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DNA BARCODING OF RARE AND ENDANGERED PLANT SPECIES OF THE REPUBLIC OF KAZAKHSTAN

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ABSTRACT

The ability to identify vegetation types is very important for the study of flora biodiversity. DNA barcoding makes it possible to standardize the identification procedure by reference DNA regions. The most popular sequences for molecular identification and phylogenetic studies are Internal Transcribed Spacer (ITS) sequences. ITS are widely used as a phylogenetic marker to classify plants at various taxonomic levels, including genus, species, and subspecies. 29 samples of rare and endangered plant species were collected from Aksu-Zhabagly State Nature Reserve, Sayram-Ugam National Park and Berikkara Tract State Nature Reserve. DNA was isolated by the CTAB method, oligonucleotides flanking the ITS region of the nuclear DNA region were selected and synthesized. Sequencing was carried out and ITS sequences of the region were obtained for 29 species of rare and endangered plant species in Kazakhstan.

Key words: Flora, ITS, fauna, DNA barcoding, identification, CTAB method

INTRODUCTION

Genomic technologies, new sequencing algorithms and the development of bioinformatics methods of analysis make it possible to obtain genomic sequences, transcriptome and exome sequences for model and cultivated plant species. It has improved the understanding of plant genomics and allows to draw deep conclusions about plant biology. Reduction of sequencing costs and improved methods for assembling and analyzing genomes lead to the constant increase in the number and quality of sequenced plant genomes. The high information content of DNA molecules contributes to the development of accurate plant identification method, based on the sequence of conservative loci. This is especially true for endemic, rare and/or endangered plant species. From the perspective of identifying such plants, it is preferable to use a single conservative locus that is present in all representatives, but at the same time has a good determining trait.

The ability to determine the species of plants is very important for the study of flora biodiversity. Molecular genetic methods cannot replace the classical methods of species identification, but the use of molecular markers is a good addition to phylogenetic studies. Molecular approaches are based on the basic pattern according to which the degree of relationship between plants is correlated with the level of similarity in homologous nucleic acid sequences.

The idea of DNA barcoding formulated in 2003 [1] makes it possible to standardize the identification procedure by reference DNA regions. As such reference loci, the following are reported to be the promising candidates: nuclear sequences ITS1 and ITS2, plastid sequences *rpoB*, *rpoC1, rbcL, matK, psbK-psbI, trnH-psbA, atpF-atpH* [2-6].

The most popular sequences for molecular identification and phylogenetic studies are those of the internal transcribed spacers of ribosomal genes (ITS) [7]. The ITS region is located between the structural genes of ribosomal RNA 18S, 5.8S, and 26S, which represent a single cluster of nuclear genes organized as tandem repeats. Each cluster of ribosomal genes consists of a transcribed region (genes 18S, 5.8S and 26S), internal transcribed spacers located on either side of 5.8S, called ITS1 and ITS2, respectively, and flanking external transcribed spacers ETS1 and ETS2 [8].

ITS are widely used as a phylogenetic marker to classify plants at various taxonomic levels, including genus, species, and subspecies [9,10]. The advantage of ITS markers is: versatility, high variability, the presence of conservative boundaries, high copy number (up to 30,000 copies per cell) [11]. The length of the ITS region is up to 800 bp in angiosperms, which corresponds to the features of DNA barcoding. Despite such disadvantages as: the observed difference between copies of ITS within the same genome in the form of deletions/insertions and the similarity in some cases in ITS in different species, in general, the advantages of ITS sequencing outweigh these disadvantages.

The aim of the work was to sequence the ITS of the region in samples of rare and endangered plant species of Kazakhstan. The tasks were: collection of plant samples from the National Parks of Kazakhstan, isolation of complete DNA, optimization of conditions for amplification and sequencing of the ITS region.

