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Study of diversity in a set of lentil RILs using morphological and molecular markers

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Abstract

We propose the use of SRAP and SSR markers along agronomic or morphological traits in genotypes' differentiation during breeding programs. 25 F_5 recombinant inbred lines (*microsperma* and *macrosperma*) with good quality characteristics and high yield and a tester were used. All materials were sowed in a randomized block design with 3 replications, at a density of 140 plants per m^2. Different morphological traits were recorded and analyzed using ANOVA. Euclidean distances were calculated and a cluster analysis and a principal components analysis (PCA) were carried out. A total of 20 out of 25 SRAP combinations and 9 out of 17 SSR were assayed on the basis of their high polymorphism. Genetic distances were calculated 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. The analysis of variance for morphological traits showed significant differences between genotypes for all variables. The analysis of principal components determined that two PCs explained 75% of the total variation observed among the genotypes and the cluster analysis allowed the conformation of two principal groups. The first one included four varieties belonging to the *microsperma* type, whereas the second group included all the *macrosperma* type Rils. Both groups showed differences also in yield and plant height. Molecular analysis showed that 66% out of 273 bands are polymorphic fragments from SRAP markers, indicating a high genetic diversity among Rils. The number of alleles per SSR locus ranged from 3 (SSR 33) to 7 (SSR 156) showing also the variability present. Two exclusive SRAP fragments generated by the combination me5-em1 (230 bp) were found in *microsperma* group allowing early selection of this trait and all characters associated with it. The correlation between Dice similarity index and Euclidean distance matrices was 87% indicating an excellent consistency between both types of markers and suggested that both types of traits would provide similar estimates on the variability and would be useful to differentiate Rils with similar origin.

Keywords: Genetic distance, molecular markers, morphological markers, varieties selection, SRAP, SSR.
Abbreviations: DF_Numbers of days to flowering, DH_Numbers of days to harvest, NP_numbers of pods per plant, NS_numbers of seeds per plant, PC_Principal Components, PCA_Principal Components Analysis, PH_plant height (cm), RIL_Recombinants inbred lines, SD_Seed diameter (mm), SRAP_Sequence-Related Amplified Polymorphism, SSR_Simple Sequence Repeat, WS_weigh 100 seed (g), Y_yield per plant (g).

Introduction

Lentil (*Lens culinaris Medik. ssp. culinaris*), a diploid species (2n = 2x= 14) with an haploid genome size of 4063 Mb (Arumuganathan and Earle 1991), is an important cool season legume largely consumed in the Indian subcontinent, northern Africa, western Asia, southern Europe, North and South America, and Australia (Erskine 1996). *Lens* is a small Mediterranean genus that comprises the cultivated lentil and 6 related taxa (Ferguson et al., 2000). The cultivated species, *L. culinaris*, has been divided into two subspecies (Barulina, 1930): macrosperma (with 6 to 9 mm of seed diameter) and microsperma (with 2 to 6 mm of seed diameter). Lentils are very nutritious, with protein levels ranging between 20 and 36% (Gulati et al. 2002) and, like other legume species, has the ability to fix atmospheric nitrogen (Duranti and Gius 1997) and maintain fertility in degraded soils (Gresta et al., 2009; Pacucci et al. 2009). Seed size and testa and cotyledon colors impact market prices and as long as the genetic control of many of these characters is fairly well understood, the information has been used in developing new varieties (Sharma, 2011).In Argentina is an interesting substitute to wheat in cereal rotations but its importance is low due to a lack of suitable varieties with local adaptation. Some of the major problems that Argentinian lentil breeders face are the narrow genetic base of the current cultivated germplasm (derived from four varieties) and its low yield potential. A lentil-breeding program was initiated in 2004 to develop new cultivars with adaptation to prevalent conditions in growing areas of Argentina. Germplasm was obtained from ICARDA (*International Center for Agricultural Research in the Dry Areas*) and local producers and conventional breeding methods using hybridization and selection are being carried out to develop improved varieties, broad the genetic base, and isolate superior recombinant inbred lines (Rils). The differentiation among selected experimental lines is usually based in morphological traits, but these traits 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) (Tullu et al. 2008; Roy et al. 2013). Also, cytological techniques (Goshen et al. 1982) and isozymes analyses (Zamir and Ladizinsky 1984) were used to identify different genotypes; but, the limitations of these methods are that they are laborious and they may not detect sufficient polymorphism to distinguish very closely related genotypes. These facts make the use of these traits inadequate to identify different genotypes by themselves; therefore, the use of molecular markers alongside is a better way to achieve a complete differentiation between lines. Several molecular markers systems were used to determine allelic diversity in lentil collections (Sonnante et al. 2007; Datta et al., 2007, Bavayeva et al. 2009; Bacchi et al., 2010). In this work, we propose the use of SRAP (*Sequence-Related Amplified Polymorphism)* (Li and Quirós, 2001) and SSR (*Simple Sequence Repeat*) markers for this porpouse. SRAP is a technique simpler than AFLP and more reliable than RAPD, whereas SSR are highly polymorphic, more reproducible and distributed throughout the genome (Joshi and Behera 2006). SSR are also highly variable in number of repeats in several nucleotide sequences, are co-dominantly inherited (Johansson et al. 1992). Thus, SSRs are largely used in genetic analyses of self-pollinating crops like legumes (Jin et al. 2008). The objective of the present study was to evaluate the variability present in a set of experimental lines obtained by mass selection in a current lentil breeding program using morphological traits and SRAP and SSR markers in order to differentiate one from another and determine if there is concordance between both kinds of analysis.

