Potato Variety Identification by Molecular Markers Based on Retrotransposon Analyses

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Abstract: We analyzed a set of twenty most grown potato (*Solanum tuberosum* L.) varieties listed in the Czech Variety List using the PCR-IRAP (Inter-Retrotransposon Amplified Polymorphism) method in order to distinguish fast and unambiguously the varieties. In total, 62 polymorphic alleles were amplified using the three primers P-Tst-1, P-Tst-3 and P-Tst-6. The recorded pattern of markers was stable and reproducible. The analyses were repeated three times and identical results were always obtained. The level of polymorphism varied from 11% to 79% depending on the respective primer. All analysed varieties could be reliably distinguished after multivariate statistics have been applied to the data obtained by the PCO and UPGMA analyses. The best resolution of individual varieties was obtained if all three primers were evaluated as a complex. The use of retrotransposon-based markers appears to be suitable for the differentiation of large sets of potato samples and should be an eligible complement to other molecular markers used in potato variety identification such as Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphisms (AFLP).

Keywords: molecular markers; PCR- IRAP; *Solanum tuberosum*; variety identification

The identification of varieties of agricultural and horticultural crops is important during their breeding and registration process, seed production, trade and inspection. Currently, there are more than 4200 different potato varieties which are cultivated in over 100 countries worldwide (Hamester & Hils 2003). In 2007, there were 178 potato varieties listed in the official Czech Variety List (Čermák 2007). The traditional approach to variety identification is the observation and recording of morphological characters or descriptors. This approach is precise but time-consuming. Guidelines for potatoes, for instance, consist of 50 characters, out of which 12 deal with sprouting, along with a series of characters such as plant height, leaf size and various features of flowers and tubers. Such an approach is still used for official testing of Distinctness, Uniformity and Stability (DUS) as required for the grant of Plant Breeders Rights and official variety registration. However, it is less suitable when results are required rapidly, e.g. for the variety confirmation based on tuber material identification. Furthermore, morphological characters are often multigenic, not available at all growth stages and influenced by environment, making it difficult to assess them quickly and objectively, and requiring repeated observations. This traditional or phenotypic approach is not effective for large collections, especially for identification at the level of tubers (Cooke & Reeves 1998).

Molecular markers may serve as a modern and suitable approach to variety identification. This approach can also be more rapid and cost-effective. Different molecular marker techniques were used in potato population genetics and variety identification (Gebhardt *et al.* 1994), phylogenetic and biodiversity studies (Kardolus *et al*. 1997), analysis of recombination frequencies between

genotypes (Wiliams *et al*. 1993), identification of genes for important agricultural traits (Gebhardt *et al*. 1994) and marker assisted selection (Hamalainen *et al*. 1997). Molecular markers used in potatoes include Random Amplified Polymorphic DNA (RAPD) (Demeke *et al*. 1993; Karp *et al.* 1996; Lee *et al*. 1996; Sosinski & Douches 1996; McGregor *et al*. 2000), Amplified Fragment Length Polymorphism (AFLP) (Demeke *et al*. 1993; Hosaka *et al*. 1994; Provan *et al*. 1996; Milbourne *et al*. 1997; Van Der Voort *et al*. 1998; McGregor *et al*. 2000; Van Treuren *et al*. 2004), microsatellites – analyses of Simple Sequence Repeats (SSR) (Kawchuk *et al*. 1996; Provan *et al.* 1996; McGregor *et al*. 2000; Ghislain *et al*. 2004) or Inter-simple Sequence Repeats (ISSRs) (Albani & Wilkinson 1998; Prevost & Wilkinson 1999). Notwithstanding the prospect and advantages of the application of molecular markers in variety identification, the only legal DUS testing system is based on morphology and traditional phenotypic evaluation. The molecular approach must be further examined and stabilised, because variety identification is one of the most controversial and problematic issues of molecular marker application in breeding and official variety testing (Čurn & Žaludová 2007).

