ISSR primer screening for analysis of genetic diversity among *Scutellaria tuvensis* (Lamiaceae) populations

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Abstract. Using the Diamond DNA kit, high quality nuclear DNA was isolated from dry leaves of the endemic species *Scutellaria tuvensis*. The selection of primers for ISSR analysis of genetic polymorphism of natural populations is described. During the experiment, 22 primers were tested, their effectiveness was assessed on a point scale. When assessing the primers, the number of reproducible amplified DNA fragments, the clarity and brightness of the obtained fragments, and only distinctive bands were taken into account. As a result, 10 ISSR markers were selected that are the most informative for assessing the population diversity of the species.

When developing a strategy for the conservation of rare and endemic plants, it is necessary to take into account the genetic diversity of their populations [1]. *Scutellaria tuvensis* Juz. is endemic species from the family Lamiaceae, with a narrow range that covers the southeastern part of Tuva (Russia) and the adjacent northwestern territory of Mongolia [2,3,4]. Population genetic studies of this species have not previously been conducted. Also, no studies have been conducted on sequencing the genome of *S. tuvensis*, therefore, to identify DNA polymorphism in populations of the species, PCR-based methods can be used, which do not need any prior information about the target sequences in the genome or its parts. One of the most common methods for detecting genetic polymorphism in plants that does not require knowledge of the nucleotide sequences of the genome is ISSR analysis (Inter Simple Sequence Repeats), which uses primers usually 16 −25 bp long, they consist of tandem short 2-4 nucleotide repeats and anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences [5,6,7]. The first stage of molecular genetic analysis is the selection of primers that are effective for studying genetic diversity. Selection of the optimal primer annealing temperature increases the efficiency and specificity of amplification.

The aim of this work is to screen ISSR markers and select the most informative primers for assessing the genetic polymorphism of natural populations of *S. tuvensis*.

Materials and methods

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The work was carried out in the Laboratory for the introduction of rare and endangered plants of the Central Siberian Botanical Garden SB RAS (Russia, Novosibirsk). DNA was extracted from dried leaves using a Diamond DNA kit (ABT, Russia) according to the manufacturer's protocol; the material weighed in amount was 8 mg. The purity (the ratio of the optical power values at 260 and 280 nm) and the concentration (ng/μL) of the extracted DNA were determined on a BioSpectrometer kinetic spectrophotometer (Eppendorf, Germany). For PCR, the DNA concentration of each sample was leveled to 10 ng/μL.

PCR was carried out on a C 1000 Thermal Cycler (BioRad Laboratories, USA); a mixture of HSTaq DNA polymerase was used for PCR. All reagents used for PCR production are produced by Evrogen, Russia. The reaction mixture of 15.0 μL contained: 8.6 μL of sterile H_2O; 1 unit HSTaq DNA polymerase; 0.4 mM primer; 2.5 mM $MgCl_2$; 1×Taq buffer; 1.0 mM dNTPs mixture; 2.0 μL DNA template. As negative controls, we performed PCRs containing all the PCR components and sterile H_2O instead of DNA template. The amplification program was developed based on the study by Guo et al. [5]: primary DNA denaturation – 5 min at 95 ºC; 40 amplification cycles: denaturation – 1 min at 95 ºC; primer annealing – 1 min and elongation – 2 min at 72 ºC; final elongation of the nucleotide chain – 10 min at 72 ºC. The annealing temperatures, depending on the primer, varied from 43.0 to 60.8 ºC. To check the reliability of the obtained ISSR spectra, the experiment was repeated at least two times. Amplification products were stained with SYBR-Green (Medigen, Russia) and separated by electrophoresis in 1.5% agarose gel in 1×TBE buffer. The size of ISSR fragments was determined by comparing their mobility with the molecular weight marker 100bp + DNA Ladder (Evrogen, Russia). The ISSR profile was visualized using the GelDoc XR+ (BioRad Laboratories, USA) and analyzed with the Image Lab Software (BioRad Laboratories, USA)

For the analysis of primers, a limited sample (3 DNA templates) and an annealing temperature gradient (at least 4 values) were used for each primer. When choosing more informative markers, the number of reproducible amplified DNA fragments, the clarity and brightness of the obtained fragments, and only distinctive bands were assessed on a point scale (from 0 to 3). Subsequently, the sum of points for each temperature of each sample and the average for the primer were calculated. The average score (“Point”) for the primer is shown in the Table. A total of 22 ISSR primers were analyzed.

Results

The use of the Diamond DNA kit allowed to obtain nuclear DNA with high quality: purity 1.93±0.04, concentration 125.85±15.12 ng/μL. The obtained result allows this commercial kit to be considered effective and optimal for DNA isolation from dried leaves of *S. tuvensis*.

