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ANALYSIS OF GENOMIC DIVERSITY OF SAMPLES AND CULTIVARS IN COMMON BUCKWHEAT (*Fagopyrum esculentum* Moench) BY THE ISSR-METHOD

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S u m m a r y

For an assessment of a genetic polymorphism of common buckwheat (*F. esculentum*, 27 samples of different ecologo-geographic origin, including 14 domestic cultivars) the authors determined the ISSR-primers of revealing intraspecific polymorphism and obtained the genomspecific polymorphic ISSR-spectra of DNA fragments. Genomic variability and genomic linkage groups cultivars of common buckwheat were determined for the first time. Obtained data can be used in buckwheat breeding program and further molecular characterization of investigated cultivars.

Key words: buckwheat, ISSR-method, inter-microsatellite sequences, genetic variability of cultivars.

Simple tandemly repeating DNA sequences – so-called mini- and microsatellites – are basic components of all eukaryotic genomes. In general, a total number of microsatellite repeats varies from 5×10^3 to 3×10^5 per genome, in which they are distributed fairly evenly (1). As a rule, many mini- and microsatellites are highly variable even in closely related organisms (2). The study of microsatellite polymorphism and intra-microsatellite sequences using ISSR (intersimple sequence repeats) technique (3) is based on amplification of genomic DNA with primers. The primers consist of 15-20 nucleotides homologous to a microsatellite repeat and often including a short (2-4 nucleotides) random "anchor" sequence at the 3'- or 5'-end; the anchor increases selectivity of the primer and reproducibility of ISSR-spectra obtained by DNA amplification between two closely spaced and oppositely oriented microsatellites. ISSR- and RAPD-spectra (random amplified polymorphic DNA) are similar, but ISSR-spectra usually contain a larger set of polymorphic bands (up to 97) which allows to detect a higher level of genomic polymorphism (4, 5).

At present time, ISSR technique is widely used for detection of intraspecific polymorphism, especially in closely related genotypes of cultivated plants. This work is quite actual in connection with contemporary breeding programs of obtaining the varieties of essentially new quality level (4, 6-8), which requires the creation of a wide gene pool of source material carrying multiple economically valuable parameters. This task implies the expansion of present gene pool and a preliminary assessment of natural variability of species.

The species *Fagopyrum esculentum* Moench the family *Polygonaceae* (Buckwheat fam.) is one of most valuable cereal crops becoming increasingly important as an essential dietary product; some buckwheat forms are used as a raw material for production of rutin – an important pharmaceutical preparation of flavonoid group. To create a wide genetic pool, local populations of *F. esculentum ss*p. e*sculentum* were involved into the selection of domestic buckwheat varieties (9, 10). Upon this base, more than 50 local varieties have been locally adapted by the end of 1950ies (11) still being involved in breeding work. Another buckwheat species - *F. tataricum ss*p. *tataricum* - is cultivated mainly in highlands of China, Japan, India, Nepal, as well as in several European countries. In breeding work on buckwheat, this species is used as a component of crosses providing improved cold resistance and viability of plants (12).

Extensive use of local natural populations of *F. esculentum* and samples of the cultivated species *F. tataricum* significantly increased productivity of domestic buckwheat varieties owing to more intense growth processes and resistance to environmental conditions. However, it's still unknown how much the genetic pool of modern domestic buckwheat varieties corresponds to intraspecific variability of *F. esculentum*, and how fully the potential of natural populations is used in breeding work today. The few studies of intraspecific diversity of local wild forms and varieties of *F. tataricum* and *F. esculentum* (mostly of Japanese breeding origin) using molecular techniques (AFLP - amplified fragment length polymorphism, RAPD - random amplified polymorphic DNA, SSR - simple sequence repeats) were performed in Japan (13-15). For Russian buckwheat, the only data of RAPD-analysis have been obtained earlier by the authors (16).

