Cultivar identification and relationships in *Pisum sativum* L. based on RAPD and isoenzymes

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Abstract

Two approaches were used to detect variability within 23 pea cultivars. Eight random amplified polymorphic DNA (RAPD) primers produced 163 bands (114 polymorphic), whereas five isoenzyme systems produced 25 bands (20 polymorphic). Both methods provided good results in finger-printing and identified all cultivars tested. Genetic similarity analysis (UPGMA) gave different results from RAPD and isoenzyme data. This could reflect different properties of both marker types. These data were also analysed using the bootstrap method, which supported the idea of one common progenitor of cultivated peas.

Additional key words: biochemical markers, genetic relationships, molecular markers, pea.

Introduction

Morphological and physiological characters (markers) linked to traits of interest have been used for different purposes in plant breeding and genetic research. Unfortunately, these characters have several disadvantages: in addition to their limited number, they are influenced both by the environment and the growth stage of the plant (Tanksley *et al.* 1989). Advances in biology during past few decades have provided breeders with powerful new tools - molecular markers which are largely free of the above mentioned disadvantages (Tanksley 1983).

One type of molecular markers is derived from variability of proteins, particularly storage proteins and isoenzymes. Protein markers have been used in crop characterization (*e.g.* Cooke 1984) and linkage mapping (Weeden *et al.* 1993). Many assays using protein variability have been established. Usually they do not require

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expensive equipment, are relatively simple to perform and the cost per sample is low. On the other hand, protein variability in some species is very low and thus only limited information can be obtained by this way (Gepts 1993)

In contrast, variability at the DNA level is several orders of magnitudes higher than in proteins. Almost all regions of DNA polymorphism are potentially useful as genetic markers. Among the types of DNA markers, *e.g.*, restriction fragment length polymorphism (RFLP) and microsatellites (for review see Paterson *et al.* 1991, Samec 1993), RAPD (Williams *et al.* 1990, 1993) have been frequently used in recent years (Samec 1993), due to the speed, efficiency, simplicity, and, if well established, reliability.

Pea (*Pisum sativum* L.) is one of the most important legumes. There are thousands of commercial cultivars (Gantotti and Kartha 1986) and a huge number of accessions in germplasm collections, all of which are useful as genetic resources for breeding. Detailed genetic characterization of these genotypes is desirable both for high efficiency of breeding strategies and for commercial seed production.

In peas, seed storage proteins (Hynes 1968, Thomson *et al.* 1978, Cooke 1983, Przybylska 1986, Šuška and Stejskal 1992), isoenzymes (Przybylska 1986, Swiecicki and Wolko 1987, Šuška 1993, Stejskal *et al.* 1996) as well as RAPD markers (Bagheri *et al.* 1995, Samec and Našinec 1995, 1996) have been useful for cultivar/genotype identification. Both types of molecular markers may also be used for pea phylogenetic studies (Przybylska 1986, Hoey *et al.* 1996) and for searching linkages to pathogen resistance genes in pea (Dirlewanger *et al.* 1992, 1994, Timmerman *et al.* 1994). The aim of this work was *1*) to use isoenzyme and RAPD markers for the exact identification of commercial pea cultivars, *2*) to compare sensitivity and efficiency of both assays, and *3*) to evaluate potential genetic/taxonomic relationships among pea accessions.

Materials and methods

Plants: The following 23 cultivars of *Pisum sativum* L. from *AGRITEC Pea Collection* were tested: *P. sativum* ssp. *sativum*: white flowers, smooth seeded, green cotyledons - cvs. Helka, Smaragd, Solara, Tyrkys; white flowers, smooth seeded, yellow cotyledons - cvs. Amino, Belman, Bohatýr, Jubilát, Junák, Odeon, Romeo, Schobi, Tolar; white flowers, wrinkle seeded, yellow cotyledons - cvs. Colt, Countess; white flowers, wrinkle seeded, green cotyledons - cvs. Citrina, Progreta, Vitiaz; *P. sativum* ssp. *arvense*: coloured flowers - cvs. Arvika, Dundale, Golf, Sirius, Tyla.

