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# DNA barcoding of some endangered plant species of the Altai Mountains based on five genetic markers

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## ABSTRACT

The analysis of DNA nucleotide sequences is an important source of information in the identification of plant taxa or plant components in the composition of medicines. In this work, we studied the possibility of using universal molecular markers for DNA barcoding of rare plant species distributed in the Altai Biosphere Reserve, located in the Altai Republic (South Siberia, Russia). In order to identify commercially-used endangered species, universal markers recommended for DNA barcoding were applied: ITS (Internal Transcribed Spacers) region of nuclear ribosomal DNA and four regions of chloroplast DNA: *trn*L-F, *trn*H-*psb*A *rpl*32-*trn*L, and *rpl*16. Among 61 species of rare flowering plants protected in the Altai Biosphere Reserve, ten species belonging to eight families were studied. The results of sequencing showed that ITS, *rpl*32-*trn*L and *trn*H-*psb*A core plant barcodes are the most effective markers for discriminating rare species from the Altai Biosphere Reserve.

**Keywords:** DNA barcoding, endangered species, ITS, *trn*L-*trn*F, *trn*H-*psb*A, rpl32-*trn*L, *rpl*16-*trn*L, Altai Biosphere Reserve

## РЕЗЮМЕ

**Нуждина Н.С., Ерофеева А.С., Бондарь А.А., Ковтонюк Н.К. ДНК-штрихкодирование некоторых редких видов растений Алтая по данным анализа пяти генетических маркеров.** Анализ нуклеотидных последовательностей ДНК является важным источником информации при идентификации таксонов у растений или растительных компонентов в составе медицинских препаратов. В настоящей работе нами исследована возможность применения молекулярных маркеров для ДНК-штрихкодирования редких краснокнижных видов растений, обитающих на территории Алтайского биосферного заповедника. Для идентификации видов были использованы маркерные локусы ITS ярДНК и *trn*L-F, *trn*H-*psb*A *rpl*32-*trn*L и *rpl*16 хлДНК. Результаты секвенирования показали, что наиболее эффективными для идентификации редких видов являются маркеры ITS, *rpl*32-*trn*L и *trn*H-*psb*A.

**Ключевые слова:** ДНК-штрихкодирование, редкие виды, ITS, *trn*L-*trn*F, *trn*H-*psb*A, rpl32-*trn*L, *rpl*16-*trn*L, Алтайский биосферный заповедник

The mountains of South Siberia often correlate with the northern geographic limits of many endangered species. The cross-border position of the Altai Mountains, located at the junction of the North Asia and the Central Asia, and the variety of climatic and cenotic conditions provide the abundance of species (ca. 2800 vascular plant species), landscapes and biotopes and prominent level of endemism as well (8.5–10.3 %) (Artemov et al. 2007, Erst et al. 2022). Over 1650 species of vascular plants inhabit the territory of the Altai Biosphere Reserve (ABR), among them, 250 species are endemic to Altai-Sayan floristic territory and 120 species are relicts (Zolotukhin & Erofeeva 2022).

An accurate identification of rare or endangered plant species is critical for understanding in their limits of diversity, geographic distributions and extinction threat. The research intended for DNA barcoding of rare species of the Altai Biosphere Reserve (ABR) will promote the developing a concept for the genetic resources conservation and creating a germplasm collection of endangered species that tend to decrease in numbers in Altai Republic due to unauthorized collection of vulnerable plants.

In recent years plant DNA barcoding research has grown rapidly (Cowan et al. 2006, Kress & Erickson 2007). According to the recommendation of the Consortium for the Barcode of Life (CBOL) plant barcoding studies use the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (White et al. 1990) and one or more plastid regions: *rbc*L, *trn*H-*psb*A (Kress & Erickson 2007), *mat*K (Johnson & Soltis 1994) or the non-coding spacers *trn*L-F (Taberlet et al. 1991) as universal barcodes.

In this study, ten endangered plant species were selected protected in Altai Mountain region and listed in the Red Book (Maneev 2017). Among them, nine species are naturally distributed in ABR: *Allium altaicum* Pall., *Chrysanthemum sinuatum* Ledeb. (=*Dendranthema sinuatum* (Ledeb.) Tzvelev), *Cypripedium macranthos* Sw., *Daphne mezereum* L., *Erythronium sibiricum* (Fisch. & C.A. Mey.) Krylov, *Rheum altaicum* Losinsk., *Rhodiola algida* (Ledeb.) Fisch. & C.A. Mey., *R. rosea* L., *R. stephanii* (Cham.) Trautv. & Mey. One species, *Sibiraea laevigata* (L.) Maxim. (= *S. altaiensis* (Laxm.) C.K. Schneid.) was introduced from West Altai Mountain and kept in ABR as a cultivar.