The purpose of this work was to perform ISSR-analysis of intraspecific genetic polymorphism of *Fagopyrum esculentum* and to evaluate the genetic pool of domestic buckwheat cultivars and samples (including *F. tataricum*) most frequently used in breeding work.

Technique. The molecular analysis of genomes of cultivated species was performed upon 27 buckwheat samples from the gene bank collection of the All-Russia Research and Development Institute of Plant Growing (VIR, St. Petersburg) and the Tatarstan Research and Development Institute of Agriculture (TatNIISKh, Kazan): *F. esculentum* - 14 varieties and 8 samples, *F. tataricum* - 5 samples from natural populations of different ecology-geographic origin.

A plant DNA was isolated from 8-10-day-old seedlings along a standard method (17) with additional deproteinization with the mixture of phenol and chloroform. In this work, 13 oligonucleotide sequences homologous to microsatellite repeats were used.

Polymerase chain reaction (PCR), separation and visualization of PCR products were performed by standard techniques (18). The reaction mixture (volume - 15 ul) contained 100 ng of total plant DNA, 0,5 uM primer, 0,2 mM of each dNTP, 2,5 mM $MgCl_2$, 0,3 units Taq-polymerase ("Dialat LTD", Moscow) in the corresponding 1× buffer. The amplification with preliminary denaturation (94 °C, 5 min) was performed in the thermocycler GeneAmp PCR System2700 ("Applied Biosystems", USA) under the regime: denaturation - 94 °C, 30 s, annealing of primer - 30 s: (temperature was selected individually for each primer), elongation - 72 °C, 1 min (40 cycles). Melting temperature for each primer was calculated by the formula T = 69,3 + 0,41 (GC,%) – 650/L, where L - a number of nucleotides in the primer; GC,% - a proportion of GC-nucleotides in the primer. Amplification products were separated by electrophoresis in 1,7% agarose gel (High resolution, "Sigma", MetaPhor, "Cambrex", USA) in 1 × TBE-buffer with a consequent staining with ethidium bromide.

For statistical processing of data, the binary matrixes (1/0) on each primer were composed to calculate the coefficients of genetic variation (the program STATISTICA 6.0) using groupling methods (tree clustering) and the unweighted pair group method with arithmetic mean UPGMA (http://www.statsoft.com).

Results. Characteristics of the studied samples are shown in Table 1; the samples of *F. tataricum* selected upon the results of RAPD-analysis (16).

1. The origin of studied buckwheat samples (*Fagopyrum*)

Species	№ the cultivar from VIR catalogue	Geographic origin
F. tataricum	k-73	Mongolia
F. tataricum	k-65	Republic of Tatarstan, Russia
F. tataricum	k-38	Brest province (Brestskaya Oblast'), Russia
F. tataricum	k-25	Ternopil' province (Ternopil's'ka Oblast'), Ukraine
F. tataricum	k-57	Poland
F. esculentum	k-4131	Canada
F. esculentum	k-4233	Japan
F. esculentum	k-549	Japan
F. esculentum	k-4170	India
F. esculentum	k-4117	China
F. esculentum	k-140	China
F. esculentum	k-143	China
F. esculentum	k-4350	Nepal
F. esculentum	Kama	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Chatyr Tau	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Skorospelaya 86	VNIIZBK (Orel, Russia)
F. esculentum	Krasnostreletskaya	TatNIISKh (Kazan, republic of Tatarstan, Russia), VNIIZBK (Orel, Russia)
F. esculentum	Kazanskaya krupnozernaya	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Karakityanka	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Saulyk	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Cheremshanka	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Bogatyr'	Shatilovo experimental station (Orel province (Orlovskaya Oblast'), Russia)
F. esculentum	Shatilovskaya 5	VNIIZBK (Orel, Russia)
F. esculentum	Ballada	VNIIZBK (Orel, Russia)
F. esculentum	Molva	VNIIZBK (Orel, Russia)
F. esculentum	Batyr	TatNIISKh (Kazan, republic of Tatarstan, Russia)
F. esculentum	Demetra	VNIIZBK (Orel, Russia)