Results and Discussion

Morphological traits

Analysis of variability

The analysis of variance showed significant differences between genotypes, years and genotype-year interaction for all the traits (Table 4) and this fact shows that the Rils differ in the variables evaluated. The average values for each trait are shown in Table 5. To capture maximal variance of the original variables a principal components analysis was performed and the associations among the 26 accessions were examined. In this analysis, two PCs explained 75% of the total variation observed among the varieties (Table 6). The first component (PC1) accounted for 60.0% of the variation and was characterized by days to flowering, days to harvest, number of seeds, pods per plant and diameter and weight of seed. The second component (PC2) accounted for 15.0% of the variation and was characterized by plant height and yield (Table 7). Two principal groups can be observed (Fig. 1). The first one includes four varieties with small seed diameter (*microsperma* types) whereas the second group included all the *macrosperma* types. Both groups showed differences also in yield and plant height. The dendrogram performed by Cluster Analysis using the ‘‘Ward’s minimum variance’’ method is presented in Fig 2. Significant differences between clusters were observed for all traits. The traits PH, DF, DH, NS, NP, SD, WS and Y showed high discriminating values ($F = 5.0$; $p < 0.001$; $F = 92.0$; $p < 0.001$, $F = 24.1$ $p < 0.001$, $F = 19.6$; $p < 0.001$; $F = 31.4$; $p < 0.001$, $F = 43.1$ $p < 0.001$, $F = 46.8$; $p < 0.001$ and $F = 11.3$ $p < 0.001$, respectively). The genotypes included in the *microsperma* group presented the lowest value for SD and the highest values for DF, NP and NS per plant. The *macrosperma* group can be divided into two major groups (G1 and G2), based on differences in PH, NP and NS per plant .The second group can be also divided into two subgroups (G2A and G2B), showing G2A the highest values for NP, NS and Y per plant.

Molecular data

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. We found 66% out of 273 bands are polymorphic fragments from SRAP markers indicating the variability presents between Rils. Banding patterns that were difficult to score and those that failed to amplify consistently in all genotypes were excluded. 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, Cravero et al (2007) in globe artichoke and Esposito et al. (2007) in a pea collection. The relationships between the 26 genotypes revealed by cluster analyses based on Dice distance are shown in Figure 3. Seventeen microsatellite markers were selected on the basis of their high polymorphism but only nine of them revealed successful amplifications of expected allele sizes (in bold Table 3). Therefore, the genetic diversity among the genotypes was assessed using 9 SSR markers with a total of 39 alleles. The number of alleles per SSR locus ranged from 3 (SSR 33) to 7 (SSR 156) with an average of 4.5 alleles. SSR 156 produced the largest number of alleles (7), followed by SSR191, SSR204, and SSR 19 (5 or 6 alleles each). These results were similar to those reported by Sonnante et al. (2007) (5 alleles at most using SSR primers), Fikiru et al. (2007) (9 alleles with ISSR primers) and Bavayeva et al (2009) (3 to 8 alleles). The relationships between the 26 genotypes revealed by cluster analyses based on Dice distance are shown in Figure 3. Two exclusive SRAP fragments generated by the combination me5-em1 (230 bp) were found in the *microsperma* group; on the other hand two SRAP fragments generated by the combination me2-em5 (200 bp and 205 bp) and the presence of SSR 204 (350 bp) were found only in all the Rils belonging to the *macrosperma* one. These five markers could be linked to the seed diameter and this fact would allow an early differentiation between *micro* or *macrosperma* types and select characters associated with seed size, especially considering that SD is a quantitative trait highly influenced by the environment which makes selection more difficult (especially when seed diameter of the genotypes are near the differentiation value of 6 mm). 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) and Ford (1997) with RAPD´s revealed important information on 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.

