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Molecular characterization of pea (*Pisum sativum* L.) using microsatellite markers

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Abstract: *Nineteen pea (Pisum sativum* L.*) accessions have been characterized using Simple Sequence Repeats (SSRs). The mains objectives of this study were to examine SSR polymorphism among cultivars and to assess genetic diversity among them. Eight microsatellites, from the Pisum microsatellite consortium (Agrogene ®, France) have been used. Five of the eight SSRs studied gave good electrophoretic profiles and helped us to amplify a number of alleles per locus varying from 3 (PSMPA5 and PSMPA6) to 13 (PSMPSAD126) with a total of 34 and an average number of 6.8 alleles per locus. The Polymorphism Information Content (PIC) varied from 0.18 for PSMPSAD134 to 0.85 for PSMPSAD126, with an average value of 0.62. The five microsatellites analyzed allowed us to separate 18 out of the 19 genotypes studied, and only the two most polymorphic markers (PSMPSAA205 and PSMPSAD126), permit to discriminate among the same genotypes (18) separated using the 5 SSRs. Genetic distances computed have been used to draw the corresponding dendrogram and to distribute genotypes according to their genetic relationship. The genotypes classified within the same group share several agro-morphological characters. Finally, the present study attests that SSR microsatellites are good tools for identifying genotypes and for the assessment of genetic diversity in pea.*

Keywords: *genetic distance, genetic diversity, molecular markers, Pisum sativum, SSR microsatellites.*

I. Introduction

Pea (*Pisum sativum* L.) is a member of the *Fabaceae* family. It is a diploid species with chromosome number 2n = 14. This grain legume is rich in proteins, used in rotation with cereals and oil crops, increases soil fertility by fixing atmospheric nitrogen and used as food and feed. Pea is an important winter pulse crop in West Europe, India, Australia, Pakistan, North and South America and is among the four important cultivated legumes after soybean, groundnut and beans [1].

In Tunisia, the area occupied by this crop is about 20,000 ha, divided mainly between the north and the center of the country [2]. The pea crop is linked to two main purposes; first, the food through garden pea which is a vegetable grown for dry seeds either for fresh consumption or for canning. Second, the feed used as mature seeds or forages (fodder, fresh or ensiled) [3]. Therefore, pea crop in Tunisia has agronomic and socio-economic importance and plays an important role in cereal based system and well considered in the rehabilitation program of grain legume in this system. These program aims to increase grain legume production, to research and enhance the exploitation of high yielding varieties tolerant to biotic and abiotic stresses and to improve the seed system delivery and quality. The first step of any breeding program targeting the development of improved high yielding and stress tolerant varieties must consider the evaluation of genetic material based on phenotyping (description and assessment against the most important stresses) and genotyping. The current pea variety registration system in Tunisia is based mainly on morphological and phenological characters. Although these descriptors are useful, they are limited in number and may be affected by environmental factors. Molecular markers are useful to complement the morphological and phenological characters because they are plentiful, independent of tissue or environmental effects, and allow cultivar identification in the early stages of development.

Molecular markers that reveal polymorphism at the DNA level have been shown to be a very powerful tool for genotype characterization and estimation of genetic diversity. The use of molecular markers for diversity analysis can also serve as a tool to discriminate between closely related individuals from different breeding sources [4, 5, 6].

Microsatellite markers, or simple sequence repeat (SSRs), which are codominant, and highly polymorphic markers easily detected with PCR procedure, appear as the best available choice of markers for pea diversity assessment and characterization [7]. An abundance of *P. sativum* microsatellite locus-specifc primer pairs have been generated through the construction of a genomic library enriched for repeat motifs by the Agrogène ® consortium (Moissy Cramayel, France) and were used in several studies on pea genetic diversity [7, 8, 9].

The aim of this study was to characterize nineteen pea cultivars, using eight SSRs markers in order to: (i) examine SSR polymorphism among cultivars; and (ii) to detect the level of genetic diversity among them.

This work was performed in the Field Crop Laboratory at the National Institute of Agricultural Research of Tunisia (INRAT).