Note: TatNIISKh – Tatarstan Research and Development Institute of Agriculture (*Tatarstanskii Nauchno-Issledovatel'skii Institut Sel'skogo Khozyaistva*), VNIIZBK – All-Russia Research and Development Institute of Grain Legumes and Cereal Crops (*Vserossiiskii Nauchno-Issledovatel'skii Institut Zernobobovykh Kul'tur*), VIR – All-Russia Research and Development Institute of Plant Growing (*Vserossiiskii Naucho-Issledovatel'skii Institut Rastenievodstva*).

The applied primers (Table 2) mainly consisted of dinucleotide repeats with 1-3 additional selective anchor nucleotides in the 3'-end, as well as tri-, tetra- and pentanucleotide repeats. The primers were selected upon the data on their ability to detect genomic polymorphism (6, 19, 20).

2. Characteristics of the used ISSR-primers and results of amplification

ISSR-primer	Nucleotide sequence of the primer	Amplification results (number of detected ISSR-fragments of DNA)
M1	$(AC)_8CG$	Differential spectra (35 polymorphic fragments)
M2	$(AC)_8(C/T)G$	Differential spectra (29 polymorphic fragments)
M3	$(GA)_8(C/T)C$	The absence of PCR-product
M4	$(AG)_8(C/T)C$	Low-polymorphic spectra
M5	$(AT)_8$	Smear
M6	$(CAC)_5$	Low-polymorphic spectra
M7	$(CAG)_5$	Differential spectra (25 polymorphic fragments)
M8	$(GTG)_5$	Low-polymorphic spectra
M9	$(GACAC)_4$	The absence of PCR-product
M10	$(CA)_6(A/G)G$	The absence of PCR-product
M11	$(CA)_6(A/G)$	Differential spectra (24 polymorphic fragments)
M12	$(CA)_6(A/G)(C/T)$	Low-polymorphic spectra
M13	$(AGC)_4(C/T)$	Monomorphic spectra

Testing of ISSR-primers on the limited set of samples (three for each species of buckwheat) has shown that not all of them provided clear multilocus polymorphic spectra of amplified genomic fragments. The primers M4, M12 and M13 (see Table 2) revealed an interspecific polymorphism, but the samples of one species revealed low-polymorphic (M4, M12) or monomorphic spectra (M13). Four primers showed the negative result: an absence of amplification (M3, M9, M10) or smear (M5) were obtained. Smear-detection of amplification products (the primer M5) occurs quite frequently, and this is possibly connected with either the structure of primer sequence, or with frequency of a corresponding microsatellite in the genome. The absence of amplification products when using the primers M3, M9 and M10 can be explained by low frequency of the microsatellite repeats $(GA)_8(C/T)C$, $(CA)_6(A/G)G$ and $(GACAC)_n$ in buckwheat genome. A number of studies assume the partial self-complementarity of such primers, which results in formation of double-chained primer-primer structures as a possible reason for the detected absence of amplification (8, 21).

Three most informative primers (M1, M2 and M7) providing reproducible amplification and clear polymorphic spectra consisting of no less than 20 fragments were selected for a consequent use. In general, 89 polymorphic ISSR-fragments (the length varied from 2700 bp to 320 bp) for 27 buckwheat genotypes were obtained. All the detected genome fragments were polymorphic, i.e., the degree of polymorphism revealed by the three selected ISSR-fragments was 100%, while at the RAPD-analysis - 88% (16). This fact is consistent with data on higher discriminatory ability of the first technique compared with the second one (4).