MATERIALS AND METHODS

Samples of plants

The leave samples of rare and endangered plant species of Kazakhstan, collected in the summer of 2021 on the territory of the national parks of the Republic of Kazakhstan, were used as research objects.

DNA extraction

Total DNA was extracted from frozen leaves of the samples by CTAB method [12]. Briefly, 150 mg of the frozen leaves was homogenized at the liquid nitrogen. 300 µL of warmed (65°C) extraction buffer (1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2% CTAB) and 20 mg of polyvinylpyrrolidone (PVP) were mixed. The volume was increased to 1000 µL with extraction buffer. The mixture was warmed at 65°C for 25 min. The mixture of the volume 1000 µL isoamyl alcohol/chloroform was added and was mixed by inverting the tube and centrifuging at 14,000×g, for 15 min.

Upper phase (300 μL) was mixed with isopropanol, and used in further incubation at 22°C for 20 min followed by centrifugation at 10,000×g, for 30 min at 4°C. Precipitated DNA was washed with 500 μL 70% ethanol and centrifugated 10,000×g, for 10 min at 4°C. The pellet was dried and suspended in TE buffer. The RNA was degraded by incubation the sample with RNAse A at 37°C for 1 h. DNA precipitated with 96% ethanol after RNA degradation. The quality of DNA was checked in agarose gel electrophoresis.

Sequencing

Sequencing reactions were carried out using a Big Dye Terminator v 3.1 Cycle sequencing Kit (Applied Biosystems, USA). DNA sequencing was performed with Applied Biosystems ABI 3730xl 96-capillary DNA analyzer (Applied Biosystems, USA).

Specie identification

Chromatograms after sequencing were analyzed and edited with SnapGene viewer software v.6.0.5. Edited sequences were subjected to BLAST searches [13] for preliminary analysis. Multiple sequence alignment was carried out by VectorNTI Advance 11 program (Invitrogen).

RESULTS AND DISCUSSION

Sequence analysis was carried out in GenBank to determine universal molecular genetic DNA loci for species identification of plants of the families *Caryophyllaceae*, *Caprifoliaceae*, *Liliaceae*, *Celastraceae*, *Ranunculaceae*, *Iridaceae* and *Alliaceae*. Samples of rare and endangered plant species of Kazakhstan collected in 2021 are included in these taxonometric groups. As a result, it was determined that the Internal Transcribed Spacer (ITS) region has the required discriminating ability for species identification. The 6 oligonucleotides were chosen: ITS_U1 (GGAAGTAGAAGTCGTAACAAGG), ITS_U2 (GCGTTCAAAGATTCGATGATTC), ITS_U3 (CATCGATGAAGAACGCAGC), ITS_U4 (GGTTTCTTTTCCTCCGCTTA), ITS1 (TCCGTAGGTGAACCTGCGG), ITS2 (TCCTCCGCTTAATTGATATGC), 18S1F (TACCTGGTTGATCCTGCCAGTAG), 18S1R (TAATATACGCTATTGGAGCTGG) These oligonucleotides were covered ITS region. Figure 1 depicts the scheme of the target region for ITS primers.

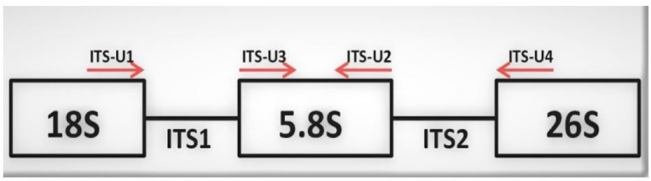

Figure 1 – The scheme of annealing primers for ITS region

From 29 plant samples collected in the summer of 2021 in: Aksu-Zhabagly State Nature Reserve, Sayram-Ugam National Park and Berikkara Tract State Nature Reserve the DNA was isolated. The amount of DNA isolated was 12.3-102.15 μg. The plant samples information Table 1 provides information of the samples, the collection locality and the amount of DNA isolated.