Table 1. Pedigree of the lines used in this assay.

Lines	Seed diameter	Cross	Coat color	Cotyledon color
Silvina	Large	Tester. Commercial variety	brown	yellow
B1051	Large	ILL 8072 x ILL6972	brown	yellow
B1052	Large	ILL 8072 x ILL6972	brown	yellow
B1053	Large	ILL 8072 x ILL6972	brown	yellow
B1054	Large	ILL 8072 x ILL6972	brown	yellow
B1055	Large	ILL 8072 x ILL6972	brown	yellow
B1056	Large	ILL 8072 x ILL6972	brown	yellow
B1058	Large	ILL 8072 x ILL6972	brown	yellow
A1061	Large	ILL 8107 x ILL 6199	brown	yellow
A1062	Large	ILL 8107 x ILL 6199	brown	yellow
A1141	Large	ILL 8107 x ILL 6199	brown	yellow
A1143	Large	ILL 8107 x ILL 6199	brown	yellow
A1144	Large	ILL 8107 x ILL 6199	brown	yellow
A1145	Large	ILL 8107 x ILL 6199	brown	yellow
A1146	Large	ILL 8107 x ILL 6199	brown	yellow
B1151	Large	ILL 7938 x ILL 6037	brown	yellow
B1152	Large	ILL 7938 x ILL 6037	brown	yellow
B1153	Large	ILL 7938 x ILL 6037	brown	yellow
B1154	Large	ILL 7938 x ILL 6037	brown	yellow
B1155	Large	ILL 7938 x ILL 6037	brown	yellow
B1156	Large	ILL 7938 x ILL 6037	brown	yellow
B1157	Large	ILL 7938 x ILL 6037	brown	yellow
B1171	Small	L 307 x L4673	brown	red
B1173	Small	L 307 x L4673	brown	red
B1181	Small	L 4671 x L 309	brown	red
B1182	Small	L 4671 x L 309	brown	red

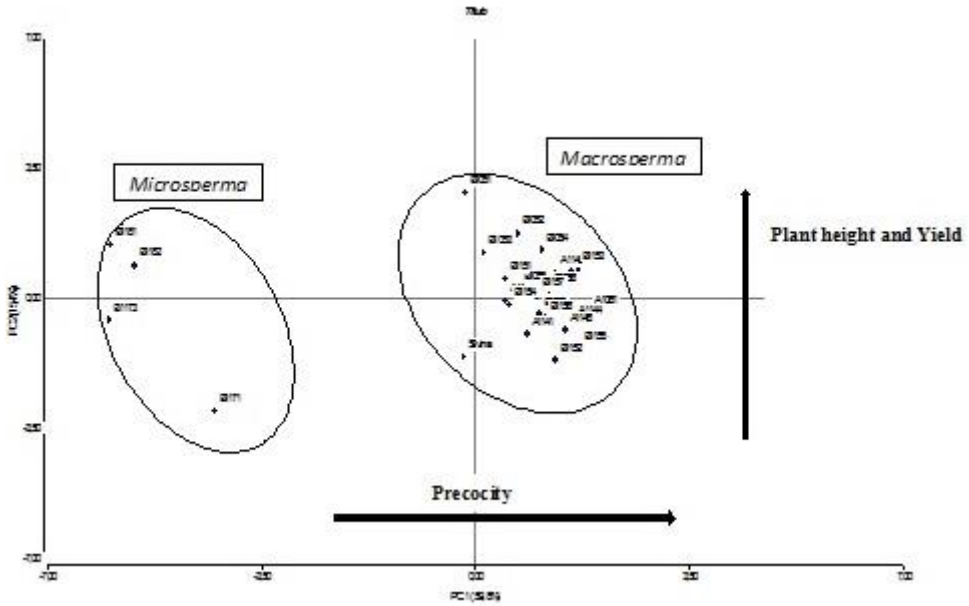

Fig 1. Scatter diagram with the distribution of varieties according to the first two principal components (PC1 and PC2).

