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IDENTIFICATION OF FIELD PEA CULTIVARS (*PISUM SATIVUM* L.) USING MICROSATELLITE MOLECULAR MARKERS

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Summary. Conventionally morphological descriptors are routinely used for establishing the identity of varieties. This kind of descriptors has some disadvantages, namely most of them are quantitative, controlled by several pairs of genes, and their expression is influenced by environmental factors. Molecular markers have a potential to facilitate this procedure, increase the reliability of decisions, and substantially save the time and space needed for experiments. In this study we intended to identify 20 cultivars of field pea (*Pisum sativum* L.), registered in the Community Catalog of Varieties, by microsatellites molecular markers. After DNA extraction, seven different *loci* were analyzed. PCR amplifications were conducted and the resulting fragments were separated on a 3.5% MS-8 agarose gel in TBE buffer. The gels were analyzed for the presence/absence of bands and a table with binary code was made. For each *locus* PIC value was calculated. The data were processed with the statistical software NTSYS-pc, using the SIMQUAL module and Jaccard similarity coefficient, followed by UPGMA cluster analysis. With the analysis of six polymorphic *loci* was possible to distinguish almost all of cultivars. The most informative *loci* were AD61 and AB53. The UPGMA dendrogram showed two main groups. The results showed a high potential and resolving power of SSR markers in distinct assessment. SSR markers might also be useful in germplasm management and genetic diversity studies. In the present research work, we have successfully employed high resolution agarose gel electrophoresis for genotyping with microsatellite markers in pea.

Keywords: cultivar identification; DNA fingerprinting; high resolution agarose gel electrophoresis; *Pisum sativum* L.; SSR markers

INTRODUCTION

Cultivar identification and estimation of genetic variability of populations are important in both variety protection and plant breeding. A new improved variety has to show distinct from other existing ones at least one characteristic, should demonstrate uniformity and stability (EC 2100/94 norm). The morphological descriptors are routinely used for variety identification. However, this kind of descriptors has some disadvantages, namely most of them are quantitative, controlled by several pairs of genes, and their expression is influenced by

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environmental factors (Smýkal, et al., 2008). Moreover, the number of morphological markers is limited and does not always allow a clear and unambiguous identification of an increasing number of cultivars. The use of markers at the DNA level allows finding solutions to these problems.

There are different types of molecular markers that permit unambiguous identification of varieties, important both in the defense of intellectual property rights and as a way of ensuring the certification of propagating material. The term DNA fingerprinting was introduced in 1985 (Jeffreys et al. 1985) and reflects the robustness of the molecular markers that allow to associate to each genotype a profile of discrete bands of DNA. A large number of molecular methods have been developed, which provide a large number of markers (RFLP, RAPD, SSR, IRAP, AFLP and RBIP, to name the most common ones).

Among the most widely used markers in crop species are simple sequence repeats (SSR) or microsatellites (Blair et al., 2007; Sarıkamış et al., 2009). Microsatellites are abundant small repetitive fragments in eukaryotic genome that display tandem repeats of a motif of two to six base pairs. They have a number of advantages over many molecular markers, including their co-dominant inheritance, relative abundance, extensive genome coverage, easy detection by PCR, as well as reproducibility and locus specificity (Nybom, 2004; Ellis and Burke, 2007).

In recent years, molecular markers have played an important role in the assessment of genetic diversity among populations. Microsatellite markers have shown a high potential for the detection of variability in pea, due to the high allelic variation detected, allowing clear identification of varieties (Smýkal, 2008; Sarikamiş, 2009). SSR markers were also used in evolutionary studies (Choumane et al., 2008; Ford et al., 2002) and to map loci responsible for the resistance to diseases or another important trait (Pilet-Nayel et al. 2002; Loridon et al. 2005).

Following a study of agronomic performance of 20 cultivars of field pea (*Pisum sativum* L.) in Castelo Branco region, east centre of Portugal (Reis et al., 2010), we decided to carry out the identification and quantify the genetic diversity of twenty cultivars of field pea using microsatellite markers. Our aim was also the optimization and establishment of a rapid and efficient DNA fingerprinting method to evaluate the efficiency of high resolution agarose gel electrophoresis in pea genotyping using microsatellite markers.

MATERIAL AND METHODS

Plant material and DNA extraction

Twenty cultivars of field pea (*Pisum sativum* L.) from European common catalogue of varieties of agricultural plant species were studied (Table 1). Young leaves from three individuals per accession were taken for DNA extraction. Genomic DNA was extracted from young leaf tissues using the DNeasy Plant Mini Kit (Qiagen, 2006).

Simple sequence repeats analysis

Seven SSR markers, originated from a pea genetic map constructed by Loridon et al. (2005) were studied to detect polymorphisms and assess inter genetic diversity of the pea cultivars (Table 2). The

Table 1. Cultivars of field pea (*Pisum sativum* L.).