The specific aims were as follows: 1) to extract and to amplify genomic DNA by the marker loci: ITS1-5,8S-ITS2 (Internal Transcribed Spacers) of nuclear ribosomal DNA and *trn*L-*trn*F, *trn*H-*psb*A, rpl32-*trn*L, and *rpl*16 of chloroplast

 

DNA; 2) to analyze the variability of five marker loci in different taxa of endangered plant species of ABR; 3) to evaluate the sequence recoverability and informativeness of five DNA markers in effective and reliable vulnerable species discrimination and detection the origin of the plant parts and plant-derived medicines which can contain medicinal commercialized plants.

## MATERIAL AND METHODS

The objects of the study were ten endangered species of high vascular plants listed in the Red Book (Maneev 2017) and distributed in ABR. The main leaf material of specimens was newly collected by the authors Natalia S. Nuzhdina and Anna S. Erofeeva in 2021–2022 from natural populations located at ABR and adjacent territories in Altai-Sayan Mountains (Altai and Tuva Republic, Russia). The specimen of *Sibiraea laevigata* was collected from the cultivar located at the experimental plot of ABR. Newly collected voucher specimens were deposited in M.G. Popov Herbarium collection (NSK) of the Central Siberian Botanical Garden SB RAS. Specimen *Rhodiola algida* (NSK0089762) from NSK collection was involved into the study.

Genomic DNA was isolated from dry material using CTAB method of Doyle & Doyle (1987). The nrDNA ITS region was amplified using the primers ITS5F and ITS4R (White et al. 1990). The PCR profile consisted of an initial denaturation at 94ºC for 5 min; 38 cycles with denaturation at 95ºC for 50 s, annealing at 50ºC for 50 s and elongation at 72ºC for 1 min; and a final extension of 5 min at 72ºC. The *trn*L-*trn*F region was amplified using the universal primers *trn*L(c) and *trn*L(f) (Taberlet et al. 1991) and the program: initial denaturation at 94ºC for 5 min 31 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 1 min and elongation at 72ºC for 2 min (Amirahmadi et al. 2010). The *trn*H-*psb*A region was amplified using the primers *trn*HGUG (Tate & Simpson 2003) and *psb*A (Sang et al. 1997). The PCR conditions were: initial denaturation at 94ºC for 5 min; 35 cycles with denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and elongation at 72ºC for 1 min; a final extension of 10 min at 72ºC (Shaw et al. 2005, Kress et al. 2005). The *rpl*32-*trn*L region was amplified using the primers *rpl*32-F and *trn*LUAG according to the PCR program: initial denaturation at 94ºC for 5 min; 30 cycles with denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min and elongation at 65ºC for 4 min; a final extension of 5 min at 65ºC (Shaw et al. 2007). The locus *rpl*16 coding the ribosomal protein L16 was amplified using the primers *rpl*16_71F (Jordan et al. 1996) and *rpl*16-1515R (Kelchner & Clark 1997) with a little modification (Table 1). The PCR conditions for *rpl*16 intron were set at an initial 4 min at 94°C, followed by 29 cycles of 92°C for 45 s, 58°C for 45 s, and 72°C for 2 min, with a terminal incubation of 10 min at 72°C.

The five candidate DNA barcodes were amplified by polymerase chain reaction (PCR) in 40 µl mixtures containing 2 µl of DNA template (10 ng/µl ), 3.6 µl of 25 mM MgCl, 1.1 µl of each primer (10mM), 0.5 µl of dNTPs (20 mM of each dNTP), 4 µl of 10x Taq-PCR buffer and 1.7U of Taq polymerase (BioSan, Russia). All PCR reactions were done in a Thermal Cycler T1000 (Bio-Rad, USA). Taq-polymerase and sterile water. PCR products (4 µl) were stained with 1 µl of loading buffer containing 30x SYBR-Green (Lumiprobe, Russia) and separated by electrophoresis on 0.8 % agarose gel. Visualization of amplified DNA fragments was performed using Gel-Doc XR+ documentation system equipped with the ImageLab Software (Bio-Rad, USA). The rest volume of PCR products (35 µl ) were sent for Sanger sequencing in the SB RAS Genomics Core Facility (ICBFM, Novosibirsk).

