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Phenotype and molecular diversity evaluation of some wild 2*n* *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 (H_o) 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 (H_{in} = 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

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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. microdontum* Bitter (EVIGEZ-00049), *S. mochiquense* Ochoa (EVIGEZ-00050), *S. sparsipilum* (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 Havlíčkův Brod Ltd., Havlíčkův 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 × buffer KCl, 1.5 mM $MgCl_2$, 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.

Morphological characters were evaluated using Plant Genetic Resources Documentation in the Czech Republic (EVIGEZ) descriptor list for the genus *Solanum* L. (Vidner et al., 1987). Totally 26 characters of tuft were evaluated (Table 1) in 2 yr evaluating period in field trial in experimental field of Czech University of Life Sciences Prague (CULS, 50°07'37" N; 14°22'36" E; 276 m a.s.l.), Prague-Suchdol, Czech Republic.

Statistical analyses

Molecular diversity was calculated for each locus. The average observed heterozygosity per locus was calculated as $H = N/N\text{-}1(1\text{-}\sum p_i^2)$, where N is the number of samples and p_i is the frequency of i^{th} 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*.

1. Plant-tuft form: (2) stem thin, (3) stem, (4) stem thick, (5) intermediate, (6) leafy thin, (7) leafy, (8) leafy thick.
2. Plant-tuft shape: (1) conical, (2) umbrella, (3) plane.
3. Plant-height: (1) very short < 25 cm, (2) 25-30 cm, (3) short 31-35 cm, (4) 36-40 cm, (5) intermediate 41-45 cm, (6) 46-50 cm, (7) tall 51-55 cm, (8) 56-70 cm, (9) very tall > 70 cm.
4. Stem-erection: (1) prostrate, (5) drooping, (9) erect.
5. Stem-branching: (2) on stem base, (4) in middle of stem, (6) on the top of stem, (8) all over the stem.
6. Stem-thickness: (1) very thin, (2) –, (3) thin, (4) –, (5) intermediate, (6) –, (7) thick, (8) –, (9) very thick.
7. Stem-color: (1) light reddish, (2) blue-violet, (3) red-brown, (4) red, (5) blue-violet-striped, (6) dark green, (7) green, (8) light green, (9) green striped.
8. Stem-number per plant: (1) very small < 2, (2) 2.0-3.0, (3) small 3.1-4.0, (4) 4.1-5.0, (5) intermediate 5.1-6.0, (6) 6.0-7.0, (7) large 7.1-8.0, (8) 8.1-9.0, (9) very large > 9.0
9. Leaf-shape: (1) rotundum, (3) wide oval, (5) long oval, (7) oblong, (9) linear.
10. 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.
11. Leaf-shape of lateral leaflets: (1) linear, (2) narrow lanceolate, (3) lanceolate, (4) wide lanceolate, (5) narrow oval, (6) oval, (7) wide oval, (8) eliptical, (9) rotundum.
12. Leaf-leaflets presence – dissection of second order: (1) absent, (2) irregular, (3) regular.
13. Leaf-lobation: (1) close leaf, (2) intermediate leaf, (3) open leaf.
14. Leaf-concrescence type of terminal: (1) one-side, (2) bilateral.
15. Leaf-surface: (3) smooth, (5) undulate, (7) strong undulate to curly.
16. Leaf-size: (1) very small, (3) small, (5) intermediate, (7) large, (9) very large.
17. Leaf-color: (1) grey-green, (3) brown-green, (5) light-green, (7) green, (9) dark-green.
18. Leaf-luster: (1) opaque, (5) slight shinning, (9) shinning.
19. Inflorescence-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.
20. Inflorescence-anthocyanin color of pedicel articulation: (1) absent, (3) weak, (5) intermediate, (7) strong, (9) very strong.
21. Inflorescence-diparacola presence: (1) not present, (5) irregularly present, (9) regularly present.
22. Inflorescence-corolla radius: (1) very small < 8 mm, (2) 8-11 mm, (3) small 12-15 mm, (4) 16-19 mm, (5) intermediate 20-23 mm, (6) 24-27 mm, (7) large 28-31 mm, (8) 32-35 mm, (9) very large > 35 mm.
23. Inflorescence-corolla color: (1) white, (2) pink, (3) light red-violet, (4) red-violet, (5) dark red-violet, (6) blue, (7) light violet, (8) grey-violet, (9) dark-violet.
24. Inflorescence-degree of flowering: (1) absent, (2) very weak, (3) weak, (4) weak to intermediate, (5) intermediate, (6) intermediate to intensive, (7) intensive, (8) very intensive, (9) extreme intensive.
25. Inflorescence-buds throwing off: (1) frequent, (5) medium frequent, (7) infrequent.
26. Berries-number per plant: (1) absent, (3) rare, (5) small < 10, (7) large 10-20, (9) very large > 20.