II. Materials And Methods

2.1, Plant material and DNA extraction

Nineteen accessions of pea, from different origins were studied (Table 1), fifteen of them were kindly provided by ICARDA and 4 ("PP-1", "PP-2", "PP-3", and "PP-4") are from the collection of the National Institute of Agricultural Research of Tunisia (INRAT).

Fresh young leaves were ground to powder with liquid N2 using a mortar and pestle. Genomic DNA was isolated from leaf samples using CTAB (cethyltrimethyl ammonium bromide) extraction method described by [10] and slightly modified. DNA quality was examined by electrophoresis in 1% agarose and estimated by visual comparison of DNA bands on gel with known concentrations of phage lambda DNA.

Table 1: Cultivars studied and their origin

Cultivars	Origin
DDR-11	India
Spring pea3	Australia
P12/95	Romania
P7/79	Romania
L-selection-1690	Syria
88P101-10-2	Australia
88P007-2-1	Australia
Argon	Russia
Sprut2	Russia
Marina	Romania
88P038-4-3	Australia
Syrian.L.Alleppo-1	Syria
88P038-10-18	Australia
88P001-4-9	Australia
88P090-5-16	Australia
PP-1	Unkown
PP-2	Unkown
PP-3	Unkown
PP-4	Unkown

2.2, DNA amplification

Eight microsatellite markers (Table 2) were selected for genotyping. Amplification reaction were carried out in a final volume of 20 µl containing 30-50 ng of template DNA, 1 X reaction buffer (20 mM (NH4) SO4, 75 Mm Tris-HCl pH 8.8), 2 mM $MgCl_2$, 0.1 mM of dNTPs (50uM of each), 0.2 µM of forward and reverse primers each, and 0.5 U of Taq DNA polymerase. The PCR amplification were carried out on a thermocycler (MuliGene OPTIMAX) using the following temperature cycles: 1 cycle of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at the annealing temperature 50°C and 1 min at 72°C. The last cycle was followed by a final incubation for 5 min at 72°C and the PCR products were stored at 4°C before analysis. The DNA amplification products were loaded on 8% non-denaturing polyacrilamide sequencing gels in 1X TBE buffer. Gels were run for 2h at 180 V, stained by ethidium bromide and visualized under UV; the fragment sizes were estimated with the 25 bp DNA step Ladder of Promega DNA sizing markers (Fig.1).

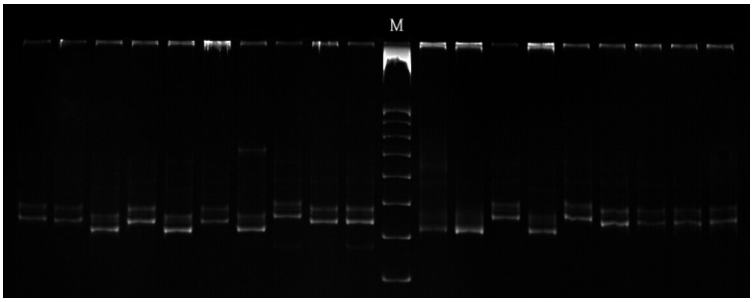

Fig.1: Electrophoretic profil of the 19 pea cultivars amplified with PSMPA6 marker. M: molecular size marker (25 bp DNA step Ladder of Promega).

2.3, Data analysis

To evaluate the information obtained with the 8 SSRs studied, we calculated the following parameters: (i) the number of alleles per locus (N) was counted from the gel profile analysis, (ii) the effective number of alleles (Ne) per locus (Ne = $1/\sum p_i^2$, where p_i is the frequency of the i^{th} allele), (iii) the polymorphism information content (PIC) for each marker was also determined, using the following equation of [11]:

where $\mathbf{p_i}$ is the frequecy of the i^{th} allele in the set of 16 genotypes. Those parameters served to evaluate the information given by the microsatellites markers (Table 1).

The binary matrix was obtained from the reading of the electrophoretic patterns corresponding to all the microsatellites analyzed. Amplified fragments for each locus were scored as present allele (1) or absent allele (0). The binary matrix was used to calculate the genetic distance between each pair of accession using the coefficient of Dice [12]. A dendrogram was constructed from the genetic distance matrix using the unweighted pair group method average (UPGMA) clustering [13]. Data were analysed using the NTSYS-pc, version 2.1 program [14].