Retrotransposon-based markers are a novel group of molecular markers used for genotype description and identification, and they are also useful in potato breeding. Mobile genetic elements, retrotransposons, generally show widespread chromosomal dispersion, variable copy number and random distribution in the genome (Kumar *et al*. 1997; Kalendar *et al.* 1999). The dispersion (Katsiotis *et al*. 1996; Suoniemi *et al*. 1996), ubiquity (Flavell *et al*. 1992; Voytas *et al*. 1992) and prevalence (Pearce *et al*. 1996, 1997) of retrotransposon-like elements in plant genomes can be exploited for DNA-fingerprinting. The application of IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon Microsatellite Amplified Polymorphism) has been demonstrated to provide suitable polymorphic markers for variety identification or breeding purposes (Kalendar *et al*. 1999; Vicient *et al*. 2001), mapping of resistance genes in cereals (Manninen *et al*. 2000; Boyko *et al*. 2002) and gene diversity detection in potatoes (Lightbourn *et al.* 2007, Spooner *et al.* 2007). For all above-mentioned applications, specific species-derived retroelement LTR sequences had to be isolated, which fact represents a certain limitation of their use. Locus-specific RBIP (Retrotransposon-Based Insertion Polymorphism) approach was developed by Flavell *et al*. (1998) for the high throughput marker analysis of *Pisum* genotypes. Retrotransposons consist of two subclasses of elements, long-terminal repeat (LTR) and non-LTR retrotransposons, and the former are further subdivided into two groups – Ty1/*copia* type and Ty3/*gypsy* type (Xiong & Eickbusch 1990). Ty1/*copia* LTR retrotransposons contain open reading frames (ORFs) corresponding to retroviral *gag* and *pol* genes and have functional domains in *pol* ordered protease (PR), integrase (in), RNA-dependent DNA polymerase (RT) and RNase H (RH). In this context, LTR-retrotransposons possess unique properties that make them appropriate for investigating the relationship between closely related species and populations (Kumar & Hirochika 2001). LTR-retrotransposons appear to evolve at significantly higher rates than conventional nuclear loci (Purugganan & Wessler 1995). The genomic organisation and diversity of the *Ty1-copia* group retrotransposon have been investigated in several crop plants and their relatives from both dicotyledonous and monocotyledonous families, including *Solanum tuberosum*, *Vicia faba*, *Vicia melanops*, *Vicia sativa*, *Hordeum vulgare*, *Secale cereale* and *Allium cepa* (Kumar *et al*. 1997). Retrotransposon-based techniques were mainly used for genetic-diversity studies (Kalendar *et al.* 1999; Flavell *et al.* 1998; Porceddu *et al*. 2002; Vitte *et al*. 2004).

Alternatively, retrotransposons can be used in an AFLP-type reaction (Vos *et al*. 1995), called Sequence-Specific Amplified Polymorphism SSAP (Waugh *et al*. 1997). In the SSAP technique, selective bases added to primers reduced the complexity of the amplified DNA, depending on the copy number of retrotransposon targets.

The study was aimed to answer following questions:

(1) Are retrotransposon-based markers suitable markers for the differentiation of large sets of potato samples and do they provide a reproducible and stable pattern of markers?

(2) Are retrotransposon-based markers easy to use, without special requirements for DNA quality/quantity and are standard DNA isolation procedures sufficient?

(3) Are retrotransposon-based markers suitable to distinguish individual potato varieties and are

retrotransposon-based markers useful complements to SSR or AFLP markers?