Depending on the primer and on the annealing temperature, from 0 (M 14) to 7 (UBC 856) DNA fragments were amplified, and their size varied from 200 to 2000 bp. ISSR spectra obtained with primers UBC 810, UBC 823, UBC 826 and UBC 857 are shown in the figure.

Primers UBC 807, 819, 823, 835, HB 14, M 9, M 14 were not informative for the molecular genetic analysis of *S. tuvensis* (0–3 points), the obtained profiles were characterized by a small number or absence of amplified fragments, poor visibility of amplicons. 10 primers – UBC 811, 825, 826, 830, 834, 836, 840, 855, 856 and 857 showed the best results, gaining an average of 5.5 to 8.8 points. These markers are characterized by the largest number of reproducible distinctive clarity bands, no background luminescence of the track, and these markers also revealed genetic polymorphism between the analyzed samples (Figure). These markers were selected for further studies to assess the genetic

polymorphism of natural populations of the endemic species *S. tuvensis*. The other primers can be used as additional primers for subsequent studies.

Table. The general characteristics of the ISSR primers

Primer	Sequence (5'→3')	Temperature range,°C	Annealing temperature, °C	Point	Band size, bp
UBC 807	$(AG)_8T$	48.0–51.0	51.0	3.0	350–800
UBC 810	$(GA)_8T$	51.0–53.2	53.0	5.4	200–800
UBC 811	**$(GA)_8C$**	**51.0–54.0**	**53.0**	**5.5**	**350–2000**
UBC 819	$(GT)_8A$	48.0–51.0	50.0	3.0	1100–1400
UBC 823	$(TC)_8C$	54.7–60.8	55.0	1.5	500–1000
UBC 825	**$(AC)_8T$**	**55.0–58.6**	**57.0**	**8.3**	**250–1000**
UBC 826	**$(AC)_8C$**	**54.7–60.8**	**56.0**	**7.0**	**400–1400**
UBC 830	**$(TG)_8G$**	**51.3–52.9**	**52.0**	**5.8**	**350–1100**
UBC 834	**$(AG)_8Y*T$**	**48.0–51.2**	**49.0**	**5.5**	**250–900**
UBC 835	$(AG)_8YA$	51.0–54.0	53.0	1.5	500–900
UBC 836	**$(AG)_8YA$**	**51.0–54.0**	**53.0**	**5.5**	**300–900**
UBC 840	**$(GA)_8YT$**	**51.3–52.9**	**51.0**	**8.5**	**300–2000**
UBC 855	**$(AC)_8YT$**	**43.1–48.2**	**43.0**	**7.0**	**350–900**
UBC 856	**$(AC)_8YA$**	**52.9–54.1**	**53.0**	**7.0**	**450–2000**
UBC 857	**$(AC)_8YG$**	**51.0–53.0**	**52.0**	**8.8**	**400–1300**
UBC 868	$(GAA)_6$	48.0–50.0	49.0	5.4	700–900
HB 10	$(GA)_6CC$	44.3–46.0	46.0	4.5	350–900
HB 12	$(CAC)_3GC$	48.0–51.2	51.0	3.5	450–1000
HB 14	$(CTC)_3GC$	48.0–51.2	49.0	1.5	900–1400
17899A	$(CA)_6AG$	48.2–50.0	48.2	5.3	700–1500
M 9	$(GACAC)_4$	55.1–58.6	56.0	3.5	1000–1500
M 14	$(GACA)_4$	43.1–48.2	—	0	—

Notes. Y = C or T.

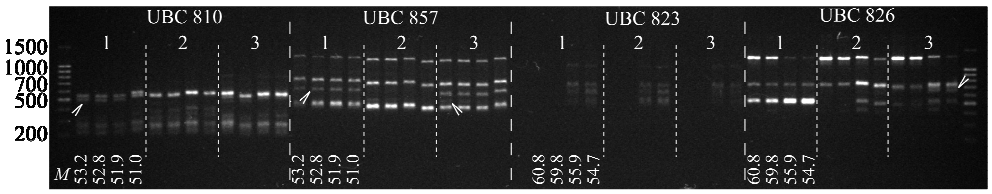

Figure. PCR amplification profiles obtained with ISSR markers (UBC 810, 857, 823, 826) of S. tuvensis. M – 100bp + DNA Ladder; 1, 2, 3 – samples; 53.2, 52.8, 51.9, 51.0, 60.8, 59.8, 55.9, 54.7 – temperature range of a primer annealing. The arrows indicate polymorphic loci.

This is the first known report on the use of ISSR markers for creating genetic fingerprints of *S. tuvensis* genotypes. The results clearly demonstrate that ISSR markers can be used in a genetic diversity study of this taxon. Thus, di-nucleotide ISSR primers with an anchor were successfully optimized in the present study for the molecular genetic analysis of *S. tuvensis* populations, 10 most informative primers were selected: UBC 811, 825, 826, 830, 834, 836, 840, 855, 856 and 857.

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