UTILIZATION OF DNA MARKERS BASED ON MICROSATELLITE POLYMORPHISM FOR IDENTIFICATION OF POTATO VARIETIES CULTIVATED IN THE CZECH REPUBLIC
VYUŽITÍ DNA MARKERŮ ZALOŽENÝCH NA POLYMORFISMU MIKROSATELITŮ PRO IDENTIFIKACI ODRŮD BRAMBOR PĚSTOVANÝCH V ČR

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ABSTRACT

In the year 2007, there were one hundred and seventy-eight potato varieties enlisted in the Czech list of registered potato varieties. The classical morphometric approach to characterization is not effective for such a number of varieties especially for identification at the level of tubers. The needfulness of variety identification at the level of tubers is important mainly for trade aspect. The Czech law no.110/1997 Sb. about the food-stuff and tobacco products and the consequential ordinance (MZe č. 332 / 1997 Sb.) require guarantee of variety declaration in commercial relation for table potato.

In this study we analyzed twenty potato varieties (Solanum tuberosum L.) cultivated in the Czech Republic. Every variety was represented by four independent replicates. This set of samples was analyzed by methods of PCR-SSR (Simple Sequence Repeats) and PCR-ISSR (Inter Simple Sequence Repeats). We discovered that both of tested methods afford sufficient polymorphism for variety identification, but the method of PCR-ISSR is not utilizable, because we observed the variability within variety. For outright identification of the whole set of potato varieties cultivated in the Czech Republic we recommend to use SSR, AFLP and retrotransposene-based markers as well as morphological markers.

KEYWORDS: PCR-ISSR, PCR-SSR, Solanum tuberosum L., variety identification

ABSTRAKT

V současné době je v České republice registrováno 178 odrůd brambor (stav roku 2007). Klasická morfometrická charakterizace odrůd přestává být v tomto objemu registrovaných odrůd účinná, obzvláště na úrovni hlíz. Potřeba identifikovat konkrétní odrůdu na úrovni hlíz je přitom nejdůležitější, hlavně z obchodního hlediska. Platný zákon č. 110 / 1997 Sb. o potravinách a tabákových výrobcích a vyhláška na něj navazující (Vyhláška MZe č. 332 / 1997 Sb.) vyžadují u konzumních brambor garanci odrůdové deklarace při obchodním styku.

Pro studii bylo vybráno 20 odrůd brambor (Solanum tuberosum L.) pěstovaných v ČR. Každá odrůda byla zastoupena čtyřmi nezávislými opakováními. Tento soubor byl analyzován metodami SSR (Simple Sequence Repeats) a ISSR (Inter Simple Sequence Repeats). Zjistili jsme, ze obě analýzy založené na polymorfismu mikrosatelitů poskytují dostatečnou variabilitu pro identifikaci odrůd, ale metoda ISSR se nejeví jako vhodná z důvodu zjištění její nestability. Pro jednoznačnou identifikaci celého spektra odrůd brambor pěstovaných v ČR doporučujeme sestavení setu markerů, který by zahrnoval více markerovacích systémů morfologických i molekulárních (SSR, AFLP a markery založené na retrotranspozonech).

KLÍČOVÁ SLOVA: identifikace odrůd, PCR-ISSR, PCR-SSR, Solanum tuberosum L.

DETAILED ABSTRACT

Kulturní brambor (Solanum tuberosum L.) je celosvětově jednou z nejdůležitějších plodin. Identifikace jednotlivých odrůd je důležitá ve všech stádiích produkce brambor, během šlechtění, procesu registrace, produkce sadby a testování. Tradičním přístupem pro identifikaci odrůd brambor je porovnávání morfologických charakteristik a znaků. Avšak morfologické charakteristiky jsou často založeny multigeně, mají průběžnou expresi a jsou ovlivňovány faktory prostředí; to vše činí z morfologických znaků obtížně opakovatelnou metodu především pro rychlé, přesné, objektivní a opakovatelné závěry.

V molekulární biologii byly vyvinuty nové techniky molekulárních a biochemických markerů. Tyto techniky se stávají užitečným nástrojem pro určení genetické vzdálenosti a umožňují charakterizaci jednotlivých genotypů.