DNA isolation and RAPD reactions were performed as described in Samec and Našinec (1996). Briefly, 10 seedlings of each genotype were cut and mixed together. DNA was isolated (Dellaporta *et al.* 1983) from 1 g of tissue. For RAPD reactions 0.025 cm^3 of total volume contained 25 ng genomic DNA, 1.6 μM primer (for nucleotide sequences see Table 1), 100 μM of each dNTP, 2.5 mM $MgCl_2$ and 0.75 U Taq polymerase (*Stratagene*, La Jolla, UAS) in reaction buffer. DNA *Thermal*

Cycler 480 (*Perkin Elmer*, Foster City, USA) was programmed for an initial 5 min denaturation at 94 °C, followed by 40 cycles of 10 s at 95 °C, 1 min at 37 °C and 1.5 min at 72 °C with the fastest possible thermal transitions. Electrophoresis was performed in 1.5 % agarose gels containing 0.2 μg cm^3 of ethidium bromide.

Isoenzyme assay: Five isoenzyme systems, useful for pea identification (Stejskal *et al.* 1996) were used. Esterase - EST (E.C. 3.1.1.2.), acid phosphatase - ACP (E.C. 3.1.3.2.) and shikimate dehydrogenase - SDH (E.C. 1.1.1.25.) were analysed in the first and second true leaves of greenhouse-grown plants, whereas amylase - AMY (E.C. 3.2.1.1.) and leucine aminopeptidase - LAP (E.C. 3.4.11.1.) were analysed in seeds.

Leaf samples (0.5 g) were ground in liquid nitrogen, incubated at -20 °C in 0.75 cm^3 of extraction buffer (50 mM Tris-HCl, pH 7.4, 5 mM 2-mercaptoethanol, 1 mM EDTA) for 2 h and then centrifuged at 30 000 *g* (4 °C) for 10 min. The supernatant was mixed with equal volume of loading buffer (125 mM Tris-HCl, pH 6.8, 10 % 2-mercaptoethanol, 20 % glycerol, 0.02 % bromophenol blue) and stored at -20 °C until electrophoresis.

Seed samples were ground to fine powder in the *Cyclotec* laboratory mill (*Tecator*, Högenäs, Sweden). Seed flour was extracted immediately before analysis in the ratio 1:10 with loading buffer (62.5 mM Tris-HCl, pH 6.8, 5 % 2-mercaptoethanol, 11.6 % glycerol, 0.01 % bromophenol blue) for 30 min and then centrifuged at 15 000 *g* (4 °C) for 5 min.

Native polyacrylamide gels (175 × 80 × 1 mm) were prepared according to the *LKB 2050 Midget Electrophoresis Unit* laboratory manual (*LKB*, Bromma, Sweden) but modified by adding 10 % D-mannitol (running gel - 7.5 % T, 3 % C; stacking gel - 5 % T, 3 % C). Electrophoresis (15 - 20 mm^3 of samples) was performed on vertical unit *VE-2U* (*Laboratory Services*, Postřelmov, Czech Republic) with a constant current 0.34 mA × cm^2 with running buffer (anode - 0.075 M Tris, 0.75 M acetate, pH 6.4; kathode - with/without 0.02 M Tris, 0.2 M Tricin, pH 7.1). Detection of enzymatic activities was performed according to Vallejos (1983).

