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Assessment of the effectiveness of ITS markers in DNA barcoding of *Betula* species in the Kazakh Altai

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Abstract. This study investigates the effectiveness of ITS (Internal Transcribed Spacer) DNA barcoding markers for identifying and differentiating eight species of *Betula* within the Kazakh Altai flora: *Betula glandulosa, B. pendula, B. pubescens, B. rotundifolia, B. rezniczenkoana, B. tianschanica, B. microphylla, and B. kirghisorum.* By extracting and sequencing DNA from herbarium specimens, the study focuses on assessing the genetic diversity and resolving phylogenetic relationships among these *Betula* species, many of which have close morphological similarities that can complicate traditional taxonomy. The results reveal that ITS markers provide clear genetic differentiation between these species, highlighting the effectiveness of ITS in distinguishing even closely related species within the Betula genus. Analysis using the Neighbor-Joining method showed two primary clusters, aligning well with known phylogenetic sections, *Apterocaryon* and *Betula*, which are established classifications within the genus. Furthermore, the study offers the first ITS sequence data for two species, *B. rezniczenkoana* and *B. kirghisorum,* contributing new genetic information to the NCBI database. These findings are crucial for ongoing conservation efforts, given the ecological and environmental pressures facing native Betula species in the Kazakh Altai. By providing reliable molecular markers, this research supports future studies on species distribution, genetic diversity, and conservation planning within this biodiverse and ecologically sensitive region.

Keywords: *Betula L.* species, ITS markers, DNA barcoding, evolutionary relationships, genetic variability

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Introduction

The genus *Betula* is an important component of biodiversity, and its study is crucial in the context of conservation and sustainable use of natural resources. In the face of anthropogenic impacts and global environmental changes, including climate change, understanding the genetic diversity and phylogenetic relationships within the Betulaceae family is essential for biodiversity conservation efforts and evolutionary research.

The genus *Betula* includes both trees and shrubs found in a wide range of habitats in the boreal and temperate zones of the Northern Hemisphere [1]. In the Kazakh Altai, Saur-Manrak, and Zaysan Depression, 15 species and 1 subspecies of the genus *Betula L.* have been identified. The Kazakh Altai is a unique and floristically diverse region, being one of the richest floristic areas in Kazakhstan. Over 2,500 species of higher plants grow here, representing almost 50% of the total flora of the Republic of Kazakhstan [2]. The species diversity is due to the region's unique climate, which has led to the formation of various ecological niches with meadows, wetlands, deserts, and semi-deserts.

In the ecosystem of the Kazakh Altai, the genus *Betula* plays a significant role, exerting an important influence on the natural environment and human activities. It contributes to landscape formation, forest resource management, biomass production, and horticulture, and also possesses high pharmacological potential [3-5]. Many birch species, being fast-growing and adaptable to various environmental conditions, can participate in the restoration of forest ecosystems in cleared or burned areas [6].

The taxonomy of *Betula* remains a subject of scientific discussion, with many issues unresolved due to the high variability of traits and hybridization. Studies by Touchette et al. [7] and Tsuda et al. [8] have examined the genetic structure and hybridization within the genus *Betula*, highlighting the complexity of *Betula* species across Eurasia.

The first author to provide a comprehensive review of the genus was Regel [9], who divided the genus into the subgenera *Betulaster* and *Eubetula.* The subgenus *Betulaster* contained only one section – *Acuminatae*. The subgenus *Eubetula* included six sections: *Costatae, Lentae, Nanae, Albae, Fruticosae*, and *Dahuricae.* Recent phylogenomic analysis of the plastome has offered a new perspective on the phylogeny and evolution of *Betulaceae*, highlighting the monophyletic relationships between genera such as *Betula* and *Alnus* [10].

The contemporary classification presented by Ashburner and McAllister is based on the analysis of phylogenetic relationships within the genus *Betula* L., morphological traits, habitat, and species ploidy. According to this classification, the genus Betula is divided into four subgenera and eight sections. These subgenera are: *Acuminata* (section Acuminatae), *Aspera* (section Asperae и Lentae), *Betula* (section Apterocaryon, Betula, Costatae, and Dahuricae), and *Nipponobetula* (section Nipponobetula). Additionally, the section Asperae is further divided into two subgroups: the section *Asperae* and *Chinenses.*

The chromosome number in *Betula* L. is n = 14. *Betula* species form a polyploid series with chromosome counts of 2n = 28, 56, 70, 84, and 112 [11]. Hybridization and introgression are common in *B. pendula* (2n = 28) and *B. pubescens* (2n = 56) due to overlapping ranges of the species [12]. Ashburner and McAllister suggest that *B. microphylla* (2n = 56) and *B. tianschanica*

(2n = 56) are hybrids of the parent species *B. fruticosa* (2n = 56), *B. pubescens, B. utilis* (2n = 56), and *B. pendula*.

Polyploidy occurs in species of different subgenera, indicating several independent polyploidizations within the genus and serving as an important criterion for distinguishing some morphologically similar species, such as the diploid B. pendula (2n = 2x = 28) and the tetraploid *B. pubescens* (2n = 4x = 56).

Traditional methods of species identification in birches often fall short due to their high phenotypic plasticity and frequent hybridizations, which blur the boundaries between different taxa [13]. Therefore, one of the methods for studying biodiversity and genetic identification is DNA barcoding [14]. Recently, this method has been considered one of the most useful and objective "tools" for species identification based on the diversity of marker gene sequences from nuclear and plastid genomes. Additionally, DNA barcoding allows for the establishment of evolutionary relationships between different *Betula* L. species and helps understand genetic diversity and evolutionary patterns within the genus.

