Phenotype and molecular diversity evaluation of some wild 2n Solanum species (super series Rotata)

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New cultivars are result of the conservation and characterization of potato (Solanum) genetic resources in secondary germplasm banks. The objectives of this study were to assess phenotype diversity of 12 clones of 10 wild diploid potato species collection super series Rotata, and to determine their genetic diversity through simple sequence repeat (SSR) markers. Totally 63 alleles of 20 cpSSR loci were detected i.e. 3.15 alleles on average per one microsatellite locus. Alleles ranged from two to six per locus. The highest polymorphism was detected in the locus ntcp9 and lowest were recorded having by two alleles in seven of loci. The average value of observed heterozygosity (Ho) was 0.61, whereas the mean of polymorphic information contents (PIC) was 0.49. Intergenic regions had highest variability (H igr = 0.65) compare with introns (Hin = 0.54) and exons (H ex = 0.45) of the chloroplast genome. Molecular analyses were complemented with tuft morphological measurements according to the descriptor list for the genus Solanum. SSR-based markers highlight a tendency to separate two groups of Rotata wild diploids and show the possibility of duplicities of wild potato genetic resources in the current Czech in vitro collection.

Key words: Heterozygozity, microsatellite, phenotype description, PIC.

INTRODUCTION

Central and South America are centers of origin and diversity of wild tuber-bearing Solanum species, and hence the primary sources of genes for disease and pest resistances lacking in modern cultivars (Bradshaw et al., 2006). The evolutionary diversity of the wild species and the comparatively narrow genetic basis of the cultivated potato make Solanum species unique materials for breeding (Carputo et al., 2013), which represents a tremendously diverse gene pool traditionally utilized as a source of various traits in potato breeding (Heřmanová et al., 2007).

Subsection Potatoe is divided into two superseries, Stellata Hawkes and Rotata Hawkes based on the corolla shape (Hawkes, 1990). Taxonomy of the genus Solanum based on the variability of morphological characters and phenology of plants had a long tradition (Correll, 1962; Okada and Clausen, 1983; Hawkes, 1990; Bradshaw and Mackay, 1994; Ochoa, 2004), but with the development of molecular analysis of nucleic acids, the polymorphism of different nuclear DNA regions i.e. mtDNA and cpDNA introns and non-coding intergenic spacers offers higher potential to clarify phylogeny of the genus (Kocyan et al., 2007; Miz et al., 2008). Also molecular markers are particularly attractive while they provide a direct estimation of genetic diversity and can help in the selection of parents that guarantee a superior genetic combination (Bisognin and Douches, 2002; Carputo et al., 2013) what is necessary in ex situ systems of biodiversity maintaining.

Microsatellites polymorphism analyses were used to study of genetic diversity in numerous crop plant species including potato (Bryan et al., 1999; Zeka et al., 2014), sunflower (Wills and Burke, 2006) and pepper (Hanáček et al., 2009). In the light of molecular research are newly presumed existence of 100 wild and only four cultivated species (Spooner, 2009; Ovchinnikova et al., 2011). Studies of species boundaries in the wild potato and their progenitors serves to illustrate the need for a variety of morphological and molecular approaches to comprehensively address complex problems of species limits (Spooner, 2009).

The main aim of this study was to evaluate wild diploid Solanum super series Rotata genotypes/species diversity through comparing molecular and phenotype characterization and possibly find duplicities preserved in Czech Gene bank collections in vitro.

MATERIALS AND METHODS

Plant material

Twelve wild tuber-bearing diploid genotypes from random seedlings belonging to 10 Solanum species were used in this
study. Wild diploid species of super series Rotata included: Solanum berthaultii Hawkes (EVIGEZ-00269), S. gourlayi Hawkes (EVIGEZ-00043 and EVIGEZ-00045), S. incamayoense K.A. Okada & A.M. Clausen (EVIGEZ-00045), S. leptophyes Bitter (EVIGEZ-00048), S. microdorum Bitter (EVIGEZ-00049), S. mochiquense Ochoa (EVIGEZ-00050), S. sparsipillum (Bitter) Juz. & Bukasov (EVIGEZ-00071), S. spegazzinii Bitter (EVIGEZ-00060), S. vernei Bitter & Wittm. (EVIGEZ-00060 and EVIGEZ-00234), and S. verrucosum Schltdl. (EVIGEZ-00299). Biological material was provided by the *in vitro* gene bank at Potato Research Institute Hvalučkův Brod Ltd., Hvalučkův Brod, Czech Republic. All genotypes were maintained as *in vitro* culture at 1x MS medium (Murashige and Skoog, 1962) with 2% sucrose and 0.7% agar in cultivation plant growth chamber (MLR-15 SANYO Electric, Osaka, Japan) at illumination of 14500 lux and photoperiod 16:8 h.