Užití molekulárních markerů pro zlepšování odrůd zemědělských plodin bylo plošně aplikováno v poslední dekádě, kdy byly molekulární markery aplikovány pro přesnou identifikaci genetické variability založené na analýzách DNA. To je důležité, protože detekce žádaných znaků není takto ovlivněna faktory prostředí.

Z celkového spektra 178 odrůd brambor pěstovaných v ČR bylo vybráno dvacet odrůd a ty byly analyzovány metodami PCR-ISSR (5 primerů) a PCR-SSR (STM1102, STM2005, STWIN12G, STM3012, STM1106, STM3015).

Výsledky získané analýzou mikrosatelitů byly transformovány do binární matice a po eliminaci monomorfních pruhů byla hodnocena genetická vzdálenost pomocí klastrové analýzy (UPGMA - Unweighted Pair Group Method Averages) a koordinační analýzy PCO (Principal Coordinates Analysis) v programu MVSP (Kovach Comp.Serv.) a STATISTICA 6.0 (Statsoft).

Metoda PCR-SSR se ukázala jako vhodná pro identifikaci odrůd brambor. Statisticky bylo hodnoceno 15 ze 17 možných pozic pruhů. Podařilo se nám odlišit osmnáct z dvaceti sledovaných odrůd (odrůdy Colette (2) a Impala (3) nelze jednoznačně odlišit). Naopak metoda PCR-ISSR se jeví jako nevhodná i přes četný poskytovaný polymorfismus amplifikovaného spektra pruhů, kdy bylo hodnoceno všech 216 možných pozic pruhů, neboť jsme zjistili polymorfismus i uvnitř odrůd.

Na souboru dvaceti odrůd registrovaných v ČR jsme získali fingrprint pomocí šesti SSR a pěti ISSR markerů. Metoda SSR analýzy se ukázala jako vhodná pro potřeby identifikace odrůd a naopak metoda ISSR analýzy, ač poskytuje vyšší polymorfismus, pro účely identifikace odrůd vhodná není, neboť vykazuje variabilitu i v rámci jednotlivých odrůd obdobně jako matoda RAPD analýzy.

INTRODUCTION

Cultivated potato (Solanum tuberosum L.) is one of the most important crops worldwide. Potato is an important food crop, it is widely used for livestock feeding, as well as for industrial processing as feedstock for many industrial and food applications. Currently, there are more than 3,200 different potato varieties that are cultivated in over 100 countries worldwide [13].

The identification of individual varieties is important at every stage of their agri-production, during their breeding, registration, seed-production, and testing processes [11]. The traditional approach to variety identification is composed of the observation and the recording of morphological characters or descriptors. The number of useful descriptors is limited in some species. Guidelines for potatoes consist of 50 characters, 12 of which are concerned with sprouting, along with a series of characters such as plant height, leaf size and various features of the flowers and tubers. Such an approach is undoubtedly successful in the process of Distinctness, Uniformity and Stability (DUS) testing. However, it is less suitable when results are required rapidly, such as for the confirmation of tuber material identification. Furthermore, morphological characters are often multigenic, continuously expressed and influenced by environmental interactions, making it difficult to assess them quickly and objectively, and requiring replication of observation [20].

DUS testing would benefit from the use of molecular markers that have been shown to be more rapid and cost-effective, and some of them have been used to assess genetic diversity in potatoes. Molecular markers in general can also be used as potential techniques for variety identification. Together with advances in molecular biology, several new molecular and biochemical marker techniques will be adopted. These techniques are a powerful tool for determining genetic distinctness and enable characterization of particular genotypes. The first groups of these approaches are techniques based on protein polymorphism. But these techniques have several disadvantages; in addition to the basic disadvantages of storage protein and isozyme analysis, they are available to marker only a limited number of genes (and potential traits), their genome coverage is low, and they are strongly influenced by plant ontogenetic stage and environmental conditions [6]. The second group of markers is based on DNA polymorphism. Restriction fragment length polymorphism (RFLP) analysis, historically the first