PCR fragments purification and sequencing were performed as described before (Nuzhdina & Kovtonyuk 2022). Analysis of the nucleotide composition of PCR fragments was carried out by automated sequencing of their primary structure on an Applied Biosystems ABI 3130XL Genetic Analyzer capillary sequencer (Genomics Center for Collective Use, Novosibirsk). The nucleotide sequences reported in this paper has been submitted to the GenBank. GC-content was calculated using Unipro UGENE v. 46.0. (Okonechnikov et al. 2012).

## RESULTS

Our results confirm that CTAB method is a universal for DNA extraction in different taxa of Angiosperms. The dataset includes 7 accessions (species) for nrDNA ITS, 8 accessions for *trn*L-F, 9 accessions for *trn*H-*psb*A, 10 accessions for *rpl*32-*trn*L and 4 accessions for *rpl*16. The primers for five selected DNA barcodes were found to be applicable for species endangered in ABR.

Totally 38 barcode sequences were generated from the study and deposited in the GenBank database with accession numbers shown in the Table 2. Of those, eleven sequences (29 % of total number of nucleotide data obtained) marked with an asterisk were newly sequenced (Table 3). The PCR amplification and sequencing success rates consistently exceeded 63.6, 81.8, 90.9 and 100 % consequently for the markers ITS, *trn*L-F, *trn*H-*psb*A and *rpl*32-*trn*L except *rpl*16 which has the lowest level of PCR amplification success. Thus, only four *rpl*16 sequences (40 %) available for identification were obtained and analyzed (OQ628437, OQ628438, OQ628440, and OQ628441). Remarkably, *Rhodiola* species have not provided any readable *rpl*16 sequence.

The total sequence recoverability of DNA barcoding was of 78 %. This result is somewhat lower than in study of endemic species of Altai Mountain Country (80 %; Erst et al. 2022) or in DNA badcoding of Canadian arctic flora (87 %; Saarela et al. 2013).

In some cases, PCR product was not obtained, or the amplified fragment (ITS or *rpl*16) characterized by overlapping signals were unsuitable for direct reading and require the use of cloning technologies, which complicates the DNA barcoding procedure based on the principle of convenience and simplicity. The lowest recoverability was observed in sequencing of *Rhodiola algida* specimen, the only barcode *rpl*32-*trn*L was obtained for. Here we can conclude that several samples failed in amplification with the studied markers (excluding *rpl*32-*trn*L) due to primer mismatch and therefore requires the application of alternative PCR primers.

The length of the *trn*H-*psb*A region in the dataset ranged from 300 bp in *Rhodiola rosea* to 648 bp in *Cypripedium macran-*

*thos*, with a mean length of 463 bp. The *rpl*32-*trn*L region had a length of 709 bp in average; the smallest *rpl*32-*trn*L sequence was noticed for *Rheum altaicum* (518 bp); the longest one (1010 bp) was detected for *Chrysanthemum sinuatum* (excluding partially readible *rpl*32-*trn*L region in *Cypripedium macranthos*). ITS sequence length varied from 660 in *Daphne mezereum* to 740 bp in *Cypripedium macranthos*, with a mean value of 690 bp. The PCR fragment length from 664 in *Allium altaicum* to 966 bp in *Sibiraea laevigata* (783 bp in average) was noticed for *trn*L-F marker DNA locus. The minimal length of *rpl*16 region have been registered for *Sibiraea laevigata* (656bp) and the maximum – for *Chrysanthemum sinuatum* (881 bp). Therefore, the minimal length variation was noticed for ITS (690bp ± 8.4); the most variable site in length was chloroplast *rpl*32-*trn*L region (709 bp ± 49.2).

GC content variation existed in each region, which was 45–60 % (mean value 52.5 ± 1.6) bp for nrDNA ITS, 31–36 % (33.8±0.5) for *trn*L-F, 23–36 % (29.1 ± 1.2) for *trn*H-*psb*A, 23–32 % (27.9 ± 0.9) for *rpl*32-*trn*L, and 28–34 % (30.8±0.9) for *rpl*16.

Among five studied DNA markers, ITS region was distinguished by an increased content of cytosine (C) and guanine (G) nucleotides (>50 %) connected with each over in DNA molecule by double hydrogen bonds. Among the studied species, GC-content reaches the maximum value in *Erythronium sibiricum* (up to 60 %).

