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MOLECULAR ANALYSIS OF BUCKWHEAT USING GENE SPECIFIC MARKERS

Želmíra Balážová, Zdenka Gálová, Martin Vivodík, Milan Chňapek, Radomíra Hornyák Gregáňová

ABSTRACT

Buckwheat (*Fagopyrium esculentum*) is a pseudo-cereal which has spread troughout the world and nowadays it represents cultural, economic and nutritionally important pseudocereal. It´s enviromentally friendly, characterized by high fiber, routine, protein and B vitamins, and is general-purpose. The goal of the present study was to analyze 17 genotypes of buckwheat by using 7 SCoT markers. In total, 52 fragments were detected, of which 38 were polymorphic. The average number of polymorphic fragments was 5.43. The most polymorphic fragments were detected in SCoT 26 and SCoT 29 markers, and the average percentage of polymorphism was 73.36 %. SCoT 29 reached the highest percentage of polymorphism (87.5 %) and SCoT 36 was lowest (60 %). The DI values ranged from 0.625 (SCoT 36) to 0.887 (SCoT 26) and the average DI value was 0.749. The average PIC value was 0.729 with PIC values ranging from 0.386 (SCoT 36) to 0.831 (SCoT 26). To determine the genetic diversity of 17 genotypes of the buckwheat, a dendrogram was created using the hierarchical cluster analysis. The genotypes were divided into two major clusters (I and II). Cluster I was divided into three other subgroups. Sixteen genotypes were included in cluster I and the genotype of Madawaska (USA) was genetically the farthest in cluster II. Genetically the closest were the varieties of Ballada (Russia) and Bamby (Austria). Used SCoT markers were sufficiently polymorphic, were able identify and differentiate chosen set of buckwheat genotypes.

Keywords: *Fagopyrium esculentum*; SCoT technique; genetic variability; DNA polymorphism; dendrogram

INTRODUCTION

Buckwheat (*Fagopyrium esculentum*), a diploid (2n = 16) annual, is a pseudo-cereal belonging to the family *Polygonaceae*. Buckwheat is an acient crop whose origins range from 5000 - 6000 years back in Asia. Common buckwheat is a traditional pseudo-cereal mainly grown in temperate regions of Asia, Europe, and North America. Due to short growth span, capability to grow at high altitudes, and the high quality protein of its grains it is an important crop in mountainous regions of India, China, Russia, Ukraine, Kazakhstan, parts of Eastern Europe, Canada, Japan, Korea, and Nepal **(Chrungoo et al., 2016)**. Recently common buckwheat is returning popular as a consequence of increasing gluten-free product market. The plant is a rich source of Zn, Cu, Mn, Se, vitamin B1, B2, E, and dietary proteins for gluten sensitive individuals and rich source of high biological-value proteins due to its balanced amino acids composition **(Wei et al. 2003; Stibilj et al. 2004)**. Common buckwheat seeds contain higher amounts of flavonoids, dietary fiber than cereals **(Przybylski and Gruczynska, 2009; Chen, 1999)**. As an important part of the human diet, common buckwheat seeds are a rich source of high biological-value proteins due to its balanced amino acids composition. The buckwheat grain is either consumed whole after boiling or steaming, or ground into a flour.

Despite the high nutritional and nutraceutical value of common buckwheat, the seed yield is low due to its self-incompatibility. Molecular breeding of common buckwheat has been impeded due to the lack of genomic resources and tightly linked markers for agronomically important genes. Molecular markers play an important role in genetic studies and marker-assisted selection (MAS) in crop breeding **(Shi et al., 2017)**.

Recently, the studies of genetic diversity are based mainly on the molecular analysis **(Žiarovská et. al., 2015; Vyhnánek et. al., 2015)**.