**DNA extraction, cpSSR genotyping and phenotype characterization**

Total genomic DNA was extracted from young leaves (100 mg) of single plants using DNeasy Kit (Qiagen, Germantown, Maryland, USA) according the manufacturer’s instructions. DNA samples were analyzed with 20 nuclear SSR primer pairs (Table 2) described previously by Bryan et al. (1999). PCRs were performed in a 12.5 µL volume containing 10 ng of total DNA, 1 x buffer KCl, 1.5 mM MgCl2, 0.5 units of Taq polymerase, 0.4 µM forward and reverse primers, and 0.3 mM dNTPs (Fermentas, Vilnius, Lithuania). The PCR was carried out using the following cycling profiles: preliminary denaturing for 3 min at 94 °C was followed by 27 cycles consisting from 40 s denaturing at 94 °C, 40 s annealing at 60 °C and 40 s elongation at 72 °C and closed by one final extension 10 min at 72 °C. Length polymorphisms of amplicon were detected in one capillary electrophoresis ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Ladder GeneScan 600 LIZ size standard (Life Technologies, Carlsbad, California, USA) was used for amplicons size evaluation.

**Statistical analyses**

Molecular diversity was calculated for each locus. The average observed heterozygosity per locus was calculated as

$$H = \frac{N}{N-1} \cdot \left(1 - \frac{p^2}{2}\right)$$

where *N* is the number of samples and *p* is the frequency of *p*° allele (Nei, 1987). Polymorphic Information Content (PIC) was calculated using PICCalc (Nagy et al., 2012). A binary matrix where polymorphic loci were scored as presence (1) and absence (0) of an allele was constructed and statistically processed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Dissimilarity matrix was calculated by means of Jaccard’s coefficient in 1000 replicate bootstrapping.

**Table 1. Characteristics used in the phenotype analyses of the Solanum wild species super series Rotata.**

| Characteristic | Description |
|---------------|-------------|
| Plant-tuft form | (1) conical, (2) umbrella, (3) plane. |
| Plant-tuft shape | (1) prostrate, (5) drooping, (9) erect. |
| Stem-erection | (1) not dissected, (2) 1 pair, (3) 2 pairs, (4) 3 pairs, (5) 4 pairs, (6) 5 pairs, (7) 6 pairs, (8) 7 pairs, (9) > 7 pairs. |
| Leaf-lobation | (1) close leaf, (2) intermediate leaf, (3) open leaf. |
| Leaf-pair number of primary leaflets | (1) non dissected, (2) 1 pair, (3) 2 pairs, (4) 3 pairs, (5) 4 pairs, (6) 5 pairs, (7) 6 pairs, (8) 7 pairs, (9) > 7 pairs. |
| Leaf-size | (1) very small, (3) small, (5) intermediate, (7) large, (9) very large. |
| Leaf-surface | (3) smooth, (5) undulate, (7) strong undulate to curly. |
| Leaf-concrescence type of terminal | (1) one-side, (2) bilateral. |
| Leaf-luster | (1) opaque, (5) slight shining, (9) shining. |
| Leaf-color | (1) grey-green, (3) brown-green, (5) light-green, (7) green, (9) dark-green. |
| Leaf-anchored at indument | (1) present, (5) irregularly present, (9) regularly present. |
| Leaf-position of pedicel articulation | (1) in the upper 1/4, (3) in the upper 1/3, (5) in the middle, (7) in the lower 1/3, (9) in the lower 1/4. |
| Leaf-symmetry | (1) absent, (2) 1 pair, (3) 2 pairs, (4) 3 pairs, (5) 4 pairs, (6) 5 pairs, (7) 6 pairs, (8) 7 pairs, (9) > 7 pairs. |
| Leaf-shape | (1) linear, (2) irregular, (3) regular. |
| Leaf-lamination | (1) simple, (2) intermediate leaf, (3) open leaf. |
| Leaf-concrescence type of terminal | (1) one-side, (2) bilateral. |
| Leaf-luster | (1) opaque, (5) slight shining, (9) shining. |
| Leaf-color | (1) grey-green, (3) brown-green, (5) light-green, (7) green, (9) dark-green. |
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| Leaf-shape | (1) linear, (2) irregular, (3) regular. |
| Leaf-lamination | (1) simple, (2) intermediate leaf, (3) open leaf. |
RESULTS AND DISCUSSION