Cultivar	Country of official acceptance and number of responsible for the maintenance
ALEZAN	FR 8174
ALHAMBRA	ES 225
ARTHUR	FR 11539
AUDIT	FR 13262
CARTOUCHE	FR 9295, UK 182
CORRENT	IT 2
CHEROKEE	FR 11553
ENDURO	FR 8444
GREGOR	DE 147, FR 9295, UK 6136
GRISEL	PT 2
GUIFILO	ES 9
GUIFREDO	IT 332
IDEAL	ES 2041
ISARD	FR 9504
JAMES	FR 9295
KLEOPATRA	DE 7627
LIVIA	FR 8451
LUMINA	FR 13262
ONIX	FR 9295
PIXEL	PT 2

ES – Spain; FR – France; DE – Germany; IT – Italy; PT – Portugal.

Table 2. Number of alleles and PIC generated by PCR using primers for seven pea (*Pisum sativum* L.) loci.

SSR marker	Primer F	Primer R	Number of alleles	PIC
A9	GTGCAGAAGCATTTGTTCAGAT	CCCACATATATTTGGTTGGTCA	2	0.42
AA205	TACGCAATCATAGAGTTTGGAA	AATCAAGTCAATGAAACAAGCA	1	0.00
AA355	AGAAAAATTCTAGCATGATACTG	GGAAATATAACCTCAATAACACA	4	0.46
AB25	TTTTCACTCAAAACACTCGGCT	GATGCCATTGCTGAAGGAGATT	2	0.46
AB53	CGTCGTTGTTGCCGGTAG	AAACACGTCATCTCGACCTGC	5	0.58
AD61	CTCATTCAATGATGATAATCCTA	ATGAGGTACTTGTGTGAGATAAA	3	0.56
AD270	CTCATCTGATGCGTTGGATTAG	AGGTTGGATTTGTTGTTTGTTG	3	0.52
Total			20	
Average			2.86	0.43

selection of the seven pea SSRs was based on their high polymorphism information content and the quality scores reported (Loridon et al., 2005). All analyses were performed at least twice to verify the results and check the reproducibility of allele scoring.

The polymerase chain reaction (PCR) mixture contained 20-40 ng template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 0.6 U Ampli Taq Gold DNA polymerase (Applied Byosistems), 1.5 mM $MgCl_2$, and 1X PCR reaction buffer in a total volume of 15 μl. Reaction mixtures without DNA were included as negative controls.

The amplification conditions involved an initial step of 10 min at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 51 or 56°C (Table 2) and 30 seconds at 72°C, with a final extension at 72°C for 5 min. PCR amplification was performed using the Biometra® PCR System. PCR products were separated on a 3.5 % (w/v) MS8 (Condalab) agarose gel containing 1.0x TBE buffer. Gels were run at 90 V for 2 h and were stained with ethidium bromide at a concentration of 0.5μl/mg. MS8 is an agarose for molecular screening that improves resolution of small DNA fragments and PCR products and is used for applications that require efficient separation of small DNA fragments and PCR products. DNA Ladder 100 bp (Biotools) was used for the approximate quantification of the bands. The allele numbers per locus were determined based on their relative position in the gel.

Data analysis

Results were analyzed based on the presence (1) or absence (0) of amplified fragments (amplicons) for each *locus* and cultivar. The resulting binary data matrix was processed with the statistical software NTSYS-pc version 2.2 (Rohlf F. J. 1998), using the module SIMQUAL and Jaccard similarity coefficient (GS*j*), followed by cluster analysis UPGMA (Unweighted Pair-Group Method with Arithmetic Mean). Polymorphism information content (PIC) was calculated using the formula developed by Anderson et al. (1993).

RESULTS AND DISCUSSION

By employing high resolution agarose gel electrophoresis we were successful to clearly separate alleles for the seven microsatellite markers studied. Fig. 1 shows the separation of two alleles of the AB25 locus. Within the investigated group of 20 peas cultivars the number of alleles ranged from 1 (AA205) to 5 (AB53), detecting altogether 20 alleles (Table 2). The average number of alleles per locus was 2.86. PIC ranged from 0.42 (A9) to 0.58 (AB53), the average being 0.43. The PIC value allows evaluation of the discriminatory value of each locus, based not only on the number of alleles but also on their relative frequencies. In the AA205 locus only one allele was detected and its value as a marker for distinguishing cultivars was null. It was in AB53 and AA355 loci where a large number of alleles was detected, 5 and 4, respectively. In spite of the use of a relatively simple separation system, the six polymorphic loci studied provided precise identification of almost all cultivars (Fig. 2). Only cultivars Ideal and Alezan had an identical set of bands profiles, presumably they might have been derived from the same pedigree. The similarity coefficients ranged from 0.083 to 0.857.

