeneous groups of lentil.

REFERENCES

Abo-elwafa A, Murai K, Shimada T (1995). Intra-specific and inter-specific variations in Lens, revealed by RAPD markers. Theor. Appl. Genet. 90: 335–340.

Ahmad R, Ferguson L, Southwick SM (2005). Molecular marker analyses of pistachio rootstocks by Simple Sequence Repeats and Sequence-Related Amplified Polymorphisms. J. Hort. Sci. Biotech. 80: 382-386.

Alvarez MT, García P, Pérez de la Vega M (1997). RAPD polymorphism in Spanish lentil landraces and cultivars. J. Genet. Breed. 51: 91-96.

Babayeva S, Akparov Z, Abbasov M, Mammadov A, Zaifizadeh M, Street K (2009). Diversity analysis of Central Asia and Caucasian lentil (*Lens culinaris* Medik.) germplasm using SSR fingerprinting. Genet. Res. Crop Evol. 56: 293–298.

Balzarini M, Di Renzo J (2003). Infogen. Software para análisis estadísticos de marcadores genéticos. Facultad de Ciencias Agropecuarias. Universidad Nacional de Córdoba. Argentina.

Barulina E (1930). The lentils of the USSR and other countries. Bull. Appl. Bot. Plant Breed. 40: 1–319.

Biçer BT, Sakar D (2008). Heritability and path analysis of some economical characteristics in Lentil. J. Cent. Eur. Agric. 9: 191-196.

Bishaw Z, Niane AA, Van Gastel AJG (2007). Quality seed production. In: Lentil: An Ancient Crop for Modern Time. Chapter 21. (Yadav SS, McNeil D, Philip C, Stevenson PC, Eds.). Springer. The Netherland. pp. 349–383.

Chahota RK, Kishore N, Dhiman KC, Sharma TR, Sharma SK (2007). Predicting transgressive segregants in early generation using single seed descent method-derived micromacrosperma genepool of lentil (Lens culinaris Medikus). Euphytica. 156: 305–310.

Datta S, Sourabh B, Kumari J, Kumar SH (2007). Molecular diversity analysis of lentil genotypes. Pulses news. 18: 2-4.

Durán Y, Pérez de la Vega M (2004). Assessment of genetic variation and species relationships in a collection of Lens using RAPD and ISSR. Span. J. Agric. Res. 2: 538-544.

Erskine W (1996). Seed-size effects on lentil (*Lens culinaris*) yield potential and adaptation to temperature and rainfall in West Asia. J. Agric. Sci. 126: 335–341.

Esposito MA, Martin EA, Cravero VP, Cointry E (2007). Characterization of pea accessions by SRAP's markers. Scientia Horticulturae. 113: 329–335.

Ferguson ME, Maxted N, Van Slageren M, Robertson LD (2000). A re-assessment of the taxonomy of Lens Mill. Bot. J. Linn. Soc. 133: 41–59.

Ferriol M, Pico B, Fernandez de Córdova P, Nuez F (2004). Molecular diversity of a germplasm collection of squash (*Cucurbita moschata*) determined by SRAP and AFLP markers. Crop Sci. 4: 653-664.

Ford R (1997). Diversity analysis and species identification in *Lens* using PCR generated markers. Euphytica. 96: 247–255.

Gestetner B, Assa Y, Henis Y, Birk Y, Bondi A (1971). Lucerne saponins IV. Relationship between their chemical constitution, and haemolitic and antifungal activities. J. Sci. Food Agric., 22: 168–172.

Keatinge JD, Qi A, Kusmenoglu I, Ellis R, Summerfield RJ, Erskine W, Beniwal SPS (1996). Using genotypic variation in flowering responses to temperature and photoperiod to select lentil for the west Asian highlands. Agric. For. Meteorol. 78: 53-65.

Konoshima T, Kokumai M, Kozuka M (1992). Antitumor promoting activities of afromosin and soyasaponin I isolated from Wistaria brachybotrys. J. Nat. Prod. 55: 1776–1778.

Li G, Quirós C (2001). Sequence-Related Amplified Polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in Brassica. Theor. Appl. Genet. 103: 455-461.

Murray JM, Thompson W (1980). Rapid isolation of high-molecular weight plant DNA. Nucl. Acids Res. 8: 4321-4325.

Pejic IP, Ajmone-Marsan M, Morgante V, Kozumplick P, Castiglioni G, Taramino M, Motto M (1998). Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. Theor. Appl. Genet. 97: 1248-1255.

Ruiz RG, Price KR, Rose ME, Fenwick GR (1997). Effect of seed size and testa colour on saponin content of Spanish lentil seed. Food Chem. 58: 223–226.

Sharma SK, Dawson IK, Waugh R (1995). Relationships among cultivated and wild lentils revealed by RAPD analysis. Theor. Appl. Genet. 91: 647–654.

Sidhu GS, Oakenfull DG (1986). A mechanism for the hypocholesterolaemic activity of saponins. Br. J. Nutr. 55: 643–649.

Smutkupt S, Peyachoknagul S, Kowitwanich K, Onto S, Thanananta N, Julsrigival S, Kunkaew W, Punsupa V (2006). Varietal determination and genetic relationship analysis of highland legumes using SRAP markers. J. Genet. Breed. 38: 19-27.

Solh M, Erskine W (1984). Genetic resources of lentil. In: Witcombe, J.R. and W. Erskine (Eds), Genetic resources and their exploitation – Chickpeas, faba beans and lentils, pp. 205-224. Martinus Nijhoff/Dr. W. Junk Publishers for ICARDA and IBPGR, The Hague.

Tatineni V, Cantrell RG, Davis DD (1996). Genetic diversity in elite cotton germplasm determined by morphological characteristics and RAPDs. Crop Sci. 36: 186-192.

Williams JT, Sanchez AMC, Jackson MT (1974). Studies on lentils and their variation I. The taxonomy of the species. SABRAO J. 6: 133–145.

Zohary D (1972). The wild progenitor and place of the cultivated lentil. *Lens culinaris*. Econ. Bot. 26: 326–332.