A total of 63 SSR alleles were observed within the genotypes analyzed, highest polymorphism with a maximum of 6 alleles and observed average heterozygosity ($H_o$) value 0.89 generated by the marker ntcp9 (Table 2). Despite this, a couple of loci had by 2 alleles with range of $H_o = 0.30$ (ntcp28) till $H_o = 0.55$ (ntcp4). The size of amplicons ranged between 110 and 287 bp (Table 2). Mean value of observed average heterozygosity was 0.61, whereas the mean of PIC was 0.49. It is possible to compare $H_o$ values for SSRs in coding and non-coding regions. Intergenic regions had highest variability ($H_{igr}$ = 0.65, n = 12) compare to introns ($H_{in}$ = 0.54, n = 6) and exons ($H_{ex}$ = 0.45, n = 2) of the chloroplast genome. Studies of wild potato species (Bryan et al., 1999; Zeka et al., 2014) and cultivars (Martyrosyan et al., 2007) by ntcp markers found that locus ntcp9 showed the highest polymorphism. The high value of $H_o$ and PIC implies high level of dissimilarity within the studied species. A radial dissimilarity dendrogram on the basis of Jaccard’s coefficient in the analyzed Solanum genotypes (Figure 1) based upon molecular analyses highlights a tendency to separate two differing groups of Rotata wild diploids - (i) S. berthaultii, S. mochiquense and two accessions of S. vernei, and (ii) two accessions of S. gourlai, S. incamayoense, S. leptophyes, S. microdontum, S. sparsipilum, S. spegazzinii and S. verrucosum. Nevertheless, any intraspecific polymorphism was not found between S. gourlai accessions and both of them resulted in one unique haplotype (Figure 1). The clustering patterns are in good agreement with results obtained from previous phylogenetic Solanum genus studies; in example Spooner (2009) found that most of these species did not form species-specific clades.

Phenotype clustering separated two groups also, but with different outline: (i) S. berthaultii, two genotypes S. gourlai, S. incamayoense, S. mochiquense, S. spegazzinii and two genotypes S. vernei, and (ii) S. leptophyes, S. microdontum, S. sparsipilum, and S. verrucosum (Figure 2). Morphological and molecular differences were observed between S. vernei accessions (EVIGEZ-00060

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**Table 2. Characteristics of microsatellite primers and results.**

| Locus | Gene location | Expected size | Allele size range (bp) observed | Nr of alleles | Average heterozygosity | PIC |
|-------|---------------|---------------|---------------------------------|--------------|------------------------|-----|
| ntcp3 | trnK in       | 196           | 191-193                         | 3            | 0.62                   | 0.48|
| ntcp4 | trnK/rps 16 1g | 162           | 156-157                         | 2            | 0.55                   | 0.38|
| ntcp6 | rps16/trnQ 1g  | 176           | 167-171                         | 5            | 0.82                   | 0.71|
| ntcp7 | ORF98/trnS 1g | 175           | 168-169                         | 2            | 0.48                   | 0.35|
| ntcp8 | trnG in       | 251           | 248-250                         | 3            | 0.68                   | 0.55|
| ntcp9 | trnG/trnR 1g  | 237           | 246-278                         | 6            | 0.89                   | 0.79|
| ntcp10| atpF in       | 120           | 110-112                         | 3            | 0.62                   | 0.50|
| ntcp12| rps2/Rf862 1g | 126           | 117-120                         | 3            | 0.56                   | 0.55|
| ntcp14| pslB/trnD 1g  | 152           | 143-148                         | 5            | 0.85                   | 0.74|
| ntcp18| psbC/trnS 1g  | 186           | 185-188                         | 4            | 0.71                   | 0.60|
| ntcp20| ycf3 in       | 122           | 112-114                         | 3            | 0.62                   | 0.50|
| ntcp23| rps4/trnT 1g  | 122           | 108-110                         | 3            | 0.68                   | 0.55|
| ntcp24| atpB ex       | 157           | 148-149                         | 2            | 0.48                   | 0.35|
| ntcp27| trnP/trnP 1g  | 166           | 160-162                         | 3            | 0.71                   | 0.58|
| ntcp28| rpl20/rps12 1g| 170           | 153-154                         | 2            | 0.30                   | 0.24|
| ntcp29| clpP in       | 157           | 150-151                         | 2            | 0.48                   | 0.35|
| ntcp30| clpP in       | 158           | 149-150                         | 2            | 0.41                   | 0.30|
| ntcp33| rpoA ex       | 149           | 145-146                         | 2            | 0.41                   | 0.30|
| ntcp39| trnR/trnS 1g  | 156           | 149-151                         | 3            | 0.44                   | 0.36|
| ntcp40| rpp12/trnH 1g | 163           | 263-287                         | 5            | 0.80                   | 0.70|
| Mean  | -             | -             | 3.15                            | 0.61         | 0.49                   |     |