**Table 1.** The sequences of primers used in the PCR amplifications.

| Primer name | Primer sequence (5'-3') |
|---|---|
| ITS-5 | GGAAGGAGAAGTCGTAACAAGG |
| ITS-4 | TCCTCCGCTTATTGATATGC |
| *trn*L(c) | CGAAATCGGTAGACGCTACG |
| *trn*L(f) | ATTTGAACTGGTGACACGAG |
| *trn*H$^{GUG}$ | CGCGCATGGTGGATTCACAATCC |
| *psb*A | GTTATGCATGAACGTAATGCTC |
| *rpl*32-F | CAGTTCCAAAAAAACGTACTTC |
| *trn*L$^{UAG}$ | CTGCTTCCTAAGAGCAGCGT |
| *rpl*16_71F | GCTATGCTTAGTGTGYGACTCGTTG |
| *rpl*16-1515R | CCYTTCATTCKTCCTCTATGTTG |

## CONCLUSIONS

In this study, our results confirmed that chloroplast *rpl*32-*trn*L and *trn*H-*psb*A marker DNA loci are the best DNA barcodes for discriminating the species and can compete with universal ITS loci in terms of their sequence recoverability and variability in distinct plant taxa. The obtained original data are in full agreement with the phylogenetic relationship of the studied taxa and could be used in evolutionary reconstructions. ITS, *rpl*32-*trn*L, and *trn*H-*psb*A DNA barcodes can be successfully employed to assess the level of genetic diversity in endangered and protected plant species. The above results obtained by nucleotide analysis of informative and universal DNA barcodes may provide useful information for species identification and pedigree analysis between widely commercialized medicinal plants like *Rhodiola* sp. and adulterants. Accurate species identification and proof of the provenance of commercialized herbs is an important step towards the protection of endangered plant resources and natural populations located at ABR. In this regard, DNA fingerprinting or barcoding belongs to the promising methods, which need reliable molecular references. A global barcoding database including nucleotide data from different populations of the same species are successful for the identification of endangered species of ABR.

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**Table 2.** Accessions sampled for DNA barcoding, together with related GenBank accession number.

| Taxon | Voucher | GenBank accession no. | | | | |
|---|---|---|---|---|---|---|
| | | ITS | *trn*L-*trn*F | *trn*H-*psb*A | *rpl*32-*trn*L | *rpl*16-*trn*L |
| *Allium altaicum* | NSK0144698 | – | OQ672771 | PP557258 | PP557267 | – |
| *Chrysanthemum sinuatum* | NSK0144709 | OQ423187* | OQ672772 | PP557259 | PP557268 | OQ628437 |
| *Cypripedium macranthos* | NSK0144699 | OQ423188 | OQ672773 | PP557260 | PP557269 | – |
| *Daphne mezereum* | NSK0144710 | OQ423189 | OQ672774 | PP557261 | PP557270* | – |
| *Erythronium sibiricum* | NSK0144708 | OQ423190 | – | PP557262 | PP557271 | OQ628438 |
| *Rheum altaicum* | NSK0144706 | – | OQ672776 | PP557263* | PP557272* | OQ628440* |
| *Rhodiola algida* | NSK0089762 | – | – | – | PP557273* | – |
| *Rhodiola rosea* | NSK0144687 | OQ423193 | OQ672778 | PP557264 | PP557274 | – |
| *Rhodiola stephanii* | NSK0144690 | OQ423199 | OQ672783* | PP557265* | PP557275* | – |
| *Sibiraea laevigata* | NSK0144700 | – | OQ672784 | PP557266* | PP557276* | OQ628441 |

* Newly sequenced region for this taxa

**Table 3.** Interspecific genetic divergences inferred from the five DNA marker loci examined

| | ITS | *trn*L-*trn*F | *trn*H-*psb*A | *rpl*32-*trn*L | *rpl*16-*trn*L |
|---|---|---|---|---|---|
| PCR effectiveness (%) | 7 (63.6) | 9 (81.8) | 10 (90.9) | 11 (100) | 5 (45.4) |
| Region length (bp) | 660–740 | (276*) 664–966 | 300–648 | (247*)518–1010 | 656–881 |
| Average region length (bp) | 690±8.4 | 783±24.9 | 463±38.0 | 709±49.2 | 784±25.3 |
| CG content, % | 45–60 | 31–36 | 23–36 | 23–32 | 28–34 |
| Average CG content (mean±st.d.) | 52.5±1.6 | 33.8±0.5 | 29.1±1.2 | 27.9±0.9 | 30.8±0.9 |
| Interspecific distances | 0.032–0.674 | 0.013–0.892 | 0.033–1.357 | 0.014–1.052 | 0.044–0.488 |
| Average interspecific distances | 0.497 | 0.523 | 0.817 | 0.750 | 0.307 |

*Partial sequences; not included in calculation of average meanings of length and GC content

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