molecular marker system, has been shown to be a valuable tool for detecting patterns of DNA polymorphism among and within Solanum species and for potato variety identification [9]. However, this procedure is laborious, expensive, only a few loci are detected per assay and automation is difficult. The recent DNA marker systems are based on PCR technology, and for this reason are more suitable for routine cultivar identification, due to the small amount of DNA required, and generally fast and simple tests. Several methods were recommended for potato variety identification. These methods include Random Amplified Polymorphic DNA (RAPD) [5, 16, 18, 21, 27], Amplified Fragment Length Polymorphism (AFLP) [5, 14, 21, 22, 25, 29, 30], microsatellites – analyses of Simple Sequence Repeats (SSR) [10, 17, 21, 25] or Inter-simple Sequence Repeats (ISSRs) [1, 24] and in recent period also analysis of retrotranspozones – Inter-Retrotransposon Amplified Polymorphism (IRAP), Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) and Retrotransposon-Based Insertional Polymorphism (RBIP) techniques [2, 3, 7, 8, 12, 15, 19, 23, 31, 32, 33].

MATERIALS AND METHODS

Plant material. We used set of twenty registered potato varieties: Adora (1), Anosta (7), Cicero (8), Cinja (9), Colette (2), Desirée (17), Ditta (Lenka) (15), Impala (3), Javor (18), Karin (10), Komtesa (4), Korneta (11), Kuras (19), Magda (5), Marabel (12), Pacov (20), Provento (16), Rosara (6), Secura (13),Vineta (14). DNA was extracted by commercial kit Invisorb Spin Plant Mini Kit (INVITEK) from potato tuber juice [28].

PCR-SSR analyses. For PCR-SSR, six primer pairs were selected: STM1102 (5´- GGA AGA ATT TTG TAG GTT CAA – 3´, 5´- AAA GTG AAA CTT CCT AGC ATG – 3´), STM2005 (5´- TTT AAG TTC TCA GTT CTG CAG GG – 3´, 5´- GTC ATA ACC TTT ACC ATT GCT GGG – 3´) [22], STWIN12G (5´- TGT TGA TTG TGG TGA TAA – 3´, 5´- TGT TGG ACG TGA CTT GTA – 3´) [25], STM3012 (5´- CAA CTC AAA CCA GAA GGC AAA – 3´, 5´- GAG AAA TGG GCA CAA AAA ACA – 3´), STM1106 (5´- TCC AGC TGA TTG GTT AGG TTG – 3´, 5´- ATG CGA ATC TAC TCG TCA TGG – 3´), STM3015 (5´- AGC AAT AAA GTC AAC ACT CCA TCA – 3´, 5´- AAT GAA TTA GGG GGA GGT GTG – 3´) [10].

PCR condition: Reaction was performed in total reaction volume 25 µl of following composition: 75 mM Tris-HCl, pH 8.8, 20mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM DTP, 200 µM dGTP, 200 µM dTTP, 2.5 U Taq purple DNA polymerase, 10 pM primer and 25 ng template DNA. Altogether 35 PCR cycles run under the following condition: 30 s denaturation at 94˚C, 30 s annealing (according the primer*), 30 s elongation at 72˚C, initial denaturation for 3 minutes at 94˚C and final elongation for 5 minutes at 72˚C. PCR products were visualized by ethidium bromide after the electrophoresis in a 3% Synergel/agarose gel in TBE buffer.

PCR-ISSR analyses. For PCR-ISSR five primers were selected: P1 ($(AC)_8$G: 5´- ACA CAC ACA CAC ACA CG – 3´), P2 ($(AG)_8$YT: AGA GAG AGA GAG AGA GYT), P3 ($(GA)_8$YC: 5´- GAG AGA GAG AGA GAG AYC – 3´), P4 ($(AC)_8$YG: 5´- ACA CAC ACA CAC ACA CYG – 3´) (Y = C or T) [24] and B1 ($(CA)_6$GT: 5´- CAC ACA CAC ACA GT – 3´) [2].