Table 2: Characteristics of microsatellite primers and results.

*Ex: exon, in: intron, igr: intergenic region.*

PIC: Polymorphic information contents.

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Figure 1. Jaccard’s tree based on dissimilarity matrix of 12 Solanum genotypes examined with 20 microsatellite pair pairs.
Molecular single-nucleotide polymorphism revealed in loci ntcp8, ntcp10, ntcp28, ntcp29, and ntcp39; 2bp polymorphism occurred in loci ntcp14 and ntcp27, whereas 30 bp and 21 bp polymorphism was observed in loci ntcp9 and ntcp40, respectively. Moreover, S. vernei genotypes were distinct in seven of 26 traits of phenotype description (Table 3). Contrarily, both accessions of S. gourlai (EVIGEZ-00043 and EVIGEZ-00045) resulted in one SSR haplotype (Figure 1), and were slightly distinct only in stem color and leaf shape of lateral leaflets on phenotypic description (Table 3 and Figure 2). If we compare these two different observations and accept the hypothesis of conservativeness of cpDNA, we can postulate that accessions of S. vernei are not members of the same species. This is supported by distance of S. vernei clade from base line Figure 1 (less than 20% similarity). On the contrary, the clustering of morphological descriptors confirms relatively high morphological similarity of both S. vernei accessions, but detailed view on crucial morphological description informs us about significant differences between them. This is also in good congruence to findings of RAPD analysis including both accessions done by Sedláková et al. (2009). With the respect to this finding we can responsibly notify that our results of morphological evaluation (Table 3) of wild 2n Solanum species super series Rotata generally corresponded to the previous studies of tuft, stem, and leaves and inflorescence traits (Correll, 1962; Okada and Clausen, 1983; Ochoa, 2004).

SSR analysis classified S. gourlai and S. incamayoense accessions to the same cluster. This result was supported by phenotypic description also. Similarly S. leptophyes and S. sparsipilum were identically classified to common