Table 1 – Plant samples information

Sample	Plant identification	Collection locality	DNA, μg
1	*Tulipa gréigii*	Aksu-Zhabagly State Nature Reserve	53.4
2	*Tulipa gréigii*	Aksu-Zhabagly State Nature Reserve	47.7
3	*Tulipa kaufmanniana*	Aksu-Zhabagly State Nature Reserve	40.8
4	*Tulipa kaufmanniana/gréigii hybrid*	Aksu-Zhabagly State Nature Reserve	48.3
5	*Juno orchioides*	Aksu-Zhabagly State Nature Reserve	48.9
6	*Tulipa kaufmanniana*	Aksu-Zhabagly State Nature Reserve	37.05
7	*Allium karataviense*	Aksu-Zhabagly State Nature Reserve	11.4
8	*Tulipa turkestanica*	Aksu-Zhabagly State Nature Reserve	55.0
9	*Tulipa bifloriformis*	Aksu-Zhabagly State Nature Reserve	54.6
10	*Sorbus persica*	Aksu-Zhabagly State Nature Reserve	98.25
11	*Lonicera karelinii*	Aksu-Zhabagly State Nature Reserve	102.15
12	*Aconitum talassicum*	Aksu-Zhabagly State Nature Reserve	18.0
13	*Euonymus koopmannii*	Aksu-Zhabagly State Nature Reserve	16.3
14	*Morina kokanica*	Aksu-Zhabagly State Nature Reserve	41.9
15	*Scutellaria subcaespitosa*	Aksu-Zhabagly State Nature Reserve	22.3
16	*Astragalus turczaninowii Kar. et Kir*	Sayram-Ugam National Park	22.0
17	*Ephedra equisetina Bunge*	Sayram-Ugam National Park	40.5
18	*Hypericum scabrum L. (Hypericum perforatum L.)*	Sayram-Ugam National Park	12.3
19	*Astragalus lasiopetalus Bunge*	Sayram-Ugam National Park	48.2
20	*Allium delicatulum Siev. ex Schult. &Schult.f. (Allium sewerzowii Regel)*	Sayram-Ugam National Park	51.2
21	*Scorzonera austriaca Willd.*	Sayram-Ugam National Park	61.0
22	*Primula kaufmanniana Regel*	Sayram-Ugam National Park	96.8
23	*Sedum tetramerum Trautv.*	Sayram-Ugam National Park	16.2
24	Allium karataviense	Sayram-Ugam National Park	48.5
25	*Alchemilla sibirica Zam.*	Sayram-Ugam National Park	27.5

26	*Rosa platyacantha Schrenk*	Sayram-Ugam National Park	21.6
27	*Moehringia lateriflora (L.) Fenzl (Caryophyllaceae)*	Sayram-Ugam National Park	22.2
28	*Spiraea lasiocarpa Kar et. Kir*	Sayram-Ugam National Park	55.0
29	*Acer semenovii Regel&Herder*	Berikkara Tract State Nature Reserve	12.8

Oligonucleotides ITS_U1, ITS_U2, ITS_U3, ITS_U4, ITS1, ITS2, 18S1F, 18S1R were synthesized by the phosphoramidite method on an ASM-800 apparatus (Biosan, Russia). The programs for ITS amplification were adjusted using gradient PCR to determine the optimum annealing temperature for different pairs of primers on an Eppendorf Nexus Gradient Thermal Cycler (Germany). The following temperature regimes were tested: temperature gradient from 40°C to 52°C with step of 2°C. As a result, the optimum annealing temperature for the use of ITS_U1, ITS_U4, and 18S1F oligonucleotides is 48°C. Sequencing directly from plant genomic DNA was ineffective and it is preferable to pre-amplify 18S rRNA with the ITS region, followed by purification of the PCR product. The purified PCR product was used to sequence the reaction for the ITS region using dideoxynucleotides according to the Sanger method [14]. The optimal conditions for the PCR reaction were: DNA - 1 µL (150 ng); 18S fw primer (10 µM) – 1 µL; ITS_U4 primer (10 µM) – 1 µL; dNTP (10 mM of each) – 0.5 µL; 10X Pfu Buffer – 2.5 µL; Pfu DNA Polymerase - 2 µL; nuclease free water – 17 µL. The program had the following temperature regimes: +98°C (1 min) – 1 cycle; +98°C (30 s), +48°C (1 min), +68°C (2 min) – 25 cycles; +68°C (10 min) – 1 cycle; +4°C (15 min) – 1 cycle. Amplification products were separated by electrophoresis in 1% agarose gel in TAE buffer with ethidium bromide (10 µg/mL). Figure 2 shows the results of PCR amplification using 18S fw/ITS_U4 oligonucleotides.