PCR condition: Reaction volume was 25 µl, PCR was performed in 75 mM Tris-HCl, pH 8.8, 20mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 µM dATP, 200 µM DTP, 200 µM dGTP, 200 µM dTTP, 2.5 U Taq purple DNA polymerase, 10 pM primer and 25 ng template DNA. Altogether 40 PCR cycles run over under the following condition: 60 s denaturation at 94˚C, 60 s annealing at 55˚C, 2 min elongation at 72˚C, initial denaturation 2 minutes at 94˚C and final elongation 7 minutes at 72˚C. PCR products were visualized by ethidium bromide after the electrophoresis in a 2% agarose gel in TBE buffer.

RESULTS AND DISCUSSION

Microsatellite fingerprint patterns were transformed into a binary character matrix with 1 for presence or 0 for absence of a band at a particular position in a lane. After removing monomorphic bands, genetic distance matrices were generated using Gower General Similarity metrics and Cluster analysis (UPGMA – Unweighted Pair Group Method Averages) and PCO (Principal Coordinates

Primer pair	Annealing temperature (*)
STM1102	55˚C
STM2005	54˚C
STM3012	57˚C
STM1106	57˚C
STWIN12G	54˚C
STM3015	57˚C

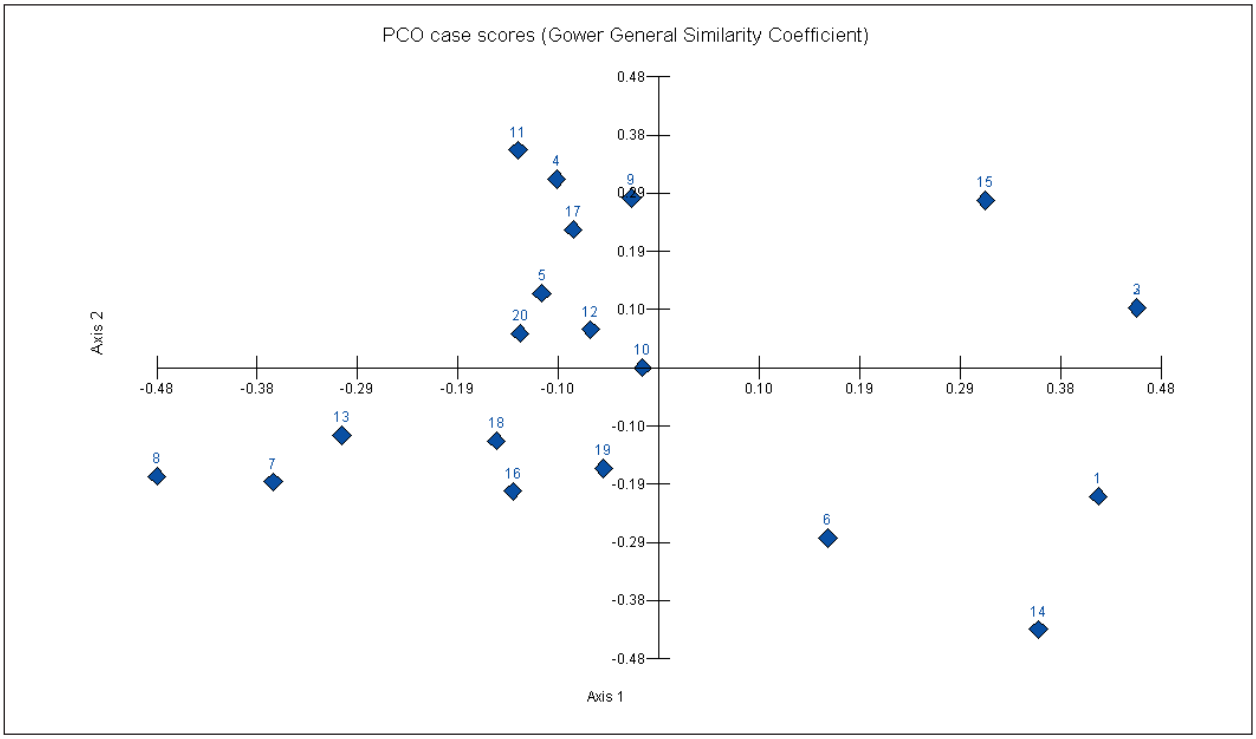

Fig.1. Results of coordinate analyses – PCO analysis obtained by analyses of six SSR markers.
Obr.1. Výsledky ordinační analýzy – PCO získané analýzou šesti lokusů SSR.