Worldwide collections of buckwheat were described by several types of dominant molecular markers, for example AFLP **(Yasui et al., 2004)**, **RAPD (Sharma and Jana, 2002)** and ISSR **(Kishore et al., 2013)**. Of the various DNA marker systems, start codon targeted (SCoT) polymorphism **(Collard and Mackill, 2009)** is gaining popularity for its superiority over other dominant DNA marker systems like RAPD and ISSR for higher polymorphism and better marker resolvability **(Gorji et al., 2011; Que et al. 2014; Satya et al., 2015; Zhang et al., 2015)**. A novel marker system called SCoT **(Collard**

and Mackill, 2009) was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT markers are generally reproducible, and it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility. They are dominant markers like RAPDs and could be used for genetic analysis, quantitative trait loci (QTL) mapping and bulk segregation analysis **(Collard and Mackill, 2009)**. In principle, SCoT is similar to RAPD and ISSR because the same single primer is used as the forward and reverse primer **(Collard and Mackill, 2009; Gupta et al. 1994)**. Suitability of SCoT markers system has been successfully employed in genetic diversity analysis and fingerprinting of a number of agricultural and horticultural crop species, such as peanut **(Xiong at al., 2011)**, tomato **(Shahlaei et al., 2014)**, citrus **(Mahjbi et al., 2015)**, date palm **(Al-Qurainy et al., 2015)**, ramie **(Satya et al., 2015)**, castor **(Kallamadi et al., 2015; Vivodík et al., 2018)**, maize **(Vivodík et al., 2016)**, rye **(Petrovičová et al., 2017)**, mango **(Gajera et al., 2014)** and Indian jujube **(Singh et al., 2017)**, plantago **(Rahimi et al., 2018)**, taxus **(Hao et al., 2018)** and rose **(Agarwal et al., 2018)**.

Scientific hypothesis

The aim of our study was to detect genetic variability among the set of 17 buckwheat genotypes using 7 SCoT markers and to testify the usefulness of a used set of SCoT primers for the identification and differentiation of buckwheat genotypes. Molecular analyses are important source for crop breeders and can be useful for gene identification for crops improvement.

MATERIAL AND METHODOLOGY

Seventeen buckwheat (*Fagopyrium esculentum*) genotypes were used in the present study. Seeds of buckwheat were obtained from the Gene Bank of the Slovak Republic of the Plant Production Research Center in Piešťany.

Isolation of DNA

Genomic DNA of buckwheat cultivars was isolated from 100 mg freshly-collected leaf tissue according to GeneJET^{TM} protocol (Thermo Scientific, USA). The concentration and quality of DNA was checked up on 1.0 % agarose gel coloured by ethidium bromide and detecting by comparing to λ-DNA with known concentration.

PCR analysis

For analysis 7 SCoT primers were chosen (Table 2) according to the literature **(Collard a Mackill, 2009**). Amplification of SCoT fragments was performed according to **(Collard a Mackill, 2009)** (Table 2). Polymerase chain reaction (PCR) was performed in 15 μl mixture in a programmed thermocycler (Biometra, Germany). Amplified products were separated in 1 % agarose gels in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system UVP PhotoDoc-t®. Size of amplified fragments was determined by comparing with standard lenght marker Quick-Load® Purple 2-Log DNA ladder (New England Biolabs, Inc).

Statistical analysis

For the assessment of the polymorphism between castor genotypes and usability of SSR markers in their differentiation diversity index (DI) **(Weir, 1990)**, the probability of identity (PI) **(Paetkau et al., 1995)** and polymorphic information content (PIC) **(Weber, 1990)** were used. The SCoT bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. The binary data generated were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands and to prepare a dendrogram. A dendrogram based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with the SPSS professional statistics version 17 software package was constructed.

RESULTS AND DISCUSSION

In plant molecular genetic research, DNA markers have abundant usage for crop improvement in plant breeding **(Bhawna et al., 2017)**. DNA markers are commonly used for the assessment of genetic diversity in crop germplasm, population structure analysis **(Chen et al., 2012; Zhang et al., 2011)**, quantitative trait loci (QTL) or the linkage map construction for mapping genes **(Bhawna et al., 2017)**. For detecting polymorphisms a new molecular marker system called SCoT **(Collard a Mackill, 2009)** was developed which tag coding sequences of the genome. SCoT marker system had initially been validated in the model species rice (*Oryza sativa*) **(Collard and Mackill 2009)**.

For the molecular analysis of 17 buckwheat genotypes 7 SCoT primers were used. PCR amplifications using 7 SCoT primers produced total 52 DNA fragments that could be scored in all genotypes. The selected primers amplified DNA fragments across the 17 genotypes studied with the number of amplified fragments varying from 5 (SCoT36) to 11 (SCoT12) and the amplicon size varied from 200 to 3000 bp. Of the 52 amplified bands, 38 were polymorphic with an average of 5.43 fragments per primer (Table 3). The percentage of polymorphic bands ranged from 54 % (SCoT12) to 87.5 % (SCoT26 and SCoT29) with an average of 73.43 %. The polymorphic information content (PIC) values varied from 0.586 (SCoT36) to 0.831 (SCoT26) with an average of 0.729 and index diversity (DI) value ranged from 0.625 (SCoT36) to 0.837 (SCoT26) with an average of 0.749 (Tab.3). SCoT marker with the highest percentage of polymorphism (SCoT29) is showed on Figure 2.