Five ISSR-fragments specific for all the studied samples of *F. esculentum* were marked, as well as three fragments - for *F. tataricum* and the number of fragments typical for certain samples and varieties. In particular, the unique fragments were found in the sample of Chinese cultivar *F. esculentum* (k-143) – the three fragments of 780 bp, 990 bp (primer M1) and 1500 bp (primer M7) length, in the varieties Bogatyr' (1000_{M2}, 1600_{M2}), Skorospelaya 86 (780_{M7}, 970_{M7}), Demetra (850_{M1}, 1700_{M1}) Karakityanka (1000_{M1},

650_{M2}) and Batyr (910_{M2}). These fragments can be used to design gene-specific DNA markers for buckwheat cultivars, samples and species.

3. Coefficients of intra- and interspecific genetic variation in the studied buckwheat samples (*Fagopyrum)*

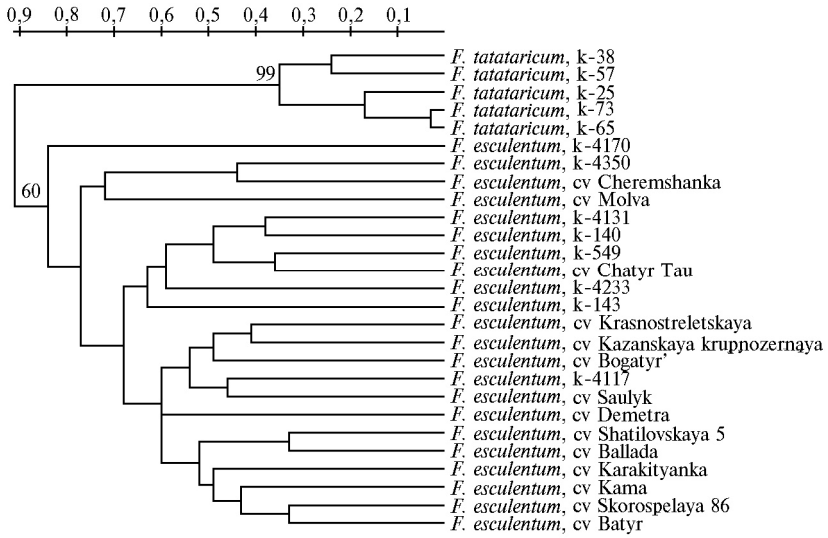
Analyzed group	Coefficient of genetic variation (average)	Standard error	Standard deviation	*Min*	*Max*
F. esculentum (cultivars)	0,169	0,005	0,051	0,056	0,303
F. esculentum (samples)	0,191	0,009	0,048	0,101	0,281
F. esculentum	0,182	0,003	0,051	0,045	0,315
F. esculentum (cultivars)–*F. esculentum* (samples)	0,190	0,005	0,050	0,045	0,315
F. tataricum	0,099	0,014	0,044	0,011	0,169
F. esculentum (samples)–*F. tataricum*	0,304	0,012	0,073	0,112	0,438
F. esculentum (cultivars)–*F. tataricum*	0,292	0,008	0,063	0,135	0,427
F. esculentum–*F. tataricum*	0,300	0,010	0,070	0,110	0,440

Note: Coefficients of genetic variation were calculated in the program STATISTICA 6.0 using groupling methods (tree clustering) and unweighted pair group method with arithmetic mean UPGMA (http://www.statsoft.com).

The obtained ISSR-spectra and data on polymorphism of DNA fragments were used to calculate pairwise coefficients of genetic variation in the genus *Fagopyrum*, which were found to range from 0,002 to 0,440. The narrowest limits of interspecific diversity (0,135-0,427) have been established in the pair *F. esculentum* - *F. tataricum* (Table 3).

Intraspecific genomic variability in *F. esculentum* (0,045-0,315) almost twice exceeded that in *F. tataricum* (0,010-0,169) (see Table. 3), which fact was earlier shown by RAPD-analysis (16).