Data analysis: Data matrices were created from photographs of gels by assigning 1 to visible bands and 0 to absent bands. Estimates of genetic similarity were calculated according to Jaccard (1908). Both DNA and isoenzyme analyses were performed at least twice. The resulting data were processed by cluster analysis using the unweighted pair group average method (UPGMA) and plotted in the form of dendrograms using *MVSP Plus ver. 2.1* software (*Kovach Computing Services*, Anglesey, UK). The correlation between RAPD and isoenzyme similarity matrices was measured by *MXCOMP* command of *NTSYS* software (*Applied Biostatistics Inc.*, Ithaca, USA). The phylogenetic relationships were checked using bootstrap analysis (Felsenstein 1985): 100 bootstrap replications were evaluated by Wagner parsimony using *PHYLIP ver. 3.57c* software (distributed by Felsenstein, University of Washington).

Results and discussion

RAPD amplification: Selection of primers in RAPD assays seems to be a crucial problem. In our experience an extensive screening using a number of primers under standard conditions is better than complicated optimisation of conditions. The main criteria for primer selection is the reproducible amplification of banding patterns. Sharp bands, whose nucleotide lengths and relative intensities are convenient for simple detection on agarose gel are essential. We performed RAPD reactions using the protocol for routine identification in peas with eight selected primers (see Table 1; Samec and Našinec 1996).

Table 1. Results of RAPD amplification.

Primer	Sequence 5′ - 3′	Bands produced total/polymorphic	Number of subsets identified	Range of Jaccard's coefficients
OPW08	GAC TGC CTC T	28/24	23	0.950-0.530
OPW01	CTC AGT GTC C	23/18	23	0.941-0.582
P14	AGG GCG TAA G	22/17	23	0.952-0.694
0PW02	ACC CCG CCA A	22/11	20	1.000-0.731
P10	TGG TCA CAG A	19/15	22	1.000-0.436
P9	TGC TCA CTG A	19/12	21	1.000-0.622
OPW09	GTG ACC GAG T	16/10	21	1.000-0.673
OPW04	CAG AAG CGG A	14/7	12	1.000-0.750

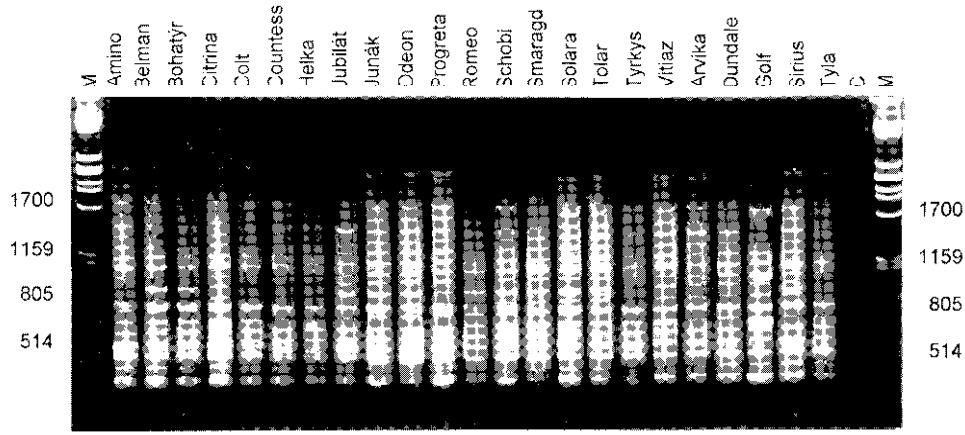

Fig. 1. Genomic DNAs of 23 *Pisum* sp. cultivars amplified with primer OPW08 and separated on 1.5 % agarose. Lanes M contain DNA digested with *PstI*. Lane C contains negative control (no genomic DNA was added to the amplification reaction).

Using 23 genomic templates, the individual primers produced 14 (primer OPW04) to 28 (primer OPW08) band positions (Table 1). Altogether, 163 band positions were detected, of which 114 were polymorphic (70 %). The average number of bands in the profile was 20.4. Nucleotide lengths of the amplification products varied from about 0.3 to more than 2 kb. The number of polymorphic fragments per primer ranged from 7 (primer OPW04) to 24 (primer OPW08, Fig. 1) with an average of 14.3 (Table 1). No RAPD fragment was exclusively present in one pea subspecies and absent in the others.