Based the genetic distance matrix using profiles of the 7 SCoT primers and hierarchical cluster analysis using the unweighted pair-group method with the arithmetic average (UPGMA) method a dendrogram was constructed. According to analysis, the set of 17 diverse accessions of buckwheat was clustered into two main clusters (I, II) (Figure 1). Sixteen buckwheat genotypes were included in cluster I and the genotype of Madawaska (USA) was genetically the farthest and created cluster II. Cluster I was further subdivided into three other subgroups (Ia, Ib and Ic). Majority (87.5 %) of Polish genotypes grouped in the subgroup Ia. Genetically the closest were the varieties Ballada (Russia) and Bamby (Austria) and grouped along side in the subgroup Ia.

Table 1 List of analyzed genotypes of buckwheat.

No.	Genotype of buckwheat (*Fagopyrium esculentum*)	Country of origin
1.	Aiva	LVA
2.	Alex	DEU
3.	Ballada	RUS
4.	Bamby	AUT
5.	Bogatyr	RUS
6.	Branszczyk	POL
7.	Czerwone orzeszki	POL
8.	Darja	SVN
9.	Emka	POL
10.	Gema	POL
11.	Hruszowska	POL
12.	JANA C1	CZE
13.	Kora	POL
14.	Madawaska	USA
15.	Pulawska	POL
16.	Pyra	CZE
17.	St Jacut	FRA

Note: RUS – Russia, AUT – Austria, POL – Poland, CZE – Czech Republik, LVA – Latvia, FRA – France, SVN – Slovenia.

Table 2 List of used SCoT markers.

SCoT primer	Sequence of primers (5´-3´)	Anealing temperature [°C]
SCoT 12	ACGACATGGCGACCAACG	50 °C
SCoT 23	CACCATGGCTACCACCAG	50 °C
SCoT 26	ACCATGGCTACCACCGTC	50 °C
SCoT 28	CCATGGCTACCACCGCCA	50 °C
SCoT 29	CCATGGCTACCACCGGCC	50 °C
SCoT 30	CCATGGCTACCACCGGCG	50 °C
SCoT 36	GCAACAATGGCTACCACC	50 °C

Table 3 Statistical characteristics of the SCoT markers used in buckwheat.

SCoT marker	Number of all fragments	Number of polymorphic fragments	Percentage of polymorphic bands (%)	DI	PIC	PI
SCoT12	11	6	54.0	0.751	0.722	0.022
SCoT23	7	5	71.0	0.702	0.694	0.039
SCoT26	8	7	87.5	0.837	0.831	0.005
SCoT28	7	5	71.0	0.730	0.696	0.024
SCoT29	8	7	87.5	0.809	0.805	0.009
SCoT30	6	5	83.0	0.786	0.770	0.011
SCoT36	5	3	60.0	0.625	0.586	0.071
Average	**7.43**	**5.43**	**73.43**	**0.749**	**0.729**	**0.026**
Total	**52**	**38**				

Note: DI- diversity index, PIC- polymorphic information content, PI- probability of identity