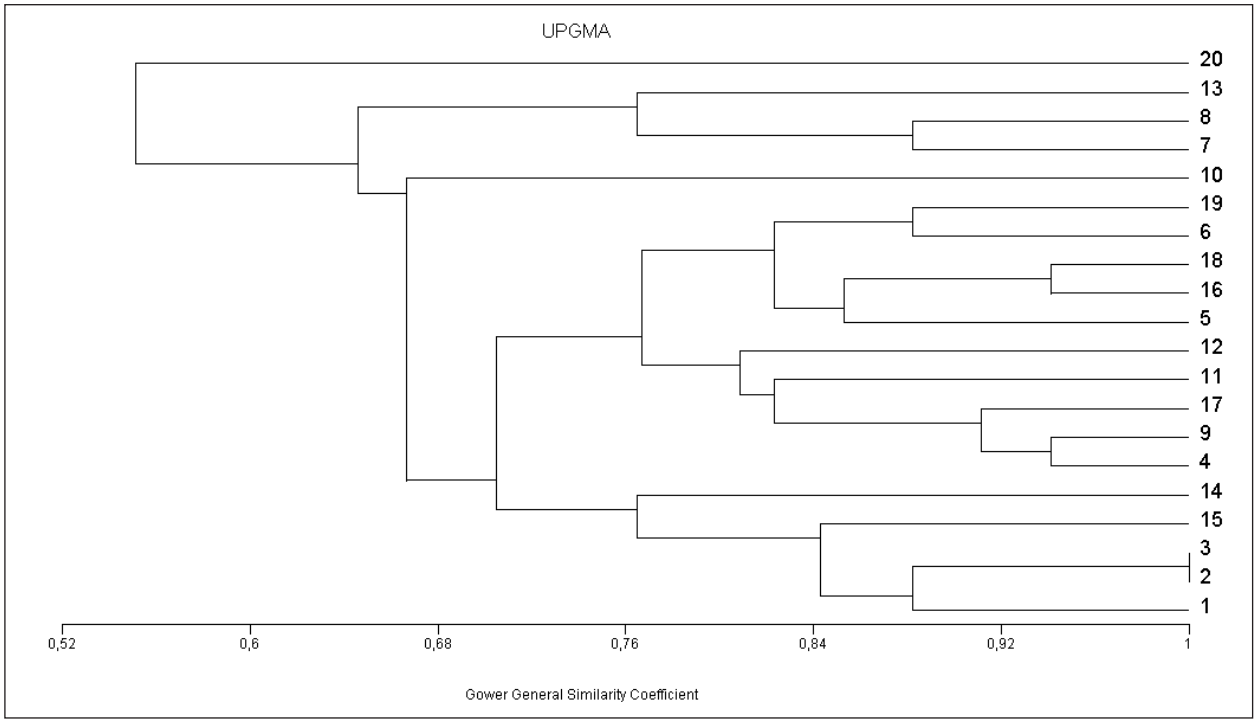

Fig.2. Dendrogram based on results of cluster analysis obtained by analyses of six SSR markers.
Obr.2. Dendrogram sestavený na základě výsledků klastrové analýzy získané analýzou 6 SSR lokusů.