Table 2: Description of the 8 microsatellite markers used for the amplifications of the studied pea lines

Primers	Sequences	Annealing temperature (°C)	Reference
PSMPA5	**F 5'-gtaaagcataaggggttctcat-3'** **R 5'-cagcttttaactcatctgaca-3'**	**50**	Tar'an et al. 2005
PSMPA6	**F 5'-cttaagagagattaaatggacaa-3'** **R 5'-ccaactcataataaagattcaaa-3'**	**50**	Tar'an et al. 2005
PSMPA9	**F 5'- gtgcagaagcatttgttcagat -3'** **R 5'- cccacatatatttggttggtca-3'**	**50**	Tar'an et al. 2005
PSMPD23	**F 5'- atggttgtcccaggatagataa -3'** **R 5'- gaaaacattggagagtggagta-3'**	**50**	Tar'an et al. 2005
PSMPSAD126	**F 5'-tggctttgcagagtgtttgagtr-3'** **R 5'-ggcttcaacagcgatccataat-3'**	**50**	Tar'an et al. 2005
PSMPSAD134	**F 5'-tttatttttccatatattacagaccgr-3'** **R 5'-acacctttatctcccgaagacttag-3'**	**50**	Tar'an et al. 2005
PSMPSAD146	**F 5'-tgctcaagtcaatatatgaagar-3'** **R 5'-caagcaaatagttgttttgtta-3'**	**50**	Tar'an et al. 2005
PSMPSAA205	**F 5'-tacgcaatcatagagtttggaa-3'** **R 5'-aatcaagtcaatgaaacaagca-3'**	**50**	Tar'an et al. 2005

III. Results And Discussion

3.1, Microsatellite diversity

Nineteen pea accessions were analyzed with 8 SSRs, five from them generated good amplifications and were polymorphic. The remaining primer pairs gave bands which are difficult to resolve and were consequently discarded from the analysis. The 5 SSRs studied were single-locus and produced alleles that could be scored, with a total of 34 alleles ranging from 3 to 13 alleles per locus, with a mean value of 6.8 alleles per locus (Table 3). [9] found a total of 63 alleles using 21 SSR markers to evaluate the genetic diversity across 12 geographically diverse pea genotypes ranged from one to four, with a mean value of 3 alleles per locus. [15] reported, for a set of 28 pea cultivars with 32 SSRs, a total of 44 alleles with a mean value of 2.1 alleles per locus. Our results showed higher variability in the genetic material used than results recorded by these authors.

All the SSRs employed in this study were polymorphic and it was possible to distinguish unambiguously 18 from the 19 genotypes studied. The most informative locus of this work was PSMPSAA205, with a PIC value of 0.86, while the less informative one was PSMPSAD134 with a PIC value of 0.18. The highest number of alleles (N), the highest number of effective alleles (Ne) and the highest number of different genotypes detected were observed for PSMPSAD126. This SSR also showed very high PIC value (0.85). Our results indicate that theses parameters are very useful for the evaluation of adequate SSR markers to distinguish unambiguously related pea accessions. Therefore the number of loci necessary to characterize pea collection can be reduced effectively by selection of the most informative ones.

According to [11] if the PIC value is greater than 0.5 this corresponds to a very informative marker. PIC values between 0.5 and 0.25 correspond to informative marker, and a peak value less than or equal to 0.25 reflects lesser informative marker. Therefore, and according to Table 3, the markers used in this study, with the exception of one (PSMPSAD134), were highly informative markers. The most informative ones (PSMPSAD126 and PSMPSAA205) can be considered as good markers. In fact, they allowed distinguishing unambiguously the same 18 pea genotypes identified using the 5 SSR markers from the 19 cultivars studied. These 2 SSRs could be promising to study pea genetic diversity.