MATERIALS AND METHODS

Plant material. The evaluated set of the twenty most grown potato varieties officially registered in the Czech Republic is listed in Table 1, together with their origin and EVIGEZ (Plant Genetic Resources Documentation in the Czech Republic) reference number. The plant material (tubers) was kindly supplied by the Czech Plant Variety Office (workplace Lípa u Havlíčkova Brodu). DNA was isolated from individual tubers, four samples from each variety, and all analyses were repeated three times to confirm the stability and reproducibility of analysed markers. DNA was extracted from a lyophilized tuber tissue by the modified CTAB DNA extraction method (Rogers & Bendich 1994) and polyvinylpyrrolidone (PVP-40000-360000, SIGMA, St. Louis, USA) was added to the extraction buffer (50 mg per sample).

IRAP analysis. For IRAP analyses, three primers P-Tst-1 (5-ATG ACT AAA TCT GCC TAC TCA TTC AAC A-3), P-Tst-3 (5-ACT AAA AAT CTG CCT ACT CAT TCA ACA CTC-3) and P-Tst-6 (5-ACT AAA TCT GCC TAC TCA TTC AAC ACT C-3) previously used by Bežo *et al.* (2006) and Hrubíková *et al.* (2006) were tested.

PCR conditions for analyses of all retrotransposon-based markers. The reaction was performed in a total reaction volume of 25 µl of the following composition: 10mM Tris-HCl, pH 8.3, 50mM KCl, 3mM $MgCl_2$, 200µM dNTPs, 1 U Taq DNA polymerase (TaKaRa, Shiga, Japan), 10pM primer (GIBCO, Carlsbad, USA) and 40 ng template DNA. After initial denaturation for 3 min at 94°C, thirty-five PCR cycles were performed, with 60 s of denaturation at 94°C, 60 s of annealing at 55°C, and 120 s of polymerisation at 72°C, followed by

Table 1. List of analysed potato varieties

Variety name	Number in gel or plots	Reference numbers of EVIGEZ databases	Origin (breeder)
Adéla	1	07S0101965	Selekta Pacov, a.s., Pacov, CZ
Adora	2	07S0101678	HZPC Holland B.V., Joure, NL
Agria	3	07S0101354	AGRICO B.A., Emmeloord, NL
Asterix	4	07S0101590	HZPC Holland B.V., Joure, NL
Colette	5	07S0101780	Kartoffelzucht Böhm KG Lüneburg, D
Dali	6	07S0101721	Kweekbedrijf Ropta-ZPC, Metslawier-St. Annaprochie, NL
Desirée	7	07S0100243	HZPC Holland B.V., Joure, NL
Ditta	8	07S0101601	Niederösterreichische Saatbaugenossenschaft reg. GmbH, Windigsteig, A
Filea	9	07S0101781	Nordkartoffel-Zuchtgesellschaft mbH, Lüneburg, D
Impala	10	07S0101538	AGRICO B.A., Emmeloord, NL
Karin	11	07S0101171	Sativa Keřkov, a.s., Přibyslav, CZ
Laura	12	07S0101917	EUROPLANT Pflanzenzucht GmbH., Lüneburg, D
Magda	13	07S0101979	Vesa Velhartice, a.s., Kolinec, CZ
Marabel	14	07S0101730	Kartoffelzucht Böhm KG Lüneburg, D
Rosara	15	07S0101670	SAKA-RAGIS Pflanzenzucht GbR Hamburg, D
Samantana	16	07S0101xxx	Selekta Pacov, a.s., Pacov, CZ
Santana	17	07S0102015	Handelmaatschappij VAN RIJN B.V., Poeldijk, NL
Secura	18	07S0101434	SAKA-RAGIS Pflanzenzucht GbR, Hamburg, D
Solara	19	07S0101583	Nordkartoffel-Zuchtgesellschaft mbH, Lüneburg, D
Velox	20	07S0101737	SAKA-RAGIS Pflanzenzucht GbR Hamburg, D

final elongation for 5 min at 72°C. PCR products were visualised by ethidium bromide staining after electrophoresis on 2% agarose gel (INVITROGEN, Carlsbad, USA) in 1 × TBE buffers, examples of the pattern are summarized in Figure 2. Bands were recorded using Epson Ultra Cam 3100Z Imaging System (EPSON Inc., Long Beach, USA).