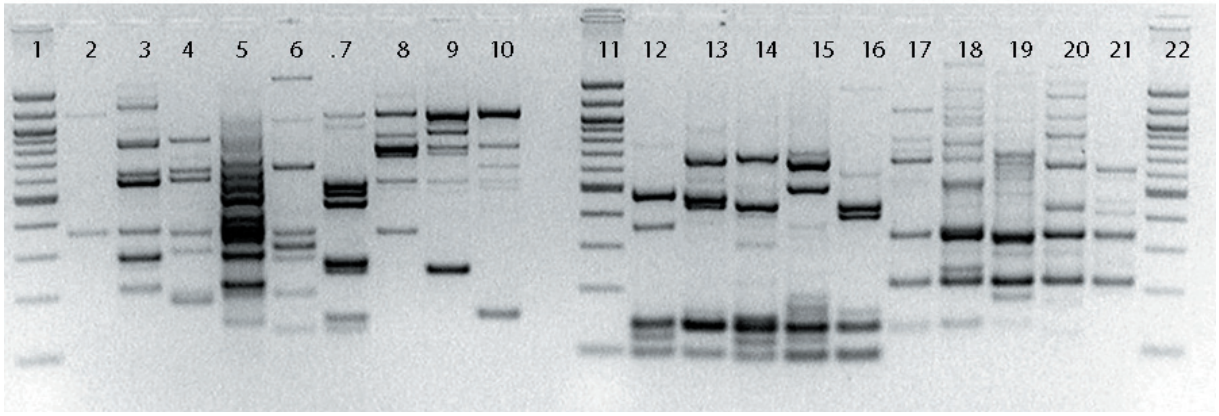

Fig.3. Example of electrophoreogram - 2% agarose gel. ISSR analysis. Variety Adora tubers A-D, 2-5 primer P1, 6-9 primer P2, 10, and 12,13 primer P3, 14-17 primer P4 and 18-21 primer B1, 1,11 and 22 DNA ladder marker 100bp.
Obr.3. Ukázka elektroforeogramu - 2% agarosový gel. ISSR analýza. Odrůda Adora hlízy A-D, 2-5 primer P1, 6-9 primer P2, 10, a 12,13 primer P3, 14-17 primer P4 a 18-21 primer B1, 1,11 a 22 DNA hmotnostní marker 100bp.