Based on data from all primers, the similarity matrix of Jaccard's coefficients was calculated (data not shown). The UPGMA procedure clustered genotypes into a dendrogram (Fig. 3*A*), in which all cultivars were clearly separated. The most related appeared to be pairs of cultivars Solara - Smaragd (similarity 0.877), Schobi - Romeo (0.872) and Tyrkys - Amino (0.858). Relatively low similarity coefficients indicate that the RAPD assay is suitable for cultivar identification.

UPGMA analysis did not divide genotypes into strictly separate clusters according to their botanical origin. However, 4 out of 5 cultivars *P. sativum* ssp. *arvense* were located in one part of the dendrogram and showed the lowest genetic similarity with the cultivars of *P. sativum* ssp. *sativum*. In contrast, cultivar Progreta (ssp. *sativum*) seemed more related to *P. sativum* ssp. *arvense* cultivars than to the other ssp. *sativum* genotypes (Fig. 3*A*).

Bootstrap analysis was performed to check the phylogenetic relationships among cultivars. Results of Wagner parsimony were processed into one consensus tree (not shown). The phylogenetic separation of cultivars into two subspecies was not confirmed. Confidence values were generally low (mostly below 20), indicating that relationships must be interpreted with caution. Similar results for peas were reported recently (Samec and Našinec 1996).

When parsimony analysis includes hybrid plant material, it usually contrasts to distance matrix methods, such as UPGMA (Felsenstein 1988, Caetano-Anollés *et al.* 1995). In our case, when higher confidence limits were set, the results of UPGMA and Wagner parsimony agreed with each other: Amino - Tyrkys (confidence 89.7), Smaragd - Solara (63.6) and Vitiaz - Citrina (57.8) (Fig. 3*A*).

Individual primers are not equally capable of producing DNA polymorphisms in pea cultivars (Table 1). The most useful primers were P14, OPW0l, and OPW08, which could distinguish between each tested cultivar in a single reaction. On the other hand, primer OPW04 can only distinguish 12 subsets of cultivars. Note that combinations of any two primers identified each genotype (data not shown). To achieve maximum reliability of cultivar identification, however, we recommend RAPD assay with at least 3 individual primers.

Isoenzymes: Five isoenzyme systems, proven useful for pea identification (Šuška 1993, Stejskal *et al.* 1996) were used. Altogether, 25 protein bands were detected (Table 2). Of those, 20 band positions (80 %) were polymorphic (Table 2). Similarity matrix of Jaccard's coefficients was created from isoenzyme data (data not shown). UPGMA analysis identified each of 23 cultivars as individual subsets with Jaccard's coeffcients ranging from 0.93 (Belman - Bohatýr) to 0.52. Three main clusters were

apparent (Fig. 3*B*), but no relation between botanical origin of cultivars and their location in the dendrogram was found. Cultivars of *P. sativum* ssp. *sativum* and *P. sativum* ssp. *arvense* are not separated in the dendrogram.

Table 2. Results of isoenzyme analysis.

Isoenzyme	Bands produced total/polymorphic	Number of subsets identified	Range of Jaccard's coefficients
EST	14/13	14	1.000-0.335
SDH	4/2	3	1.000-0.528
LAP	3/2	3	1.000-0.444
AMY	2/2	2	1.000-0.000
APH	2/1	3	1.000-0.000

Wagner parsimony (not shown) revealed relations consistent with UPGMA data in the following genotypic groups: a) Progreta - Countess - Vitiaz; b) Junák - Romeo - Solara - Smaragd - Schobi; c) Helka - Colt; and d) Odeon - Tyrkys. The remaining Wagner confidence values were very low (25-0.6) and no other consensus was apparent using UPGMA.