At the same time, intervarietal differences in *F. esculentum* were very high (coefficients of pairwise identity - 0,056-0,303) and corresponded to genomic variability of wild samples of *F. esculentum* (0,101-0,281) from Japan, China, Nepal, thereby indicating a wide genetic basis of domestic buckwheat varieties. Previously, the results of RAPD test suggested a similar conclusion (16).



Dendrogram showing the degree of genetic identity of studied buckwheat species, cultivars and samples (*Fagopyrum)* (built using the UPGMA hierarhical culuster analysis based on ISSR-labeling).

The dendrogram built upon data of cluster analysis revealed a clear interspecific differentiation of *F. tataricum* and *F. esculentum*: the studied samples were divided into two main groups with reliable bootstrap values (Fig.). The representatives of *F. tataricum* formed a relatively low-polymorphic group, while the cluster of *F. esculentum* included three main groups of samples with different similarity levels. The first clade (basal and most polymorphic) was represented by local wild populations of *F. esculentum* from Nepal and India, as well as two varieties - Cheremshanka and Molva. The second clade was formed by the samples originated from China and Japan, as well as the domestic variety Chatyr Tau. The third clade was fully represented by the studied cultivars. Despite the fact that intervarietal groupings of this clade were supported by low bootstrap values, there was a certain correlation between their degree of identity and morphobiological properties of varieties from these groups. Thus, the group Karakityanka – Kama – Skorospelaya 86 - Batyr was represented by early-ripening and drought-resistant varieties with limited ramification of plants. In this group, the variety Batyr has a longer growing season, but its pedigree includes an ultrafast-ripening cultivar Kazanskaya 309. The group Shatilovskaya 5 - Ballada have close origin, economical and biological properties. The pedigree of cultivar Kazanskaya krupnozernaya showed the presence of material selected from the cultivar Krasnostreletskaya. The variety Demetra formed a separate branch, which may be associated with its significant morphobiological features.

Thus, for the first time ISSR-method was used for genome analysis of buckwheat the genus *Fagopyrum*. The ISSR-primers capable to detect intraspecific polymorphism have been established. For each sample of *F. esculentum* and *F. tataricum*, the unique ISSR-spectra were obtained and the sample- and variety-specific fragments were found, which can be used to develop gene-specific DNA markers for buckwheat. Upon the basis of microsatellite variability and inter-microsatellite sequences, the authors have determined the degree of genetic differences between the samples. Domestic cultivars of *F. esculentum* have shown high level of intervarietal polymorphism comparable to genetic variability of wild forms. The results of this study can be used for identification of varieties and hybrids in breeding programs.