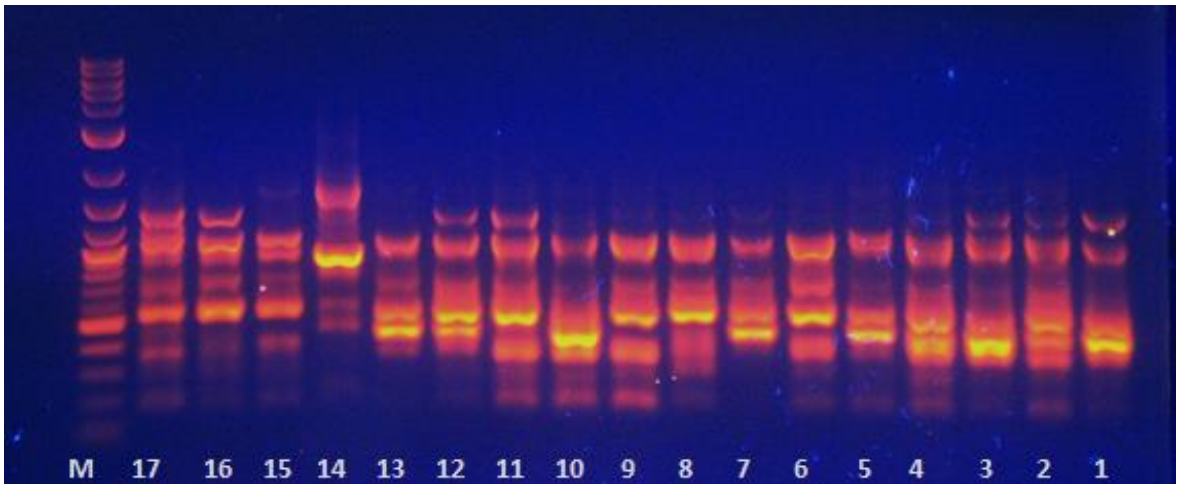

Figure 1 Electrophoreogram of SCoT29 marker.
Note: 1-17 are genotypes of buckwheat (tab.1), M is Quick-Load® Purple 2-Log DNA ladder.

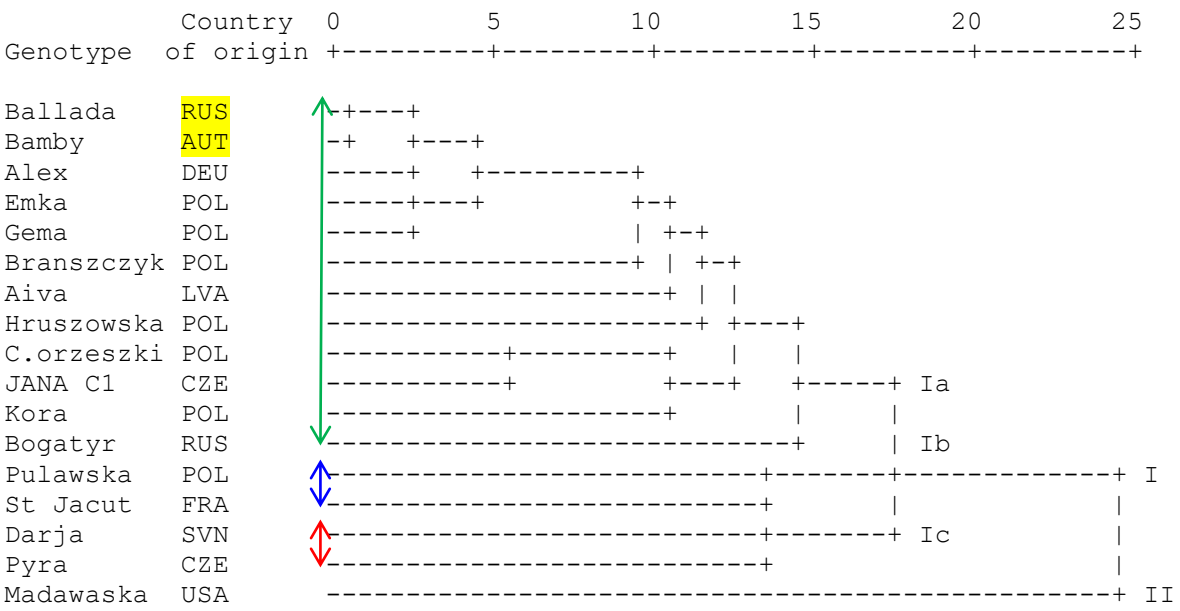

Figure 2 Dendrogram of 17 buckwheat genotypes prepared based on 7 SCoT markers.
Note: RUS – Russia, AUT – Austria, POL – Poland, CZE – Czech Republik, LVA – Latvia, FRA – France, SVN – Slovenia.

Lower average percentage of polymorphism (21 %) obtained **Kallamadi et al. (2015)** who analysed molecular diversity of castor (*Ricinus communis* L.) by SCoT technique. Out of 36 SCoT primers tested, all primers produced amplification products but only 10 primers resulted in polymorphic fingerprint patterns. Out of a total of 108 bands, 23 (21%) were polymorphic with an average of 2.1 polymorphic bands per primer. The total number of bands per primer varied from 5 and 20 in the molecular size range of 100–3000 bp. The PIC/DI varied from 0.06 for SCoT28 to 0.45 for SCoT12 with an average of 0.24.