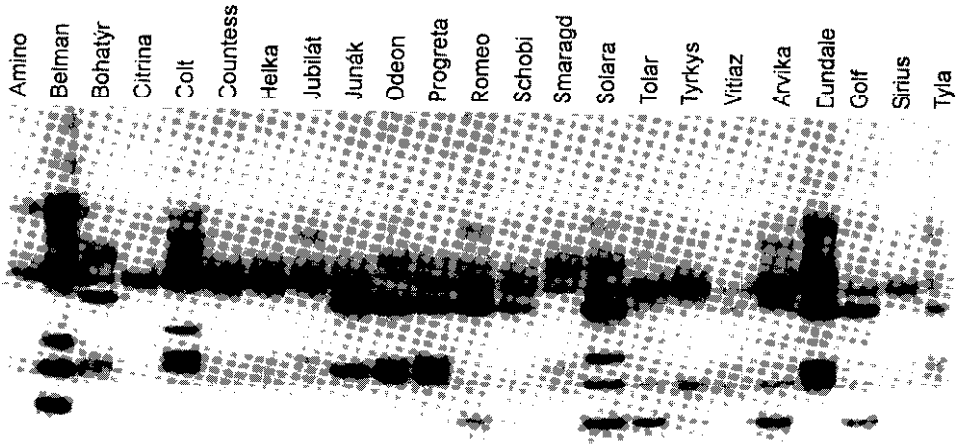

Fig. 2. Variability of leaf esterase among 23 pea cultivars.

Of the five isoenzymes, leaf esterase showed the highest variability: 13 out of 14 loci detected were polymorphic (Fig. 2). Esterase alone was able to distinguish 19 of 23 cultivars. Very little variability was apparent in the other isoenzymes, with only 1 or 2 polymorphic bands in the spectra (Table 2). Individual isoenzyme assays are therefore unable to unambiguously identify cultivars. When higher number of

isoenzyme systems are used, however, this method provides excellent results for fingerprinting.

Comparison of results and approaches: Both methods presented here clearly distinguish amongst all cultivars. RAPDs have the potential to produce informative DNA polymorphisms. This polymorphism is useful either for fingerprinting or as a source of genetic markers. Three primers (of 40 tested) produced high number of polymorphisms and were able to distinguish each cultivar in one reaction, while no isoenzyme system itself was able to do so. RAPD analysis is very rapid - complete results can be obtained within one day. If whole plant tissue is used directly in RAPD reactions, the procedure could be further accelerated (Klimyuk *et al.* 1993). It is also important for breeders, that small amounts of plant material (*e.g.* part of single seed) is sufficient for the analysis (Chunwongse *et al.* 1993). A disadvantage of RAPD analysis is the inconsistency, especially when analyses are performed in different laboratories. This problem can be solved by improving the methodology and by carefully standardising the conditions. Automatic reading and statistic evaluation of electrophoretic gels can substantially improve the results.

More isoenzymes are necessary to obtain sufficient protein variability for exact discrimination of pea cultivars. Isoenzymes as gene products may be modified or altered during plant development - thus sample preparation for leaf enzyme analysis must be highly standardised and also intracultivar variation must be thoroughly examined. Nevertheless, the reproducibility of our isoenzyme analyses was sufficient for routine cultivar identification.

Statistical analyses (Jaccard coefficient) revealed higher genetic diversity in isoenzymes than in RAPD (compare Fig. 3*A,B*). This probably reflects a higher proportion of polymorphic positions in isoenzymes (80 %) than in RAPDs (70 %). Evaluation of genetic relationships using UPGMA gave quite different results between RAPD and isoenzyme analyses (Fig. 3*A,B*). Better correspondence between the results can be obtained by increasing the number of isoenzyme systems studied. Thus, Chase *et al.* (1991) found good correlations between RFLP and isozyme markers in *Phaseolus vulgaris* and they were able to separate two genetically distinct groups differing in the composition of seed storage protein phaseolin. The individual dendrograms were not absolutely equal, but using a combination of DNA and isoenzyme data the authors were able to generate precise dendrogram.