Data analysis. Molecular data files were analysed by the UltraQuant 6.0 software (UltraLum, Claremont, USA) with manual correction. Fingerprint patterns were transformed into a binary character matrix with 1 for the presence or 0 for the absence of a band at a particular position in a lane. After removing monomorphic bands, genetic distance matrices were generated using Nei and Li (1979) and Gower's General Similarity matrix and cluster analysis (UPGMA – Unweighted Pair Group Method Averages) and PCO (Principal Coordinates Analysis) were performed. These statistical analyses were calculated using the MVSP (Kovach Comp. Serv., Pentraeth, UK) and STATISTICA 6.0 software package (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

The 20 potato varieties were analysed using 3 IRAP primers. Number of amplified bands, number of polymorphic bands per primer and variety, primer differentiation ability indices and similarity indices are given in Tables 2–3. The highest number of amplified and polymorphic bands was obtained using primer P-Tst-6. The highest number of bands (15) was amplified in the variety Velox with primer P-Tst-3. The average number of amplified bands was 10. Primer P-Tst-6 revealed the highest number of polymorphic bands and also analysis using this primer was the most informative for cluster analysis and PCO analyses, all cluster and PCO analyses are summarized in the Figure 3.

In all twenty approved varieties all analyses were performed on four individual tubers and

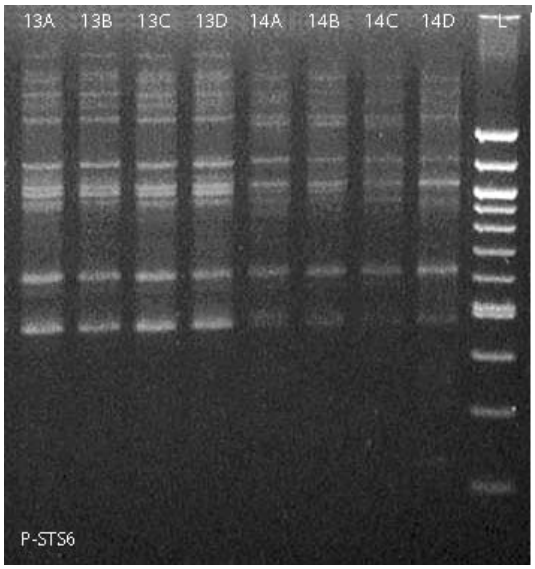

Figure 1. Stability of analysed retrotransposon-based markers; results of analysis with P-Tst-6 primer, 2% agarose gel, varieties 13 and 14 (replicates A-D represent individual tubers), L – 100 bp

the analyses were repeated three times. The aim of the study was to verify the assumed stability of retrotransposon-based markers and reproducibility of these markers. The number of amplified bands and the character of banding patterns were stable and identical in all analyses of particular genotypes as shown in Figure 1. On the basis of these results, we can positively answer the posed questions and suggest that IRAP technique generates stable and reproducible patterns of markers in potato. The stability of IRAP markers is conditioned and predetermined by optimal DNA quantity and quality. The generation of scorable and reproducible pattern of these markers seems to be strongly influenced by the quality of DNA template and by the DNA isolation method employed. The yield and purity of isolated DNA were a crucial problem. DNA was isolated from a lyophilised tuber tissue. The isolation of DNA from potato tuber juice or

Table 2. The number of amplified polymorphic bands in a set of 20 potato varieties

	Variety																			
Primer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Number of amplified polymorphic bands for particular primers																			
P-Tst-1	9	10	11	12	7	5	11	9	10	10	9	4	7	13	5	6	15	13	8	12
P-Tst-3	8	14	13	11	7	9	12	5	9	14	8	8	13	12	6	11	13	10	11	15
P-Tst-6	10	12	10	10	6	11	10	5	9	8	13	8	8	10	6	8	9	8	7	9