and graphically interpreted by means DARwin software v. 5.0 (Perrier and Jacquemoud-Collet, 2006).

Morphological dissimilarities between genotypes were analyzed by the Complete Linkage method. Cluster analysis was performed as Euclidean distance.

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, 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

Table 2. Characteristics of microsatellite primers and results.

Locus	Gene location[1]	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 igr	162	156-157	2	0.55	0.38
ntcp6	rps16/trnQ igr	176	167-171	5	0.82	0.71
ntcp7	ORF98/trnS igr	175	168-169	2	0.48	0.35
ntcp 8	trnG in	251	248-250	3	0.68	0.55
ntcp 9	trnG/trnR igr	237	246-278	6	0.89	0.79
ntcp10	atpF in	120	110-112	3	0.62	0.50
ntcp12	rps2/RF862 igr	126	117-120	3	0.56	0.55
ntcp14	psbM/trnD igr	152	143-148	5	0.85	0.74
ntcp18	psbC/trnS igr	186	185-188	4	0.71	0.60
ntcp20	ycf3 in	122	112-114	3	0.62	0.50
ntcp23	rps4/trnT igr	122	108-110	3	0.68	0.55
ntcp24	atpB ex	157	148-149	2	0.48	0.35
ntcp27	trnP/psaJ igr	166	160-162	3	0.71	0.58
ntcp28	rpl20/rps12 igr	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/rrn5 igr	156	149-151	3	0.44	0.36
ntcp40	rp12/trnH igr	163	263-287	5	0.80	0.70
Mean		-	-	3.15	0.61	0.49

[1]ex: exon, in: intron, igr: intergenic region.
PIC: Polymorphic information contents.

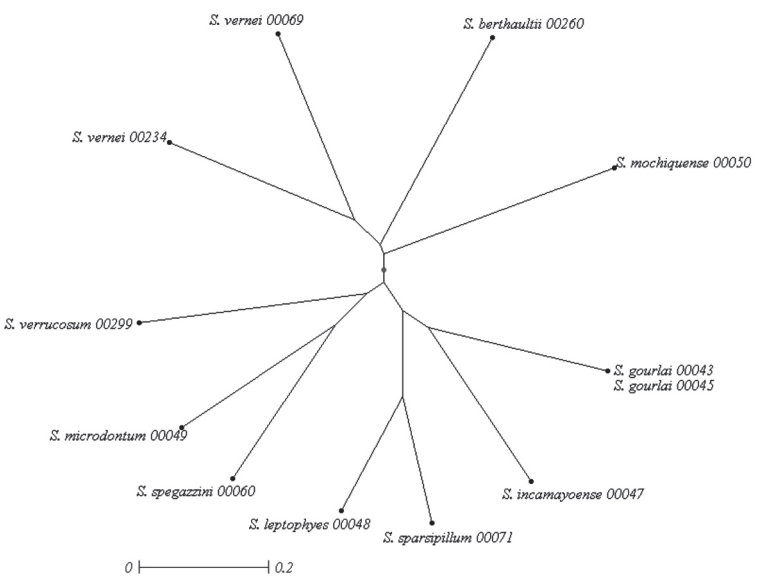

Figure 1. Jaccard's tree based on dissimilarity matrix of 12 *Solanum* genotypes examined with 20 microsatellite pair pairs.