Table 3: List of 5 SSR primers and their variability parameters

Locus	Number of loci	N	Ne	# Genotypes	PIC
PSMPSAD134	1	6	1.22	6	0.18
PSMPSAA205	1	9	7.37	9	0.86
PSMPSAD126	1	13	6.70	15	0.85
PSMPA6	1	3	2.50	3	0.60
PSMPA5	1	3	2.56	3	0.60
Total	5	34.0	20.33	36.0	
Moyenne	1	6.80	4.1	7.20	0.62

N: number of alleles; Ne: Effective number of alleles; # Genotypes: different genotypes per locus; PIC: polymorphism information content.

3.2, Genetic relationships among cultivars

The genetic similarity among the 19 pea genotypes included in this study is graphically represented by an UPGMA dendrogram (Fig. 2) using 5 SSRs. The dendrolgram was used to observe the distribution of the 19 cultivars into several clusters according to the original data obtained in the similarity matrix, and based on the genetic distance among them. The dendrogram also allow the estimation of relationships among the cultivars studied based on shared alleles across them. At genetic similarity of 0.2, the dendrogram showed two main groups. The first group was composed of "DDR-11", "Spring pea 3", "P7/79", "88P101-10-2", "PP-3", "PP-4", "Marina" "PP-2", "Sprut 2", and "PP-1". The second group included the rest studied genotypes.

The majority of the lines belonging to the first group share some morphological characters such as leaf color, flower color, pod color and seed color with the exception of a few lines as "PP-1" and "PP-3" which were distinguished by the leaf and the immature pod colors darker than the other lines. Most of the lines in this group share the same intensity curvature of pods which is low except "DDR-11" showed a high intensity, "PP-1" lines with an average intensity, while "88P101-10-2" and "PP-2" lines showed a very weak intensity or absent curvature. These results indicate that there is no always correlation between morphological traits and genetic similarity among genotypes using SSR markers.

We can see a number of lines in this group such as "DDR-11", "Spring Pea 3", "P7/79" and "PP-4" share similar agronomic characteristics such as the physiological maturity date, total number of branches, number of fruiting branches, number of seeds per pod and pod size, which reflect concordance between genetic and agronomic traits similarity.

Regarding the second group of the dendrogram, it includes a number of lines that also share the same morphological characters with some exceptions.

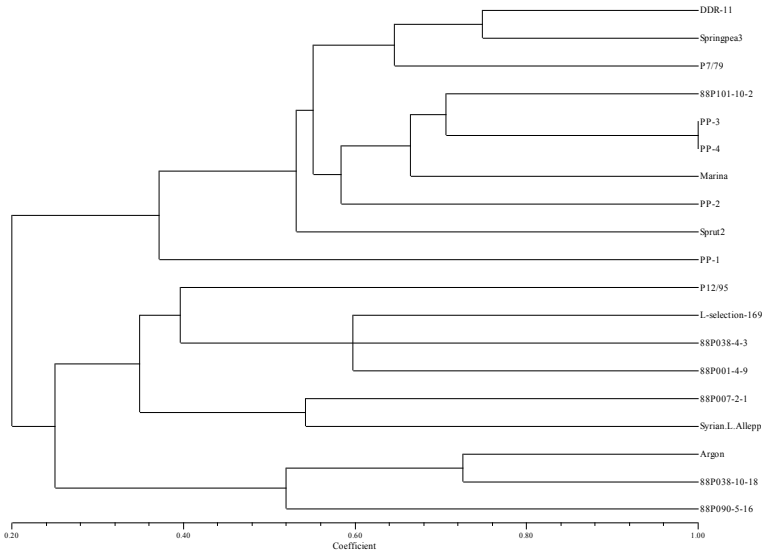

Fig. 2: UPGMA Dendrogram of 19 pea cultivars based on their variation at 5 SSR loci using the Dice coefficient (Nei et Li, 1979).

IV. Conclusion

Further study of morphological characters and evaluation of agronomic traits, conjointly with the use of a higher number of markers distributed throughout the genome of pea and having a high level of polymorphism, allows more robust and conclusive correlations between genetic similarity and agro-morphological traits. It

would even be possible to identify markers linked to genes or QTLs controlling some morphological and agronomic traits. This could be very useful later in marker assisted selection programs and helpful to the breeding of pea crop.

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