As can be seen from Figure 1, for all samples, amplification of the product with a length of ~2600 bp was observed, which corresponds to the locus consisting of the 18S gene of the ribosomal RNA subunit (~1800 bp) and ITS region (800 bp). PCR products were purified by chloroform-ethanol extraction. For verification, PCR was performed on the amplified and purified fragments using the ITS_U1 and ITS_U4 oligonucleotides flanking the ITS region. The conditions for PCR: purified DNA of 18SrRNA-ITS - 1 µL (150 ng); ITS_U1 primer (10 µM) – 1 µM; ITS_U4 primer (10 µM) – 1 µM; dNTP (10 mM of each) – 0.5 µL; 10X Taq Buffer – 2.5 µL; Taq DNA Polymerase - 2 µL; nuclease free water – 17 µL. The program had the following temperature regimes: +95°C (1 min) – 1 cycle; +95°C (30 s), +48°C (1 min), +72°C (2 min) – 25 cycles; +72°C (10 min) – 1 cycle; +4°C (15 min) – 1 cycle. Figure 3 shows the results of PCR amplification using ITS_U1/ITS_U4 oligonucleotides.

As can be seen from the figure, for all samples, amplification of the product was observed with a length of ~800 bp, which corresponds to the ITS region. Based on the results obtained, the reaction sequence was set using all four oligonucleotides: ITS_U1, ITS_U2, ITS_U3, ITS_U4, overlapping the ITS region (figure 1). The resulting sequences were analyzed by the BLAST program using NCBI data. The results of identification by ITS sequencing, overlap, and percent identity are presented in Table 2.

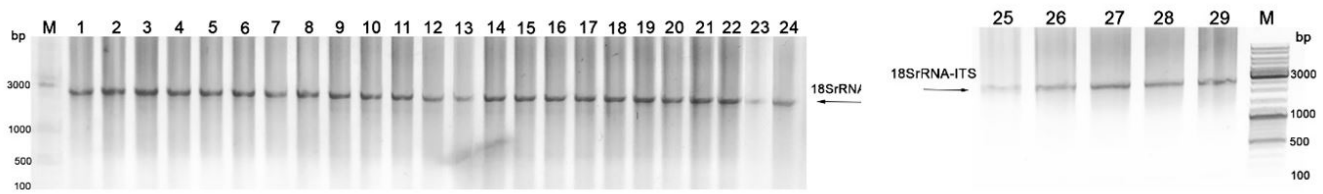

Figure 2 –Results of 18S rRNA and ITS amplification by using 18S fw and ITS_U4 olygonucleotides

