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Detection of DNA polymorphism among pea cultivars using RAPD technique

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Abstract

An influence of some Random Amplified Polymorphic DNA (RAPD) reaction factors on resulting banding pattern and the ability of RAPD technique to detect DNA polymorphism among six economically important pea cultivars was tested. Relatively high level of DNA polymorphism among peas was observed, using polyacrylamide/urea gels and silver staining. Altogether 13 arbitrarily designed primers produced 313 amplification products. In addition 59 polymorphisms were found. These polymorphisms can serve as potential genetic markers. RAPD data were processed using cluster analysis and plotted as dendrogram. Each tested cultivar was clearly distinguished from the others. Moreover, *Pisum sativum* and *P. sativum* subsp. *arvense* cultivars were separated into 2 different clusters, according to their systematic relationships.

Key words: arbitrary primers, molecular markers, *Pisum sativum*

Introduction

Over the last few years, the polymerase chain reaction (PCR, Saiki *et al.* 1988) has led to the development of several assays independent of preliminary knowledge of DNA sequence to reveal DNA polymorphism. These methods all use single arbitrary primers to generate multiple amplification products (Williams *et al.* 1990, Welsh and McClelland 1990, Caetano-Anollés *et al.* 1991).

One of them is Random Amplified Polymorphic DNA or RAPD (Williams *et al.* 1990), which has rapidly become a powerful tool for production of DNA markers (for reviews see Samec 1993, Tingey and Tufo 1993). The main advantage of this method is its speed - the RAPD assays are over 10-fold more effective in time and labour compared to those based on restriction fragment length polymorphism (Reiter *et al.* 1992). However, the optimisation of reaction conditions is necessary to achieve

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reliable results which can be time consuming.

In the group of autogamous legumes, RAPD has been used in linkage mapping of faba bean (Torres *et al.* 1993) and pea (Dirlewanger *et al.* 1994). The objectives of this work were to show effects of selected reaction factors on resulting banding pattern and then to assess the level of total RAPD variability among economically important pea cultivars with prospects of finding genotype-specific DNA markers.

Materials and methods

Plant material: Cultivars of pea included *P. sativum* L. cv. Bohatýr, Trille, Smaragd, Solara and *P. sativum* L. subsp. *arvense* cv. Ina and Tyla. Seeds were obtained from Technical Crops and Legumes Research Institute, Šumperk (Czech Republic). Young leaves from 10 plants (4 g fresh mass) were sampled, frozen in liquid nitrogen and then stored at -70 °C. The isolation of DNA and caesium chloride purification were performed according to the method of Amasino *et al.* (1984).

DNA amplification: Sequences of 13 decamer primers (*Molecular Medicine Unit*, London, UK) are given in Table 1. The reaction mixture (50 mm^3) comprised 50 ng template DNA, 1xTaq reaction buffer, 1.6 μM primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$ and 1.25 U Ampli*Taq* polymerase (*Perkin-Elmer*, Norwalk, USA). Enzyme was added after initial denaturation (hot start). Amplifications were performed in the *GeneAmp PCR System 9600* (*Perkin-Elmer*) programmed for a preliminary 5 min denaturation at 95 °C followed by 36 cycles of 20 s at 95 °C, 20 s at 35 °C and 1 min at 72 °C.

PAGE electrophoresis and silver staining: Electrophoresis was performed in 5 % polyacrylamide gel (acrylamide:bisacrylamide 17:1) containing 6 M urea. Silver staining procedure was based on method of Merrill *et al.* (1981) with some modifications: (*a*) the gels were fixed for 2 × 10 min with 5 % acetic acid + 10 % ethanol, (*b*) 2 cm^3 of 37 % formaldehyde were added to 1 dm^3 of silver solution, and (*c*) 1.6 mg of $Na_2SO_3.5H_2O$ per dm^3 was added to the developer. The stained gels were impregnated with 10 % glycerol and air dried between two sheets of wet clear cellulose membrane in a dark place.

Data analysis: Similarly to Demeke *et al.* (1992), the RAPD gels were visually scored by assigning numbers from 0 to 4 to each band as follows: 0 - absent, 1 - faint, 2 - clearly presented, 3 - strong, 4 - very strong band. The bands in the same position which differed by the scale factor of at least 2 were considered as polymorphic. Estimates of genetic similarity were calculated according to Nei and Li (1979). The resulting similarity matrix was used to evaluate the relationships among samples with a cluster analysis using unweighted pair group average method (UPGMA - *MVSP Plus* version *2.1* software, *Kovach Computing Services*, Anglesey, U.K.).