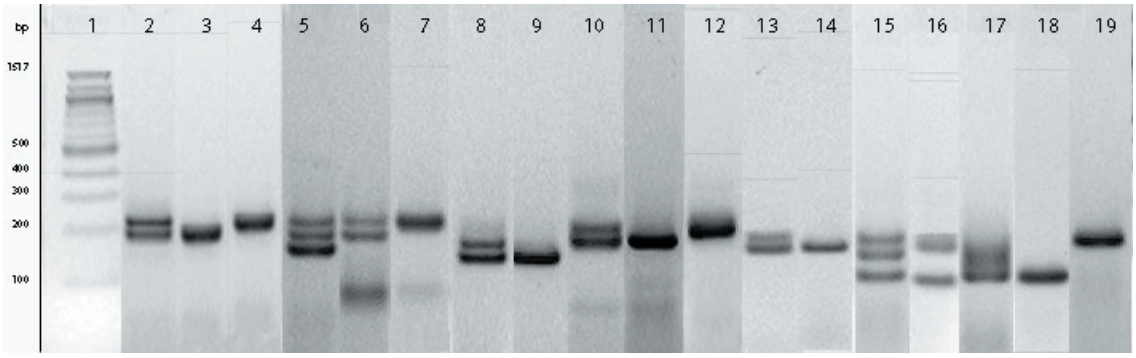

Fig.4. Example of electrophoreogram - 3% Synergel/agarose gel. SSR analysis. 1 DNA ladder marker 100bp, 2 – 4 primers STM1102, 2 (varieties 1, 6, 7, 8, 10, 12, 13, 16, 18, 19), 3 (varieties 2, 3, 4, 5, 9, 11, 15, 17), 4 (varieties 14, 20), 5 – 7 primers STM2005, 5 (variety 13), 6 (varieties 1, 2, 3, 4, 5, 7, 8, 9, 10, 17, 18, 19), 7 (varieties 6, 11, 12, 14, 15, 16, 20), 8 and 9 primers STM3012, 8 (varieties 1, 2, 3, 4, 9, 10, 12, 13, 15, 17, 20), 9 (varieties 5, 6, 7, 8, 11, 14, 16, 18, 19), 10-12 primers STM1106, 10 (variety 11), 11 (varieties 7, 8, 13, 20), 12 (varieties 1, 2, 3, 4, 5, 6, 9, 10, 12, 14, 15, 16, 17, 18, 19), 13 and 14 primers STWIN12G, 13 (varieties 1, 14, 16, 17, 20), 14 (varieties 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 18, 19), 15 – 19 primers STM3015, 15 (varieties 1, 2, 3, 14, 15), 16 (varieties 5, 7, 9, 11, 12, 13, 16, 18), 17 (variety 6), 18 (varieties 4, 8, 10, 17, 19), 19 (variety 20).
Obr.4. Ukázka elektroforeogramu - 3% Synergel/agarosový gel. SSR analýza. 1 DNA hmotnostní marker 100bp, 2 – 4 primery STM1102, 2 (odrůdy 1, 6, 7, 8, 10, 12, 13, 16, 18, 19), 3 (odrůdy 2, 3, 4, 5, 9, 11, 15, 17), 4 (odrůdy 14, 20), 5 – 7 primery STM2005, 5 (odrůda 13), 6 (odrůdy 1, 2, 3, 4, 5, 7, 8, 9, 10, 17, 18, 19), 7 (odrůdy 6, 11, 12, 14, 15, 16, 20), 8 a 9 primery STM3012, 8 (odrůdy 1, 2, 3, 4, 9, 10, 12, 13, 15, 17, 20), 9 (odrůdy 5, 6, 7, 8, 11, 14, 16, 18, 19), 10-12 primery STM1106, 10 (odrůda 11), 11 (odrůdy 7, 8, 13, 20), 12 (odrůdy 1, 2, 3, 4, 5, 6, 9, 10, 12, 14, 15, 16, 17, 18, 19), 13 a 14 primery STWIN12G, 13 (odrůdy 1, 14, 16, 17, 20), 14 (odrůdy 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 18, 19), 15 – 19 primery STM3015, 15 (odrůdy 1, 2, 3, 14, 15), 16 (odrůdy 5, 7, 9, 11, 12, 13, 16, 18), 17 (odrůda 6), 18 (odrůdy 4, 8, 10, 17, 19), 19 (odrůda 20).

Analysis) were performed. These statistical analyses were calculated using MVSP (Kovach Comp.Serv.) and STATISTICA 6.0 software package (Statsoft).

SSRs analyses. Polymorphism of SSR markers was observed for 20 selected varieties after amplification with six primer pairs. Statistical evaluation appears from matrix of presence polymorphic bands. There were evaluated 15 from 17 possible positions of bands. Microsatellite analysis allows to distinguish and identify 18 from total set of 20 varieties (Colette (2) and Impala (3) can not be distinguished) (Fig.1, Fig.2, Fig.4). Analogous results were recorded by other authors, e.g. Schneider and Douches [26] unambiguously distinguished 24 from 40 potato varieties by the usage of 6 SSR primer pairs. McGregor et al. [21] reliably identified 20 from 39 potato varieties by the usage of 5 SSR primer pairs.

ISSRs analyses. Polymorphism of ISSR markers (Inter Simple Sequence Repeats) was observed for 20 selected varieties after amplification with five primers. Statistical evaluation appears from matrix of presence polymorphic bands. There were evaluated all 216 possible positions of bands. Using of this method permits discrimination of each variety. The similarity between varieties was 65 – 80%. But we gather that this method is not utilizable, because we observed the variability within of variety (Fig.3). Although Prevost a Wilkinson [24] published the results where they staunchly determined all 34 potato varieties by usage of four ISSR primers and McGregor et al. [21] reliably identified all 39 potato varieties by usage of six ISSR primers we cannot recommend this method for variety identification not due to its resolving power but due to low stability and repeatability of ISSR technique.

CONCLUSION

We obtained pattern of six SSR and five ISSR markers for the set of twenty selected varieties registered and cultivated in the Czech Republic in the year 2007. Recorded polymorphism was appraised and the varieties were separated to the categories by the fingerprint data. The method of SSR analysis is suitable for evaluation of variability and for the purposes of variety identification. On the other hand ISSR method conveys plentiful polymorphism but the disadvantage is a polymorphism within variety and we observed also the instability of the pattern depending on the age of DNA, likewise RAPD analyses [4].

For outright identification of whole range of potato varieties cultivated in the Czech Republic we recommend to use the set of molecular and morphological markers in accordance with Ghislain et al. [10] and Bežo et al. [2].

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