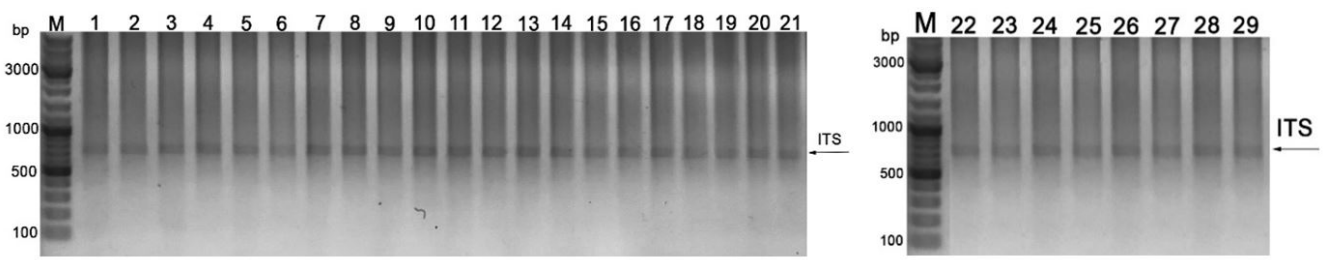

Figure 3 - Results of 18S rRNA and ITS amplification by using ITS_U1 and ITS_U4 olygonucleotides

Table 2 – Results of plant identification by ITS sequencing

Sample	Identification by ITS sequencing	Overlap, %	Identity, %
1	*Tulipa mogoltavica*	95	100
	Tulipa xtschimganica	100	99.72
2	*Tulipa mogoltavica*	96	99.67
	Tulipa xtschimganica	99	99.52
3	*Tulipa mogoltavica*	94	95.31
4	*Tulipa mogoltavica*	97	99.72

5	*Iris orchioides (syn Juno orchioides)*	97	99.72
6	*Tulipa mogoltavica*	97	99.72
7	*Allium karataviense*	67	100
8	*Tulipa turkestanica*	98	100
9	*Tulipa bifloriformis*	99	94.91
	Tulipa sogdiana	94	96.66
10	*Sorbus aria (syn Sorbus thibetica)*	100	96.47
11	*Lonicera webbiana Lonicera alpigena*	90	99.67
12	*Aconitum talassicum*	83	99.84
13	*Euonymus nanus*	91	100
14	*Lomelosia songarica*	86	98.35
15	*Scutellaria pinnatifida*	75	87.23
	Scutellaria glechomoides	33	92.66
16	*Oxytropis purpurea*	99	98.02
17	*Ephedra equisetina*	100	99.23
18	*Hypericum scabrum*	96	96.84
19	*Astragalus pellitus*	85	99.5
20	*Allium fetisowii*	78	100
21	*Scorzonera pubescens*	85	99.53
	Scorzonera austriaca	96	88.06
22	*Cortusa matthioli*	98	97.56
	Primula kaufmanniana	87	97.62
23	*Sedum subulatum*	88	93.65
24	*Allium karataviense*	78	100
25	*Alchemilla cymatophylla*	100	99.84
26	*Rosa xanthina*	100	97.30
	Rosa platyacantha	96	97.49
27	*Cerastium davurivum*	85	99.0
28	*Spiraea myrtilloides*	29	96.77
	Spiraea lasiocarpa	24	95.67
29	*Acer tataricum*	36	96.12

From the analysis of tables 1 and 2, it follows that the species identification of 15 samples (5.7-10,12,13,17,18,21,22,24,26,28,29) carried out according to the molecular genetic characteristics of the ITS region coincided with botanical identification. For 11 samples (1-4.6), identification matched to genus. For 6 samples (11,15,16,19,23,25) GenBank lacks DNA sequences. For 5 tulip samples, other regions will need to be used for sequencing. The results for 7 species of tulips (samples 1-4,6,8,9) were of particular interest to study their phylogenetic relationship. Turktas et al. noted that resolution of the ITS region for species of the genus *Tulipa* is insufficient [15], therefore for 7 tulip samples, other regions will need to be used for sequencing. To resolve controversial issues, chloroplast markers will be used, which serve as good additions to identification in the ITS region [8].