Comparisons between morphological traits and molecular data

Comparison between SRAP-SSR and morphological data was carried out using the procrustes generalized analysis. The correlation between Dice similarity index (SRAP and SSR data) and Euclidean distance (morphological data) matrices was 87% indicating good correspondence between both data sets (Tatineni et al., 1996). Rils distribution for this analysis is shown in Fig 4. Two big clusters of Rils can be observed and their 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 formed another cluster (Sharma et al., 1995, Alvarez et al., 1997, Duran and Pérez de la Vega, 2004). Our results confirm that this classification can be valid, despites the study of Williams et al. (1974) that did'nt support the separation of these two types of lentil based on seed size .The first group included 4 Rils, all of them corresponding to the *microsperma* type while the second one included twenty-one Rils and the tester (Silvina) of the *macrosperma* type. Each group can be subdivided in two subgroups. Due to the high value of consensus between

Table 2. Primer sequences of SRAP used to generate molecular data.

Primers F		Primers R	
Name	Sequence	Name	sequence
me1	5´-TGAGTCCAAACCGGATA-3´	em1	5´-GACTGCGTACGAATTAAT-3´
me2	5´-TGAGTCCAAACCGGAGC-3´	em2	5´-GACTGCGTACGAATTTGC-3´
me3	5´-TGAGTCCAAACCGGAAT-3´	em3	5´-GACTGCGTACGAATTGAC-3´
me4	5´-TGAGTCCAAACCGGACC-3´	em4	5´-GACTGCGTACGAATTTGA-3´
me5	5´-TGAGTCCAAACCGGAAG-3´	em5	5´-GACTGCGTACGAATTAAC-3´

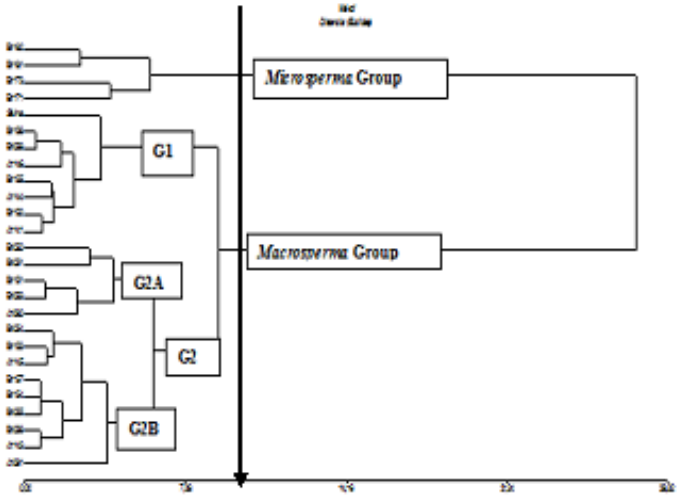

Fig 2. Dendrogram of 26 accessions based on morphological data using Euclidean distance matrix.

Table 3. Forward and reverse primer sequences with successful amplifications used for 9 microsatellite loci.

Primer	Forward (5'-3')	Reverse (5'-3')
SSR 46-2	CACTAAACATGGAAAATAGG	CTTATCTTTGTTTGAAGCAA
SSR 72	CAAACAGTACAAGGAAAGGAG	CTGACTGAGCTGCTTGAAC
SSR 90	CCGTGTACACCCCTAC	CGTCTTAAAGAGAGTGACAC
SSR 191	GCAAATTTCTTGGTCTACAC	GGGCACAGATTCATAAGG
SSR 19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG
SSR 33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC
SSR 156	GTACATTGAACAGCATCATC	CAAATGGGCATGAAAGGAG
SSR 204	CACGACTATCCCACTTG	CTTACTTTCTTAGTGCTATTAC
PSAB111	TGGAGCAGTTATGAGGAAAATTTG	TTGCATATGTGAGAATTTATAGCAATTAATG