| Trait                                    | S. berthaultii 00260 | S. gourlai 00045 | S. microdontum 00047 | S. vernei 00234 | S. incamayoen. 00047 | S. sparsipilum 00049 | S. mochiquense 00050 | S. vernei 00234 | S. verrucosum 00299 | S. leptophyes 00048 | S. spegazzini 00060 |
|------------------------------------------|----------------------|------------------|----------------------|-----------------|----------------------|----------------------|----------------------|-----------------|---------------------|---------------------|----------------------|
| 1 Plant-tuft form                        | 7                    | 5                | 5                    | 5               | 7                    | 8                    | 3                    | 2               | 8                   | 8                   | 3                    |
| 2 Plant-tuft shape                       | 1                    | 3                | 3                    | 3               | 1                   | 1                    | 1                   | 1               | 3                   | 3                   | 2                    |
| 3 Plant-high                             | 5                    | 2                | 2                    | 3                | 7                   | 2                    | 4                    | 2               | 5                   | 5                   | 5                    |
| 4 Stem-erection                          | 5                    | 5                | 5                    | 5               | 5                   | 5                    | 5                   | 5               | 5                   | 5                   | 5                    |
| 5 Stem branching                        | 8                    | 8                | 8                    | 8               | 8                   | 8                    | 8                   | 8               | 8                   | 8                   | 8                    |
| 6 Stem thickness                         | 6                    | 4                | 4                    | 4               | 6                   | 8                    | 6                    | 4               | 7                   | 7                   | 7                    |
| 7 Stem color                             | 1                    | 4                | 3                    | 4               | 9                   | 5                    | 8                    | 9               | 5                   | 5                   | 5                    |
| 8 Stem-number per plant                  | 1                    | 1                | 1                    | 1               | 4                   | 1                    | 8                    | 2               | 4                   | 3                   | 9                    |
| 9 Leaf shape                             | 7                    | 7                | 7                    | 7               | 3                   | 3                    | 7                    | 5               | 7                   | 7                   | 7                    |
| 10 Leaf pair number of primary leaflets   | 5                    | 5                | 5                    | 5               | 5                   | 4                    | 5                    | 5               | 5                   | 5                   | 5                    |
| 11 Leaf shape of lateral leaflets        | 4                    | 5                | 4                    | 5               | 5                   | 5                    | 4                    | 5               | 5                   | 5                   | 5                    |
| 12 Leaf leaflets presence                | 2                    | 2                | 2                    | 2               | 2                   | 2                    | 2                    | 2               | 2                   | 2                   | 2                    |
| 13 Leaf lobation                         | 1                    | 2                | 2                    | 2               | 2                   | 3                    | 3                    | 3               | 1                   | 2                   | 2                    |
| 14 Leaf concrescence type of terminal    | 2                    | 2                | 2                    | 2               | 2                   | 2                    | 2                    | 2               | 2                   | 2                   | 2                    |
| 15 Leaf surface                          | 5                    | 5                | 5                    | 5               | 5                   | 5                    | 5                   | 5               | 7                   | 5                   | 5                    |
| 16 Leaf size                             | 5                    | 3                | 3                    | 3               | 5                   | 5                    | 5                   | 3               | 7                   | 5                   | 7                    |
| 17 Leaf color                            | 1                    | 7                | 7                    | 7               | 9                   | 7                    | 5                   | 9               | 1                   | 7                   | 7                    |
| 18 Leafuster                             | 1                    | 5                | 5                    | 5               | 5                   | 5                   | 1                    | 5               | 5                   | 5                   | 1                    |
| 19 Inflorescence position of pedicel articulation | 3    | 5                | 3                    | 5               | 5                   | 5                    | 7                   | 5               | 7                   | 7                   | 7                    |
| 20 Inflorescence anthocyanin color of pedicel articulation | 7    | 7                | 7                    | 7               | 3                   | 1                    | 3                   | 5               | 7                   | 7                   | 7                    |
| 21 Inflorescence diparacola presence     | 1                    | 1                | 1                    | 1               | 1                   | 1                    | 1                   | 1               | 1                   | 1                   | 1                    |
| 22 Inflorescence corolla radius size     | 8                    | 7                | 7                    | 7               | 7                   | 7                    | 7                   | 4               | 7                   | 7                   | 7                    |
| 23 Inflorescence corolla radius color    | 8                    | 9                | 9                    | 9               | 9                   | 1                    | 1                   | 9               | 1                   | 7                   | 7                    |
| 24 Inflorescence degree of flowering     | 8                    | 6                | 6                    | 6               | 8                   | 6                    | 8                   | 3               | 7                   | 5                   | 7                    |
| 25 Inflorescence buds throwing off       | 7                    | 7                | 7                    | 7               | 7                   | 7                    | 7                   | 7               | 7                   | 7                   | 7                    |
| 26 Berries number per plant              | 1                    | 5                | 5                    | 1               | 7                   | 5                    | 1                   | 5               | 3                   | 1                   | 7                    |
cluster on the basis of both data sets, molecular and morphological, respectively (Figures 1 and 2). Regarding to the other species within the studied collection can be considered as being able to encompass genetically different material since we detected variability of microsatellites and phenotype traits in evaluated genotypes/species. Similar results of phenotypic description (Ochoa, 2004) and molecular characterization (Bryan et al., 1999; Spooner, 2009) of these species were reported.

CONCLUSIONS

Molecular and morphologic approach of species boundaries, in this case super series Rotata, confirms complexity and fragility in wild potato species determination taxonomy, even some of examined species were characterized as close relatives either by phenotypic and molecular analyses. Microsatellites results show the diversity and possibility of duplicities in the current Czech in vitro collection of the wild potato genetic resources.

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