CONCLUSION

In DNA barcoding of plants, the choice of the genome region used as a phylogenetic marker is more important. It must meet the requirements: be present in all plants, be universal, have variability and not exceed 600-800 base pairs. The internal transcribed spacer of nuclear DNA is one of the most commonly used DNA markers on plant phylogenetic and DNA barcoding analyses, and it has been recommended as a core plant DNA barcode. Total DNA was isolated from 29 samples of rare and endangered plant species collected in the summer of 2021 in the Berikkara Tract State Nature Reserve, Aksu-Zhabagly State Nature Reserve, Sayram-Ugam National Park. Oligonucleotides flanking the ITS region of nuclear DNA were selected. The ITS sequence of the region was determined and 29 samples of rare and endangered plant species of Kazakhstan were identified by sequencing. For 7 samples of the genus Tulipa, the genes of chloroplast DNA will be sequenced for phylogenetic analysis.

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ҚАЗАҚСТАН РЕСПУБЛИКАСЫНЫҢ СИРЕК КЕЗДЕСЕТІН ЖӘНЕ ЖОЙЫЛЫП БАРА ЖАТҚАН ӨСІМДІК ТҮРЛЕРІН ДНҚ-ШТРИХКОДТАУ

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ТҮЙІН

Өсімдіктердің түрлерін анықтау мүмкіндігі флораның биоәртүрлілігін зерттеу үшін өте маңызды. ДНҚ-штрихкодтау анықтамалық ДНҚ аймақтары арқылы сәйкестендіру процедурасын стандарттауға мүмкіндік береді. Молекулярлық сәйкестендіру және филогенетикалық зерттеулер үшін ең танымал тізбектер ішкі транскрипцияланған аралық тізбектер (ITS) болып табылады. ITS өсімдіктерді әртүрлі таксономиялық деңгейлерде, соның ішінде тұқымда, түрлерде және кіші түрлерде жіктеу үшін филогенетикалық маркер ретінде кеңінен қолданылады. Ақсу-Жабағылы мемлекеттік табиғи қорығында, Сайрам-Өгем ұлттық табиғи паркінде және Берікқара шатқалы мемлекеттік табиғи қорығында сирек кездесетін және жойылып бара жатқан өсімдік түрлерінің 29 үлгісі жиналды. ДНҚ CTAB әдісі арқылы бөлініп алынды және ядролық ДНҚ-ның ITS аймақтарын қапталдайтын олигонуклеотидтер таңдалып, синтезделді. Қазақстандағы сирек кездесетін және құрып кету қаупі төнген өсімдіктердің 29 түрі бойынша секвенирлеу жүргізіліп, аймақтың ITS тізбегі алынды.

Кілтті сөздер: Флора, ITS, фауна, ДНҚ-штрих-кодтау, сәйкестендіру, CTAB әдісі

ДНК-ШТРИХКОДИРОВАНИЕ РЕДКИХ И ИСЧЕЗАЮЩИХ ВИДОВ РАСТЕНИЙ РЕСПУБЛИКИ КАЗАХСТАН

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АБСТРАКТ

Способность идентифицировать типы растительности очень важна для изучения биоразнообразия флоры. ДНК-штрихкодирование позволяет стандартизировать процедуру идентификации по эталонным участкам ДНК. Наиболее популярными последовательностями для молекулярной идентификации и филогенетических исследований являются внутренние транскрибируемые спейсерные последовательности (ITS). ITS широко используются в качестве филогенетического маркера для классификации растений на различных таксономических уровнях, включая род, вид и подвид. 29 образцов редких и исчезающих видов растений были собраны в Государственном природном заповеднике Аксу-Жабаглы, Национальном парке Сайрам-Угам и Государственном природном заповеднике урочище Бериккара. ДНК выделяли методом CTAB, отбирали и синтезировали олигонуклеотиды, фланкирующие регионы ITS области ядерной ДНК. Проведено секвенирование и получены последовательности ITS региона 29 видов редких и исчезающих видов растений Казахстана.

Ключевые слова: Флора, ITS, фауна, ДНК-штрихкодирование, идентификация, метод CTAB