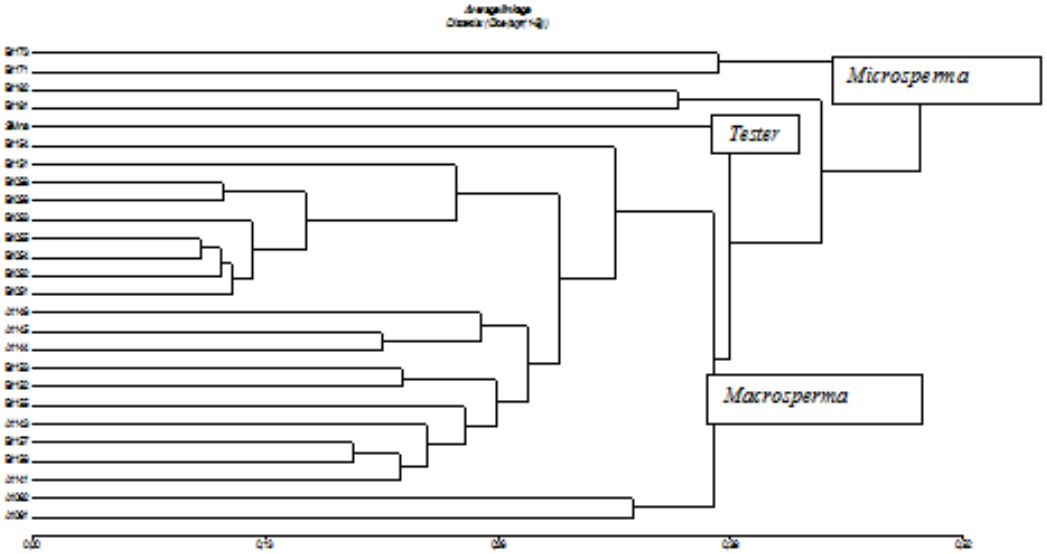

Fig 3. Dendrogram of 26 accessions based on SRAP and SSR data using Dice's genetic distance matrix.

Table 4. Analysis of variance for the different traits.

		PH		DF		DH		NP	
	df	MS	F	MS	F	MS	F	MS	F
Model.	103	194,5	7.8***	130.9	13.1***	163.4	13.7***	2023.0	2.5***
Genotypes (G)	25	116,6	4.7***	434.2	43.6***	246.5	20.6***	3753.7	4.6***
Environments (E)	3	3912,7	156.7***	34.7	3.5*	1342.5	112.2***	7391.9	9.0***
GxE	75	59,9	2.4***	30.4	3.1***	83.8	7.0***	1241.4	1.5***
Error	198	24,9		9.9		11.9		818,1	

		NS		SD		WS		Y	
	df	MS	F	MS	F	MS	F	MS	F
Model.	103	4960.89	3.1***	1.7	43.2***	7.9	13.1***	3.7	2.2***
Genotypes (G)	25	10405.4	6.4***	6.7	170.6***	15.1	24.8***	5.2	3.2***
Environments (E)	3	25757.7	15.9***	0.6	15.0***	112.1	184.0***	20.2	12.4***
GxE	75	2319.0	1.4*	0.1	1.4*	1.3	2.2***	2.4	1.5*
Error	198	1616.4		0.04		0.6		1.6	