Results and discussion

Influence of reaction factors on RAPD banding pattern: There are ambiguous descriptions published about the influence of conditions on resulting banding pattern (Devos and Gale 1992, Munthali *et al.* 1992, Ellsworth *et al.* 1993, Sobral and Honeycutt 1993, Samec 1993). Therefore we tested effects of primer, magnesium ions, template and enzyme concentrations on amplification of pea DNA.

Primer concentration substantially affected the results of RAPD reactions. The originally used primer concentration 0.2 μM (Williams *et al.* 1990) did not work efficiently in our experiments. At this concentration only few larger sized products were detected. Increased primer concentration up to 3.2 μM caused an appearance of new bands in the banding profile, mainly in the low molecular mass range (Fig. 1). We have chosen the primer concentration of 1.6 μM as a standard, since it generated a sufficient number of bands. Recently the similar primer concentration has been recommended (Sobral and Honeycutt 1993).

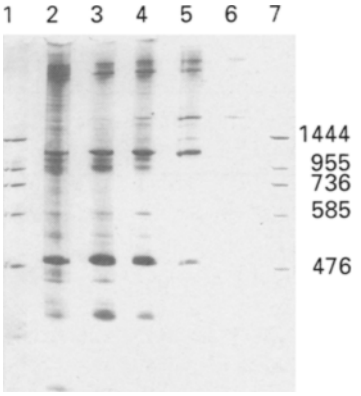

Fig. 1. Effect of decreased primer P2 concentration on amplification of DNA from cv.Bohatýr. 2 mm^3 of samples were loaded. Lanes 1 and 7 DNA size marker (pUC19/*Taq*I/*Sau*3A), lanes 2 - 7 primer P2 at concentrations 3.2, 1.6, 0.8, 0.4 and 0.2 μM, respectively.

Effect of magnesium was tested in the range of final concentrations from 0 to 4 mM. At least 1.5 mM Mg^{2+} was necessary for amplification. With increased magnesium concentration, some bands were gradually disappearing while, at the same time, some new bands appeared. We selected concentration of 2.5 mM Mg^{2+} as optimal for all primers used.

Intensity of bands increased with enzyme concentration from 10 to 50 U cm^{-3} but no new bands appeared. About 25 enzyme units per 1 cm^3 of reaction mixture was enough to amplify and detect a full-range pattern.

Optimal template concentration in RAPD is closely related to both DNA extraction method and genome size. Relatively small change in template concentration can substantially modify the resulting banding pattern (Devos and Gale 1992, Munthali *et al.* 1992). However, we observed no changes in the amplification

profiles over the range of 100 ng to 60 μg of DNA per 1 cm^3 of reaction mixture. Such stability of RAPD profile could be due to a highly purified DNA. Total failure of amplification occasionally occurred with the lowest template concentration. We therefore accepted the template concentration of 1 μg(DNA) cm^{-3}(reaction mixture) as optimal. This concentration, in combination with other optimized reaction components, yielded results of very good reproducibility.

Results of RAPD reactions: Altogether 13 oligonucleotides of arbitrary sequence were used as single primers to amplify genomic sequences from six pea cultivars. Individual primers produced from 0 to 44 amplification fragments with pea DNA template (Table 1). The length of amplification products varied from less than 200 to more than 3000 nucleotides. Each amplification profile consisted of small number (1 - 5) of strong bands and a variable number of low-intensity bands. Average number of amplification products in the profile was 26.1. Primer P18 failed to produce any amplification fragment.

Table 1. List of oligonucleotides utilized as random primers, their sequences and associated amplification fragments.

Primer	Sequence 5 - 3	Bands scored total	polymorphic
P1	AGT CAG CCA C	23	4
P2	GTG ATC GCA G	40	4
P7	ACG TAT CTG C	33	8
P8	TCG TCA CTG A	44	7
P9	TGC TCA CTG A	37	10
P10	TGG TCA CAG A	27	12
P11	CCG GCC GTC A	25	2
P12	ACC GCG AAG G	6	2
P13	GTG TGC CCC A	27	1
P15	AAT CGG GTC G	25	4
P16	TGC CGA GTC G	20	4
P17	CCG TAT ACA C	6	1
P18	GAG GCC TAT A	0	0
Total		313	59

Altogether 313 amplification fragments were generated and 59 of them were polymorphic. It means that one primer produced 4.5 polymorphism in average. This is more in comparison with previous reports. For example, the frequency of finding RAPD polymorphisms has been shown to be 0.3 per primer tested in *Arabidopsis thaliana*, 0.5 per primer in soybean, 1 per primer in maize and 2.5 per primer in *Neurospora* (Tingey and Tufo 1993). Our efficiency in polymorphism production was achieved probably due to a resolution power of urea/polyacrylamide gel, as well as the sensitivity of silver staining, which both increased total number of bands detected. Using agarose/ethidium bromide detection system, we detected only about 1/3 loci from the same amplification sample (not shown).