Comparisons of RAPD and isoenzyme similarity coefficients using the MXCOMP command of NTSYS showed no correlation between matrices ($r = 0.073$). RAPD is probably less affected by the selection during breeding than isoenzymes, because it is focused on both the coding and noncoding regions of the genome. Nevertheless, RAPD analysis appears to be more consistent with the botanical classification (based on morphological traits) than the isoenzyme assay (Fig. 3*A,B*).

Neither assay strictly separated ssp. *sativum* and *arvense*. This suggests that cultured pea forms (both coloured and white flowering) originated early in domestication from the common wild primitive coloured progenitor *P. humile* (Ben Ze'ev and Zohary 1973, Hoey *et al.* 1996) and thus no strict phylogenetic separation could be expected within cultivated subspecies. In fact, recent European cultivars

mostly originate from one, relatively narrow gene pool with common parents in many cases (Angevin 1995). A relatively high degree of mutual intercrossing exists between ssp. *sativum* and *arvense* cultivars. Thus, only a small portion of *Pisum* variability is employed in registered cultivars (Gantotti and Kartha 1986). For phylogenetic studies the genotypes should be composed with knowledge of cultivar origin and both wild and primitive cultivars should be included (Hocy *et al.* 1996). The terms *sativum* and *arvense* should be considered as agronomic, or technological rather than botanical.

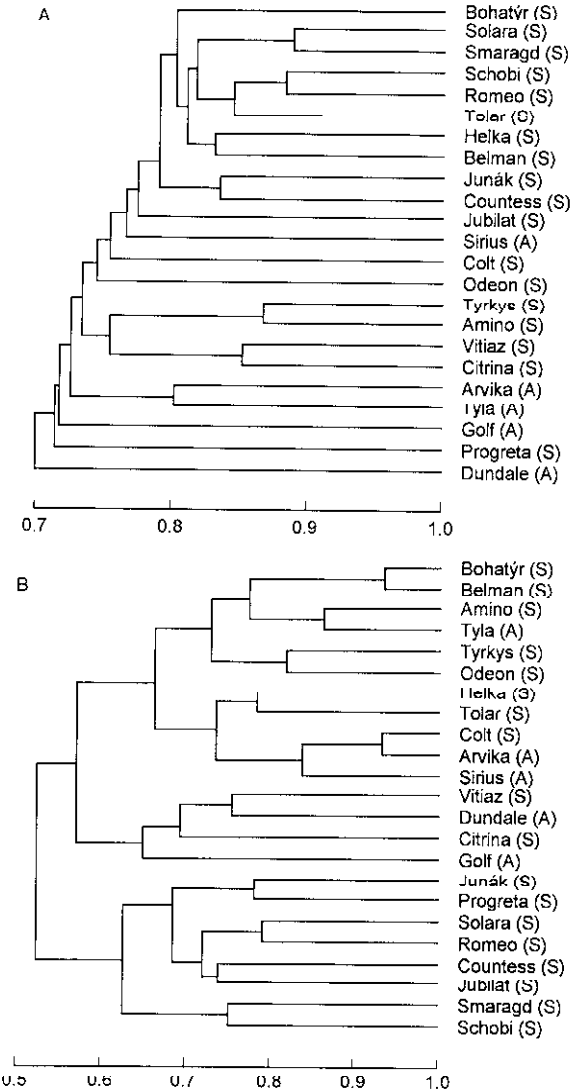

Fig. 3. Dendrograms based on Jaccard's coeffiients (S - cultivars of *P. sativum* ssp. *sativum*, A - cultivars of *P. sativum* ssp. *arvense*; *A* - dendrogram generated from RAPD data, *B* - dendrogram generated from isoenzyme data).

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