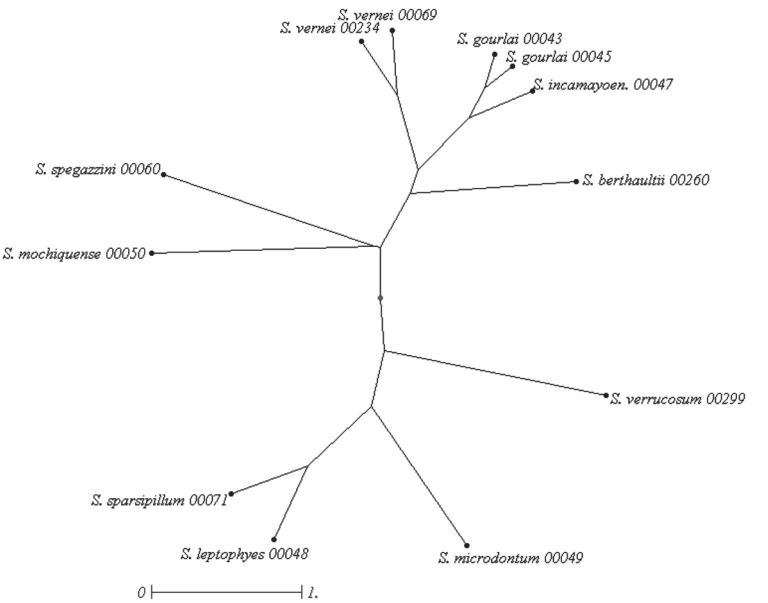

Figure 2. Dendrogram of Complete Linkage compressing tuft characteristics of 12 *Solanum* genotypes.

and EVIGEZ-00234). 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 2*n 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

Table 3. *Solanum* species phenotype traits evaluation results.

Trait	*S. berthaultii* 00260	*S. gourlai* 00045	*S. gourlai* 00043	*S. incamayoen.* 00047	*S. leptophyes* 00048	*S. microdontum* 00049	*S. mochiquense* 00050	*S. sparsipillum* 00071	*S. spegazzini* 00060	*S. vernei* 00069	*S. vernei* 00234	*S. verrucosum* 00299
1 Plant-tuft form	7	5	5	5	5	7	8	3	2	8	8	3
2 Plant-tuft shape	1	3	3	3	1	1	1	1	1	3	3	2
3 Plant-high	5	2	2	2	3	7	2	4	2	5	5	1
4 Stem-erection	5	5	5	5	5	5	5	5	5	5	5	5
5 Stem branching	8	8	8	8	8	8	2	8	8	8	8	8
6 Stem thickness	6	4	4	4	6	8	6	6	4	7	7	3
7 Stem color	1	4	3	4	9	5	8	9	5	5	5	5
8 Stem-number per plant	1	1	1	1	4	7	1	8	2	4	3	9
9 Leaf shape	7	7	7	7	3	3	3	3	7	5	7	5
10 Leaf pair number of primary leaflets	5	5	5	5	5	2	4	5	5	6	5	3
11 Leaf shape of lateral leaflets	4	5	4	5	5	6	5	7	4	5	5	4
12 Leaf leaflets presence	2	2	2	2	2	2	3	3	3	3	3	2
13 Leaf lobation	1	2	2	2	2	3	3	3	3	1	2	2
14 Leaf concrescence type of terminal	2	2	2	2	2	2	2	2	2	2	2	2
15 Leaf surface	5	5	5	5	5	5	7	5	3	7	5	3
16 Leaf size	5	3	3	3	5	5	5	5	3	7	7	3
17 Leaf color	1	7	7	7	9	7	5	9	1	7	7	7
18 Leaf luster	1	5	5	5	5	5	5	5	1	5	5	1
19 Inflorescence position of pedicel articulation	3	5	3	5	5	5	7	5	1	5	7	7
20 Inflorescence anthocyanin color of pedicel articulation	3	7	7	7	3	3	1	3	5	7	7	9
21 Inflorescence diparacola presence	1	1	1	1	1	1	1	1	1	1	1	1
22 Inflorescence corolla radius size	8	7	7	7	7	7	7	7	4	7	7	5
23 Inflorescence corolla radius color	8	9	9	9	9	1	1	9	1	7	7	9
24 Inflorescence degree of flowering	8	6	6	6	8	8	6	8	3	7	5	5
25 Inflorescence buds throwing off	7	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	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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