*p<0.05, **p<0.01, ***p<0.001

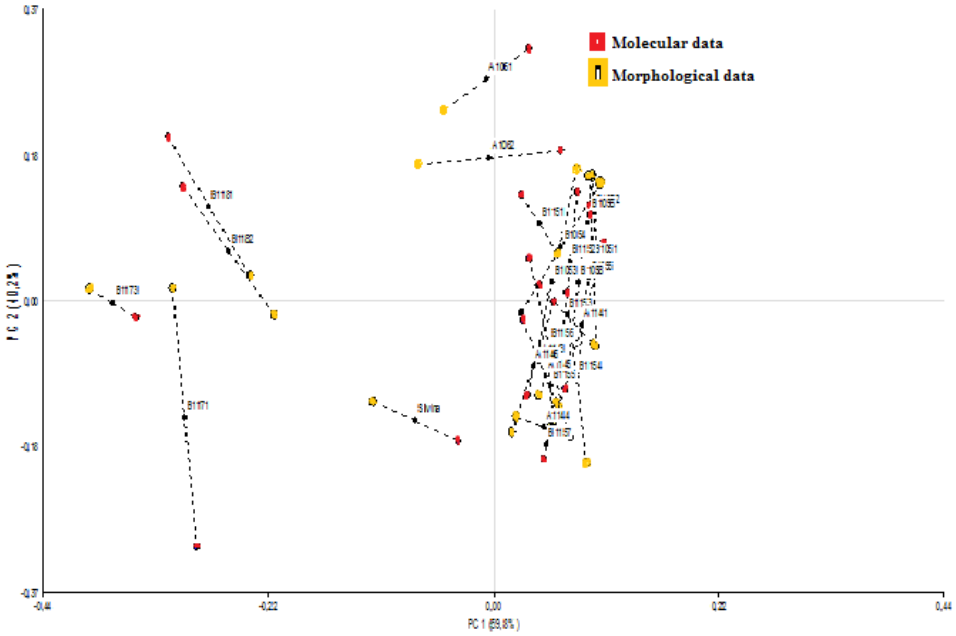

Fig 4. Consensus between Morphological and Molecular data: Consensus: 81%.

molecular and morphological data, the varieties located at the top right of the figure corresponded to those more precocious and with higher yield values. The identification of different types of lentils is one of the key steps in any lentil breeding program. The information provided by our study allowed us to establish a correlation between morphological and molecular data of r = 0.87. This value indicates an excellent consistency between both types of markers and suggests that both types of traits (morphological – molecular) would provide similar estimates on the variability between Rils.

Materials and methods

Plant materials

The present study was conducted at the research station of the Faculty of Agriculture of Rosario´s University, Argentina (33° 1 S and 60° 53 W), from 2009 to 2011. The experimental material consisted of 25 F_5 recombinant inbred lines (Rils) obtained from crosses between lines developed by ICARDA (*International Center for Agricultural Research in the Dry Areas)*. Four of them belong to the subspecies *microsperma*, while the other twenty-two belong to the subspecies *macrosperma* (Table 1). The commercial variety called "Silvina" was used as tester. All materials were sowed in a randomized block design with 3 replications, at a density of 140 plants per m^2.

Traits measurement and statistical analysis

Morphological analysis

Observations were recorded on number of pods per plant (NP), number of seeds per plant (NS), plant height (PH), days to flowering (DF), days to harvest (DH), seed diameter (SD) yield per plant (Y), and 100 seeds weight (WS). The values of the different traits were compared by an analysis of variance (ANOVA) using InfoStat for Windows (Di Renzo et al., 2012). 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 InfoStat software (Di Renzo et al., 2012). A Principal Components Analysis (PCA) was also performed.

Table 5. Mean values and standard errors for each morphological trait.