REFERENCES

1. Katti M.V., Ranjekar P.K. and Gupta V.S., Differential Distribution of Simple Sequence Repeats in Eukaryotic Genome Sequences, *Mol. Biol. Evol*., 2001, vol. 18, no. 7, pp. 1161-1167.
2. Toth G., Gaspari Z. and Jurka J., Microsatellites in Different Eukaryotic Genomes: Survey and Analysis, *Genome Res*., 2000, no. 10, pp. 967-981.
3. Zietkiewicz E., Rafalski A. and Labuda D., Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification, *Genomics*, 1994, vol. 20, pp. 176-183.
4. Sica M., Gamba G., Montieri S., Gaudio L. and Aceto S., SSR Markers Show Differentiation Among Italian Populations *Asparagus acutifolius* L., *BMC Genetics*, 2005, no. 6.
5. Muthusamy S., Kanagarajan S. and Ponnusamy S., Efficiency of RAPD and ISSR Markers System in Accessing Genetic Variation of Rice Bean (*Vigna umbellata*) Landraces, *Electronic J. Biotechnol*., 2008, vol. 11, no. 3, pp. 1-10.
6. Prevost A. and Wilkinson M.J., A New System of Comparing PCR Primers Applied to ISSR Fingerprinting of Potato Cultivars, *Theor. Appl. Genet*., 1999, vol. 98, pp. 107-112.
7. Gilbert J.E., Lewis R.V., Wilkinnson M.J. and Caligari P.D.S., Developing an Appropriate Strategy to Assess Genetic Variability in Plant Germplasm Collections, *Theor. Appl. Genet*., 1999, vol. 98, pp. 1125-1131.
8. Joshi S.P., Gupta V.S., Aggarwal R.K., Ranjekar P.K. and Brar D.S., Genetic Diversity and Phylogenetic Relationship as Revealed by Inter Simple Sequence Repeat (ISSR) Polymorphism in the Genus *Oryza*, *Theor. Appl. Genet.,* 2000, vol. 100, pp. 1311-1320.
9. Gorina E.D., Initial Material, its Biological Peculiarities and Efficiency of Selection Methods in Buckwheat, *Extended Abstract of Doct. Sci. Dissertation.,* Zhodino, 1981.
10. Fesenko N.V., Biological Basics and Breeding Methods of Buckwheat, *Extended Abstract of Doct. Sci. Dissertation,* Leningrad, 1974.
11. Krotov A.S., Buckwheat of the USSR and Improvement of Its Commercial Properties, *Extended Abstract of Doct. Sci. Dissertation,* Leningrad, 1955.
12. Campbell C.G., Buckwheat *Fagopyrum esculentum* Moench, Italy, Rome, 1997.
13. Tsuji K. and Ohnishi O., Phylogenetic Relationships among Wild and Cultivated Tartary Buckwheat (*Fagopyrum tataricum* Gaert.) Populations Revealed by AFLP Analyses, *Genes Genet. Syst*., 2001, vol. 76, pp. 47-52.
14. Iwata H., Imon K., Tsumura Y. and Ohsawa R., Genetic Diversity Among Japanese Indigenous Common Buckwheat (*Fagopyrum esculentum*) Cultivars as Determined from Amplified Fragment Length Polymorphism and Simple Sequence Repeat Markers and Quantitative Agronomic Traits, *Genome*, 2005, vol. 48, pp. 367-377.
15. Konishi T., Yasui Y. and Ohnishi O., Original Birthplace of Cultivated Common Buckwheat Inferred from Genetic Relationships among Cultivated Populations and Neutral Populations of Wild Common Buckwheat Revealed by AFLP Analysis, *Genes Genet. Syst.,* 2005, vol. 80, pp. 113-119.
16. Kadyrova G.D., Kadyrova F.Z., Ryzhova N.N. and Kochievva E.Z., RAPD-Analysis of Genomic Polymorphism of *Fagopyrum* Species and Cultivars, *Ecologicheskaya genetika*, 2008, vol. VI, no. 3, pp. 3-10.
17. Ryzhova N.N., Pyshnaya O.V. and Kochieva E.Z., Molecular RAPD-Analysis of Pepper Hybrids, *S.-kh. biol.,* no. 5, pp. 104-106.
18. Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A. and Tingey S.V., DNA Polymorphisms Amplified by Arbitrary Primers are Useful as Genetic Markers, *Nucl. Acids Res*., 1990, vol. 18, pp. 6531-6535.
19. Fang D.Q. and Roose M.L., Identification of Closely Related Citrus Cultivars with Inter-Simple Sequence Repeats Markers, *Theor. Appl. Genet.,* 1997, vol. 95, pp. 408-417.
20. Wang G., Mahalingam R. and Knap H.T., (C-A) and (G-A) Anchored Simple Sequence Repeats (ASSRs) Generated Polymorphism in Soybean, *Glycine max* (L.), *Merr. Theor. Appl. Genet*., 1998, vol. 96, pp. 1086-1096.
21. Cekic C., Battey N.H. and Wilkinson M.J., The Potential of ISSR-PCR Primer Pair Combinations for Genetic Linkage Analysis Using the Seasonal Flowering Locus in *Fragaria* as a Model, *Theor. Appl. Genet*., 2001, vol. 103, pp. 540-546.