On the other side, higher percentage of polymorphism with SCoT primers has been reported in crops like peanut **(Xiong et al., 2011)**, cicer **(Amirmoradi et al., 2012)**, mango **(Luo et al., 2010)**, ramie **(Satya et al., 2015)**, sugarcane **(Que et al., 2014)**, Chinese bayberry **(Fang-Yong and Ji-Hong, 2014)**, pepper **(Tsaballa et al., 2015)**, castor **(Kallamadi et al., 2015)**, maize **(Vivodík et al., 2016)** and taxus **(Hao et al., 2018)**.

Satya et al. (2015) used 20 SCoT markers to assess genetic diversity and population structure of indigenous, introduced and domesticated ramie (*Boehmeria nivea* L. Gaudich.). A total of 155 genotypes from five populations were investigated for SCoT polymorphism, which produced 136 amplicons with a range of 4 to 10 bands per primer, of which 119 (87.5%) were polymorphic. Polymorphism information content ranged from 0.25 to 0.93 with an average of 0.69. **Gajera et al. (2014)** used 19 SCoT primers for amplification among 20 mango cultivars which yielded a total of 117 clear and bright loci. Number of loci ranged from 4 to 10 with an average of 6.16 loci per primer. Of 117 loci, 96 loci (79.57 %) were polymorphic, the number of polymorphic loci varied from 2 to 10 with an average of 5.05 loci per primer. The detected

polymorphism per primer among the tested cultivars ranged from 50 % (SCoT26) to 100 % (SCoT-33, SCoT-40, and SCoT-51). In our study we detected by SCoT26 primer the percentage of polymorphic bands 87.5 %. **Que et al. (2014)** used 20 SCoT primers to assess the genetic diversity among 107 sugarcane accessions within a local sugarcane germplasm collection. Tventy SCoT primers amplified 176 DNA fragments, of which 163 were polymorphic (92.85%). **Fang-Yong and Ji-Hong (2014)** assessed the genetic diversity of 31 germplasm resources of *Myrica rubra* of China using 38 SCoT markers. Authors detected 298 reproducible bands of which 251 were polymorphic (84.23%). **Hao et al. (2018)** through a screening of 36 start codon targeted (SCoT) polymorphism primers, among 15 individuals of 4 *Taxus* species detected in 20 SCoT primers clear and repeatable polymorphism. The number of SCoT bands generated from *Taxus* samples was in the range of 5 – 10 for each SCoT primer. The ratio of polymorphic bands across the primers was 62.5 – 100%, with an average of 82.0%, indicating that SCoT markers provided a high level of information and could be employed for assessing genetic diversity and molecular identification of *Taxus* species.

Luo et al. (2010) found comparable percentage of polymorphism (76.2 %) using SCoT markers in analysis of diversity and relationships among mango cultivars and also **Agarwal et al. (2018)** who detected 72.49% percentage of polymorphism in the analysis of genetic diversity within 29 rose accessions using 32 SCoT markers.

To determine the level of polymorphism in analysed buckwheat genotypes polymorphic information content (PIC) was calculated (Table 3). Lower PIC values compare to our analysis (0.524) were detected by **Tsaballa et al. (2015), Kallamadi et al. (2015), Huang et al. (2014) and Hajibarat et al. (2015). Tsaballa et al. (2015)** analyzed genetic variability among the 30 landraces and one commercial Greek cultivar of pepper (*Capsicum annuum* L.) using 6 SCoT primers. They detected PIC values ranged from 0.123 (SCoT33) to 0.258 (SCoT15), with an average value of 0.232 per primer. **Kallamadi et al. (2015)** detected average PIC/DI vales from 0.06 (SCoT28) to 0.45 (SCoT12) with an average of 0.24 in analysis of genetic diversity in 31 accessions of castor representing seven geo-graphic areas by 36 SCoT markers. **Huang et al. (2014)** assessed the genetic diversity of six *Hemarthria* cultivars using seven SCoT primers. They calculated PIC values ranged from 0.471 to 0.758 with an average of 0.612. **Hajibarat et al. (2015)** used a set of 9 SCoT primers to fingerprint 48 chickpea genotypes. PIC values ranged from 0.43 to 0.47 with an average value of 0.45 per primer.