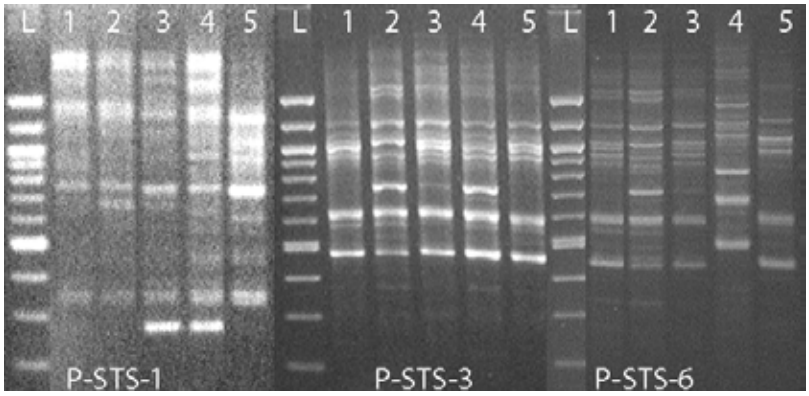

Figure 2. An example of electrophoregram – 2% agarose gel, varieties 1–5, L – 100bp, primers P-Tst-1, P-Tst-3, P-Tst-6

fresh tubers did not provide a sufficient amount (concentration) of DNA. Isolation procedures based on modified Rogers and Bendich (1994) CTAB protocol resulted in an incomparably higher yield of sufficiently pure DNA. DNA isolation using Invisorb Spin Plant Mini Kit (INVITEK, Hayvard, USA) afford maximum 25 ng DNA. By using of CTAB protocol we obtained at least 40 ng of DNA per analysed sample. An addition of PVP (40 000–360 000) to lysis buffer and replication of "chloroform steps" during DNA isolation further improved the quality of DNA template and did not negatively influence the cost and speed of DNA isolation. These results are in correspondence with findings published by Li *et al.* (2007).

In primer P-Tst-1, altogether 19 polymorphic bands were amplified, in primer P-Tst-3 20 bands and in primer P-Tst-6 23 bands were amplified (Table 3, Figure 2). All analysed samples were evaluated in two ways – based on particular single primer used in IRAP analysis and based on the combination of all three primers. Modern approaches of the digital image analysis of primary electrophoretic data combined with statistical evaluation were used. The position of particular bands was digitized, and binary data of the presence/absence of particular bands were assembled. Nei and Li (1979) and Gower's General Similarity Coefficients were calculated for the analysed pairs of varieties, both similarity indices provided comparable results, but ordinary analyses are feasible to be calculated only with GGSC (according to analytical software MVSP). Nei and Li similarity indices were in the range of 0%–86% in primer P-Tst-1, 13%–87% in P-Tst-3 and 11%–89% in P-Tst-6. The smallest recorded distance was between the varieties Asterix and Impala (11% in primer P-Tst-6), the largest recorded distance was between varieties Adora and Ditta, Adora and Impala, Adora and Magda (100% in primer P-Tst-1). Using this method allows the discrimination of the whole set of 20 varieties, the resolution power of analysed retrotransposon-based markers was higher than in microsatellites (Nováková *et al.* 2007) and differences between varieties allowed the reliable differentiation of all individual varieties. Grouping in all three individual primers and also in the complex analysis was slightly different, so there was no grouping pattern according to the recorded variety lineages. The large genetic distance of analysed varieties should be one of the explanations of this situation and the pattern of these results could be changed after the large-scale analysis of an extensive set of varieties. PCO analysis also gave similar results and no massive grouping of varieties was recorded, and analysed

Table 3. The number of amplified and polymorphic bands per particular primer in a set of 20 potato varieties

Primer	Total number of amplified bands	Number of amplified polymorphic bands	Percentage of polymorphic bands
P-Tst-1	21	19	90
P-Tst-3	22	20	90
P-Tst-6	23	23	100
P-Tst-1 + P-Tst-3 + P-Tst-6	66	62	94