Most of the polymorphic products were shorter than 2 kb. The number of polymorphic fragments substantially varied with primer from 0 to 12 (Table 1). Primers P10, P9, P7 and P8 produced the greatest number of polymorphic bands and together comprised 62.7 % of total polymorphisms. Apparently, the nucleotide sequence of primer is responsible for suitability of primer for genome fingerprinting. The primers P8, P9 and P10 are closely related (70 % homology). Note that there is no similarity in the character of resulting banding profiles.

Most of the tested primers generated enough polymorphisms for clear identification of each of the six pea cultivars (Fig. 2).

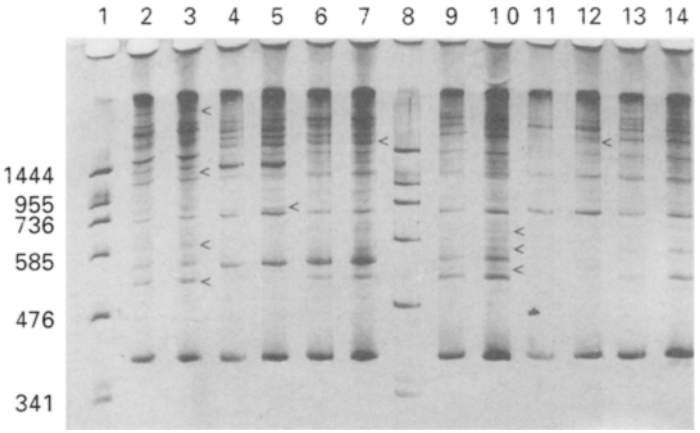

Fig. 2. Amplification of genomic DNA from 6 pea genotypes with primer P9. 1 and 3 mm^3 of each amplification mixture were loaded on 5 % polyacrylamide/6 M urea gel: Bohatýr (lanes 2, 3), Ina (lanes 4, 5), Tyla (lanes 6, 7), Trille (lanes 9, 10), Smaragd (lanes 11, 12), Solara (lanes 13, 14) and DNA marker pUC19/ *Taq*I/*Sau*3A (lanes 1, 8, 15). Polymorphisms are indicated by arrows.

Table 2. Similarity matrix for Nei and Li's coefficient: range of values from 0 to 1.0, with values closer to 1.0 indicating increasing similarity.

Cultivar	Bohatýr	Ina	Tyla	Trille	Smaragd	Solara
Bohatýr	1.000					
Ina	0.854	1.000				
Tyla	0.837	0.865	1.000			
Trille	0.857	0.836	0.863	1.000		
Smaragd	0.847	0.830	0.842	0.883	1.000	
Solara	0.873	0.825	0.847	0.876	0.892	1.000

Nei and Li's genetic similarity coefficient was used to generate a similarity matrix of 313 amplified DNA sequences (Table 2). Within the pea cultivars the proportion of shared bands (*i.e.* GS value as described in Materials and methods) ranged from 0.823 to 0.892. A cluster analysis (UPGMA) based on this similarity matrix was

performed to generate a dendrogram (Fig. 3). The dendrogram obtained by this procedure readily separated the genotypes into two main groups: Both *P. sativum* subsp. *arvense* cultivars were grouped into one cluster as closely related (similarity 0.865) and all *P. sativum* cultivars, again closely related with similarities from 0.847 to 0.892, fell to the second cluster (Fig. 3). The two main clusters differed with similarity 0.842. Such result fully corresponds with real systematic relationships between two cultivars of pea.

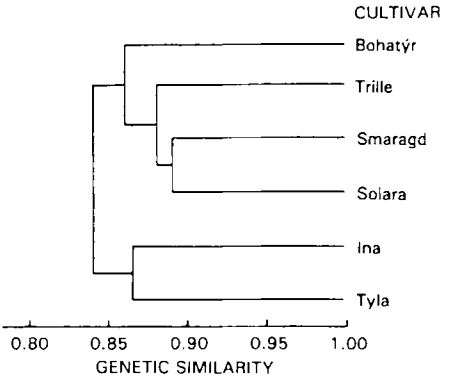

Fig. 3. Dendrogram of pea genotypes, constructed using UPGMA based on Nei and Li's similarity coefficients. Scale on bottom is Nei and Li's coefficient of genetic similarity.

This study shows that the RAPD technique can be very efficient in the production of DNA polymorphisms in pea cultivars. This is very important, because pea is an autogamous crop and phenotypic variability among cultivars is relatively low. The RAPD polymorphism can serve as a source of molecular markers for economic traits as well as for genetic mapping or cultivar identification.

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