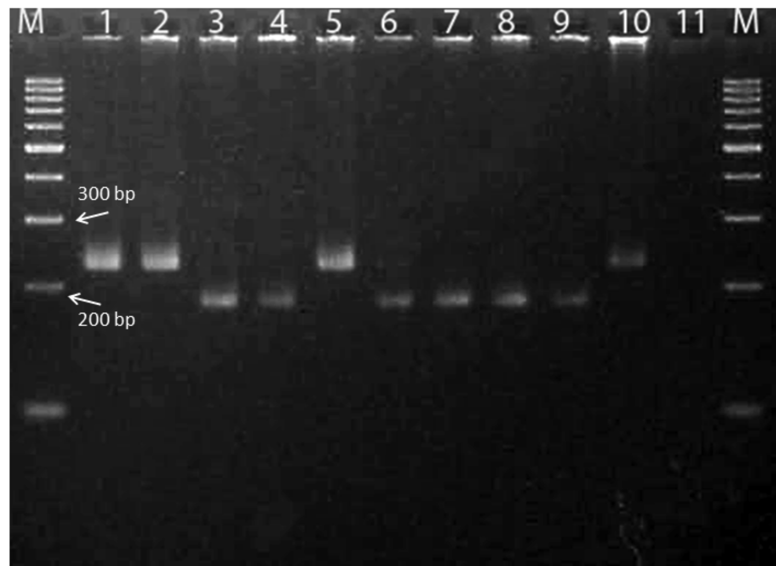

Figure 1. Agarose gel image of SSR amplification profiles for AB25 locus. M – DNA Ladder 100 bp; 1 to10 – profiles for different field pea cultivars; 11 – negative control.

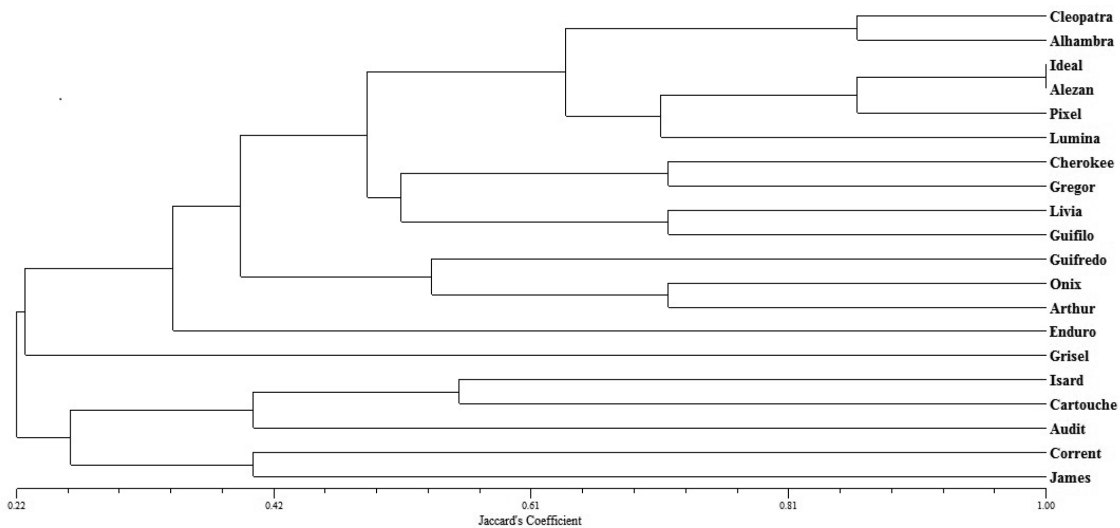

Figure 2. Phenogram based on Jaccard coefficients of similarity for 6 polymorphic SSR loci data in pea (*Pisum sativum* L.).

The cluster analysis of SSR markers separated the pea genotypes into two distinct clusters. The first cluster included the five cultivars: Isard, Cartouche, Audit, Corrent and James. The second cluster included the remaining fifteen cultivars and was further divided in two subclusters. The first subcluster included the Portuguese genotype Grisel while the second subcluster contained the remaining fourteen cultivars. In this subcluster Ideal and Alezan showed 100 percent similarity.

A high stability of cultivars was found, which can be assigned to the high degree of self-pollination in pea, as previously observed by Smýlak et al. (2008).

CONCLUSIONS

Molecular markers are useful in germplasm management as well as in genetic diversity studies as a tool for rapid variety identification. The knowledge of genetic relationships is useful in plant breeding programs since it allows making informed decisions, especially regarding the choice of genotypes to cross for the development of new populations or to facilitate the identification of parents to cross in hybrid combinations in order to maximize the expression of heterosis. In addition, molecular markers may be an important tool in plant variety protection.

In this study, molecular identification of 20 cultivars of field pea with microsatellites was carried out. By combined analysis of six polymorphic loci it was possible to distinguish almost all cultivars. However, the complete identification of 20 cultivars implied the study of few additional loci. For the seven loci studied we detected 20 different alleles, AB53 and AD61 being the most informative loci for genetic variability.

Polyacrylamide gel electrophoresis, high resolution agarose gel electrophoresis and automated capillary electrophoresis can be used for microsatellite analysis. PAGE is a tedious and time-consuming process, while capillary electrophoresis can be performed more quickly and is good for high-throughput analysis but it is more expensive and requires more sophistication and expertise. Our results demonstrate that high resolution agarose gel electrophoresis is a reliable and appropriate approach for identification of small length polymorphisms.

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