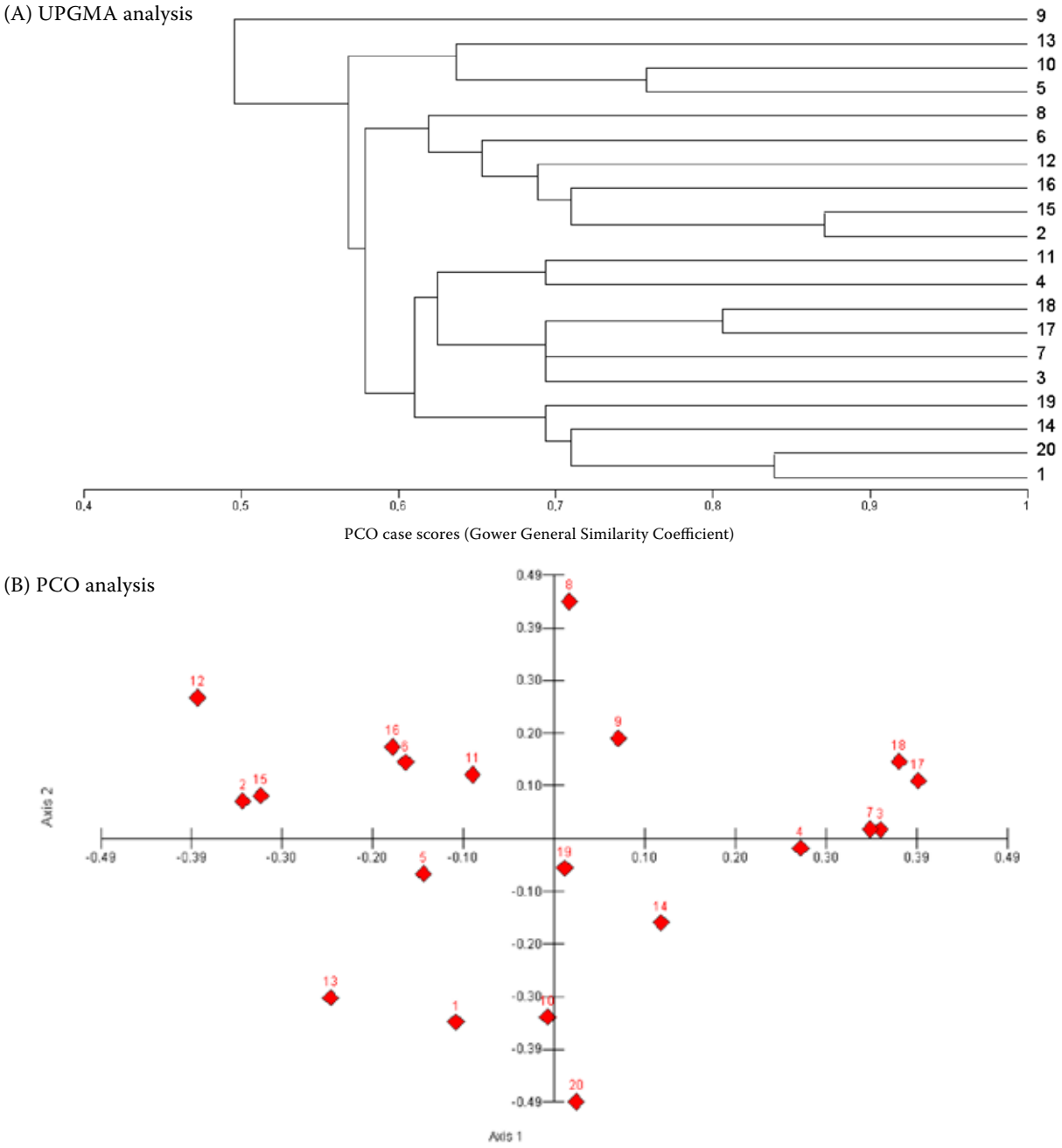

Figure 3. Results of cluster (UPGMA) and coordinate analysis (PCO analysis) obtained by IRAP marker analyses

varieties were also dispersed evenly across the ordinary diagram.