Lines	PH (cm)	DF	DH	NP	NS	SD	WS	Y
Silvina	36.9±3.1	88.7±1.2	138.3±0.8	62.3±12.6	79.9±18.0	5.7±0.08	2.9±0.2	2.2±0.4
B1051	39.6±2.5	82.8±0.9	144.9±2.8	95.9±9.2	110.9±13.1	5.9±0.04	4.7±0.6	4.7±0.5
B1052	37.9±1.8	80.9±0.9	133.7±1.7	77.1±11.9	94.8±14.3	5.8±0.04	4.6±0.4	4.2±0.7
B1053	37.9±2.1	83.7±0.6	145.6±3.7	73.8±6.7	92.0±11.4	5.8±0.06	4.7±0.5	3.9±0.3
B1054	41.6±2.4	85.4±0.4	142.5±1.9	66.5±4.9	78.4±7.1	5.9±0.06	5.0±0.5	3.9±0.3
B1055	37.9±2.7	84.5±0.7	137.8±1.6	66.8±7.8	83.5±12.2	5.9±0.07	4.4±0.4	3.2±0.2
B1056	40.5±2.3	83.4±0.4	141.9±1.5	56.5±6.8	63.9±7.8	5.9±0.05	4.8±0.4	3.1±0.4
B1058	36.3±2.5	84.0±0.6	140.4±0.7	58.7±9.7	70.9±13.9	5.9±0.07	4.8±0.4	2.9±0.3
A1061	42.8±3.2	87.5±2.0	139.5±1.9	41.9±5.9	48.67±6.0	6.5±0.10	5.4±0.3	2.5±0.2
A1062	38.8±1.5	82.9±0.9	146.7±2.7	55.3±6.8	64.06±9.2	6.6±0.06	5.9±0.5	3.6±0.4
A1141	34.3±1.9	83.3±1.1	138.9±0.9	56.3±6.3	69.1±9.0	5.9±0.06	4.4±0.5	2.8±0.4
A1143	39.2±3.2	84.6±0.7	141.0±0.5	58.8±9.8	72.5±13.7	5.8±0.07	4.5±0.5	2.9±0.4
A1144	35.2±1.9	81.1±1.1	136.4±2.4	59.3±8.0	71.9±12.0	5.9±0.08	4.8±0.7	2.8±0.3
A1145	40.2±2.6	81.3±0.9	139.5±1.9	64.4±6.9	74.9±9.3	5.8±0.08	4.9±0.5	3.3±0.3
A1146	36.5±2.8	83.9±1.1	144.1±3.3	46.3±9.8	53.3±11.3	5.7±0.05	5.3±0.5	2.8±0.7
B1151	36.7±2.6	83.3±0.6	146.7±2.9	65.3±4.6	78.4±6.7	5.8±0.06	4.7±0.4	3.8±0.4
B1152	34.4±2.7	84.9±0.4	141.4±0.9	51.6±7.6	65.5±11.2	6.0±0.06	4.4±0.4	2.5±0.3
B1153	39.7±2.7	84.5±0.7	140.9±1.3	63.6±7.6	69.2±7.9	5.8±0.06	5.4±0.6	3.6±0.5
B1154	37.8±3.2	83.7±1.1	139.7±0.7	65.6±3.7	77.8±5.9	5.8±0.09	4.1±0.5	2.9±0.2
B1155	32.5±2.3	83.5±0.9	137.2±0.9	51.0±5.5	59.5±7.9	6.0±0.05	4.8±0.2	2.8±0.3
B1156	35.3±2.7	84.9±0.6	141.5±1.1	59.2±4.9	68.6±5.9	6.0±0.06	4.7±0.5	3.1±0.3
B1157	38.1±3.4	84.4±0.3	139.8±2.1	62.8±8.6	71.7±11.1	5.9±0.07	4.6±0.4	3.2±0.4
B1171	34.4±2.9	99.4±1.9	152.6±2.4	69.9±12.3	96.7±19.7	4.0±0.08	1.7±0.2	1.3±0.2
B1173	35.4±1.5	102.7±1.9	153.5±2.8	116.9±13.9	161.1±18.9	4.0±0.06	1.7±0.2	2.4±0.2
B1181	42.6±2.9	101.2±1.2	148.8±1.2	101.1±10.1	154.9±14.8	3.8±0.05	2.3±0.2	3.4±0.4
B1182	46.3±3.7	96.6±3.3	144.6±2.6	99.6±21.5	153.3±35.3	3.8±0.05	2.1±0.2	3.4±0.9

Table 6. Eigenvalues obtained from the Principal Components Analysis.

Lambda	Value	% Total variance	% Cumulative variance
1	5.98	60	60
2	1.54	15	75
3	0.96	10	85
4	0.66	7	92
5	0.52	5	97
6	0.17	2	99
7	0.07	1	100

Table 7. Eigenvectors obtained from the Principal Components Analysis.

Traits	e1	e2
PH	-0.11	0.51
DF	-0.38	-0.17
DH	-0.28	-0.03
NP	-0.34	0.35
NS	-0.38	0.25
SD	0.40	0.07
WS	0.38	0.22
Y	0.15	0.69

Molecular analysis

To perform the DNA extraction, about 100 mg of fresh leaf tissue were grounded in liquid nitrogen and the total genomic DNA was extracted using the CTAB method (Smýkal et al, 2008). A total of 25 SRAP combinations between 5 primers Forward (F) and 5 Reverse (R) (Table 2) and 17 SSR (Table 3) were assayed on all accessions. The amplifications were carried out in a thermo-cycler MyCycler^{TM} (BIO-RAD). At the beginning of the PCR reaction, the annealing temperature was set at 35 °C and run for 5 cycles. Then the 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. The amplified fragments were separated by denaturing acrylamide sequencing gels and reveled whit silver (Li and Quiros 2001). SRAP and SSR fragments were scored for presence or absence as 1 and 0 respectively. Genetic distances were

calculated with SRAP and SSR data according to Dice's similarity index and a dendrogram was performed. A Principal Coordinate analysis was also done. A comparison between morphological and molecular data was carried out through the Procrustes Generalized analysis using the InfoStat program (Di Renzo et al., 2012).

Conclusion

SRAP and SSR markers are efficient tools in the differentiation of genetic variability and are consistent with the information provided by morphological markers because they allow the same grouping of varieties. Coupling molecular with morphological markers provides thorough starting information for programs aiming to obtain new inbred lines, especially from different genetic backgrounds.

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