Higher values of PIC were detected by other authors **(Luo et al. 2010; Gajera et al. 2014; Que et al. 2014; Gao et al. 2014; Fang-Yong et al. 2014; Jiang et al. 2014; Satya et al., 2015)** and these values presented a high level of polymorphism of genotypes detected by SCoT markers. Higher PIC values were detecte by **Que et al. (2014)** who used assessed the genetic diversity among 107 sugarcane accessions using 20 SCoT markers and calculated PIC values from 0.783 to 0.907 with a mean of 0.861.

Agarwal et al. (2018) detected comparable polymorphic information content (PIC) ranged from 0.42 to 0.92 with an average of 0.78 in the identification and characterization of genetic variation within 29 rose accessions using 32 SCoT markers.

Kishore et al. (2013) used 13 ISSR markers to analyze genetic diversity and relatedness of 15 germplasms of *Fagopyrum tataricum.* They detected comparable average PIC value of the ISSR markers (0.812) which represents high level of polymorphism.

For the revealing of the genetic relationships among the cultivars a dendrogram is constructed. **Que et al. (2014)** to assess the genetic diversity among 107 sugarcane accessions within a local sugarcane germplasm collection used 20 SCoT primers. Using UPGMA cluster analysis of the SCoT marker data divided 107 sugarcane accessions into six clusters. **Jiang et al. (2014)** analyzed the diversity and genetic relationships among 95 orchardgrass accessions by using SCoT markers. In total, 273 polymorphic bands with an average of 11.4 bands per primer were detected. The UPGMA dendrogram separated 95 accessions into 7 main clusters according to the geographical origin. **Kallamadi et al. (2015)** analysed the genetic diversity of 31 accessions of castor using 36 SCoT markers with the aim to construct the UPGMA dendrogram in which the accessions of castor separated into two major clusters (11 and 17 accessions). Three accessions failed to cluster with others accessions. **Vivodík et al. (2018)** analyzed 56 genotypes of Tunisian castor using 37 SCoT primers. In the UGMA dendrogram, the collection of 56 Tunisian castor genotypes clustered into two main clusters (1 and 2). **Rajesh et al. (2015)** constructed dendrogram using genetic similarity coefficients obtained from UPGMA analysis among the coconut accessions. Coconut accessions grouped into two main clusters. Cluster analysis supported population genetic analysis and suggested close association between introduced and domesticated genotypes. **Gajera et al. (2014)** constructed dendrogram of the 20 mango cultivars using 19 SCoT primers which clustered into two major groups based on the SCoT data analysis with UPGMA.

CONCLUSION

The objective of this study was to determine the genetic variation among 17 rye varieties using 7 SCoT markers. Values of diversity index were higher than 0.7 in 85.7 % of SCoT markers that represents high level of polymorphism of used markers. We can recommend them for further analyses. The dendrogram was prepared based on UPGMA algorithm using the Jaccard´s coefficient and divided into two main clusters where all buckwheat genotypes were distinguished. Clustering partially reflected geographic origin of studied buckwheat genotypes. Majority (87.5 %) of Polish genotypes grouped in the same subcluster Ia.

SCoT marker system is a simple and novel marker system belonging to gene-targeted and functional markers. Functional markers developed from the transcribed region of the genome have the ability to reveal polymorphism, which might be directly related to gene function. The technique is similar to RAPD or ISSR, is simple, is used simple primer which acts as the forward and the reverse. Visualisation of amplicons can be performed by standard

agarose gel electrophoresis. The higher primer lengths and subsequently higher annealing temperatures ensure higher reproducibility of SCoT markers, compared to RAPD markers. SCoTs markers are more informative and effective. Our result showed appreciably high genetic diversity among the buckwheat genotypes studied. This study showed high genetic diversity within the studied buckwheat genepool as an important source for crop breeders and indicated that there is value in sampling for useful genes for crops improvement.

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Contact address:

Želmíra Balážová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zelmira.balazova@uniag.sk

Zdenka Gálová, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: zdenka.galova@uniag.sk

Martin Vivodík, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: martin.vivodik@uniag.sk

Milan Chňapek, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: chnapek.m@gmail.com

Radomíra Hornyák Gregáňová, Slovak University of Agriculture, Faculty of Economics and Management, Department of Mathematics, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, E-mail: radomira.greganova@uniag.sk