Comparable results were published by Bežo *et al.* (2006) in studies with a wide set of Slovak potato varieties. Žiarovská (2007) also reported the suitability of retrotransposon-based markers for discrimination of potato varieties on the basis of IRAP, REMAP and microsatellite markers. Hrubíková *et al.* (2006) published similar results for retrotransposon-based markers. In that study, they analysed 26 potato genotypes and IRAP gave 52 fragments with 80% polymorphism. Smýkal (2006) using IRAP, RBIP and SSRs markers, unequivocally identified 15 out of 33 pea varieties and the others made 9 cluster groups with 2–3 members in each. Antonius-Klemola *et al.* (2006) used a retrotransposon-based approach to the identification of apple varieties. They used nine primers for IRAP analyses when one primer produced from 6 to 15 informative fragments. Further, they found

that most IRAP bands from apple samples were not shared with Japanese quince. By combination of the set of nine IRAP primers, all standard apple varieties had unique profiles, whereas all sport mutations of a single variety gave identical patterns to their mother cultivars. In addition, they obtained the identical results from analyses carried out in two geographically distant laboratories. Nair *et al.* (2005) used IRAP markers for the genome classification of banana varieties from South India. They observed that the genomic DNA of the 36 varieties showed multiple polymorphic bands with *gypsi*-IRAP primer. A specific band of about 350 bp was observed in all the varieties with the B genome, moreover, they found that the intensity of this band increased in varieties with two B genomes.

Compared to RAPD or ISSR techniques (Zietkiewics *et al.* 1994; Pattanayak *et al.* 2002; Chakrabarti *et al.* 2006) widely used in potato genetic resources evaluation, description and variety identification by retrotransposon-based markers are stable, reproducible and provide a similar or even higher level of polymorphism. Because of low polymorphism, a larger set of microsatellite markers must be analyzed for the precise description and discrimination of particular genotypes as was reported by Kawchuk *et al.* (1996), as well as by Ashkenazi *et al.* (2001) and Ghislain *et al.* (2004). AFLP, a modern technique widely used in plant genotyping, has an application also in the field of potato variety identification and germplasm description (Van Treuren *et al.* 2004). Compared to retrotransposon-based markers, this approach is much more difficult (amount and purity of template DNA, complexity and cost of analyses, demands on a separation technique) and thus retrotransposon-based markers are a suitable marker system utilizable in potato variety identification.

New techniques based on DNA profiling provide novel approaches to variety identification which offer advantages over traditional morphological comparisons. Retrotransposon-based markers are a novel group of markers not yet widely used in potato breeding and potato variety identification. In this study we have analyzed a set of the twenty most grown potato varieties officially registered in the Czech Republic using the IRAP method. Altogether 62 polymorphic alleles (bands) were amplified using three primers and all three primers provided sufficient resolution power and allow the discrimination of all analysed varieties. The best resolution of the analysed set of varieties was achieved when all markers generated by all three primers were evaluated in a complex. IRAP markers were stable and reproducible during all analyses and no instability between tubers (Figure 1) and replicated analyses (data not shown) was recorded. This approach, utilization of retrotransposon-based markers, is suitable for the differentiation of large sets of potato samples and has also specific requirements (quality of DNA) and should be an eligible complement to other molecular markers used in potato variety identification (SSRs, AFLPs).

Acknowledgements. This study was supported by Ministry of Agriculture of the Czech Republic, Project No. 1B44011, by Czech Science Foundation, Grant No. 521/08/H042 and by Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6007665806.

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Received for publication March 17, 2008
Accepted after corrections March 3, 2009

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