A significant number of studies highlight the high effectiveness of using nuclear genome markers – ribosomal internal transcribed spacers (ITS) (Figure 1). These are among the most thoroughly characterized and rapidly evolving genes, and their resolution is quite high when used together, making them a powerful tool for the identification and classification of birches [15]. Analysis of the chloroplast genome provides insights into maternal evolutionary relationships between species, while biparentally inherited nuclear DNA offers independent data for inferring evolutionary relationships [16-18].

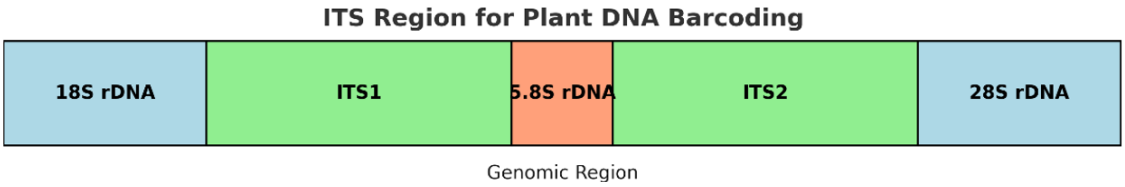


Figure 1. Diagram of the ITS Region

The effectiveness of markers for DNA barcoding in plants is determined by both the ease of amplification and the ability to distinguish species. The ITS marker shows a high amplification rate of ~90% and a strong capability for species differentiation [18]. However, species identification of *Betula* L. using barcoding faces several challenges, such as genetic variability, adaptation to environmental conditions, limited availability of reference sequences, and changing regional ecology. Additionally, the genus *Betula* L. is considered one of the most complex among all circumpolar genera [17].

These issues in the systematics of the genus underscore the relevance of the research presented in this study. This research aimed to determine the genetic diversity and establish relationships between different *Betula* L. species found in the Kazakh Altai.

Materials and research methods

The study focused on herbarium specimens of 8 *Betula* species: *Betula*: *B. glandulosa, B. pendula, B. pubescens, B. rotundifolia, B. rezniczenkoana, B. tianschanica*, and *B. microphylla, B. kirghisorum* collected from the Kazakh Altai region (Table 1). Botanical identification of the species was conducted by the staff of the RSE "Altai Botanical Garden". Taxonomic information for the samples was obtained from herbarium labels (Figure 1).

Table 1

DNA Collection of Endemic and Rare Plant Species from the Kazakh Altai

Species	Coordinates	Elevation	Geographical and Administrative Location
Betula glandulosa	50.32694 83.54556	1870	Sarymsakty Ridge, Burkhat Pass
Betula pendula	50.31933 83.89556	1180	Northwest Slope of Ivanovsky Ridge
Betula pubescens	50.32028 84.19556	1935	Koksinsky Ridge Altai Botanical Garden
Betula rotundifolia	50.1934 83.3246	774	Altai Botanical Garden
Betula rezniczenkoana	50.1934 83.3246	774	Altai Botanical Garden
Betula tianschanica	50.1934 83.3246	774	Northwest Slope of
Betula microphylla	49.18972 85.51944	820	Mount Bukhtarma
Betula kirghisorum	51.101 73.3356	101	Qaragandy region

Samples were homogenized using an automatic homogenizer TissueLyser LT (Qiagen, Germany). Genomic DNA was extracted from herbarium specimens using a modified acidic CTAB extraction buffer (2% CTAB, 2 M NaCl, 10 mM Na3EDTA, 100 mM HEPES, pH 5.3) with RNase A treatment. DNA was dissolved in 1×TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). DNA quality was assessed spectrophotometrically using a Nanodrop (Thermo Fisher Scientific Inc., Waltham, MA, USA) and by running a 1% agarose gel stained with ethidium bromide at 90 V for 20 minutes.

Amplification was conducted in a final volume of 25 µL. The amplification mixture included 10 ng of DNA, PCR buffer (2 mM MgSO4; 10 mM KCl; 10 mM (NH4)2SO4; 20 mM Tris-HCl, pH 8.8), 5 pmol of primers, 200 µM dNTPs, and Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific). PCR amplification of DNA was performed using ITS primers on a ThermalCycler (Thermo Fisher Scientific). Primer sequences are provided in Table 2.

Initial denaturation was performed at 98°C for 3 minutes, followed by 15 seconds at 98°C. The subsequent 35 amplification cycles were conducted with annealing temperatures ranging from 55°C to 58°C for 30 seconds and extension at 72°C for 60 seconds. A final extension was carried out at 72°C for 2 minutes.

Table 2

Primer Sequences for DNA Barcoding

№	Primer	Sequence 5' - 3'	GC%	Tm °C	Amplicons
1	ITS4	TCCTCCGCTTATTGATATGC	45	62	400-680bp
2	ITS5	GGAAGTAAAAGTCGTAACAAG	38	58	

Amplification results were checked using a 1.2% agarose gel stained with ethidium bromide (Figure 1). The sizes of the amplified DNA fragments were determined by comparing them to a marker (Thermo Scientific GeneRuler DNA Ladder Mix 100-10,000 bp). Fragment lengths were analyzed using the iBright 1500 Imaging System for gel documentation.

Figure 1. Herbarium samples of various *Betula* species

PCR products were purified using the ExoSap-IT PCR Product Cleanup Kit (Applied Biosystems, Inc., USA) and visualized on a 1.2% agarose gel. Sequencing was performed with ITS-specific

primers using the ABI3700 capillary sequencer (Applied Biosystems Thermo Fisher Scientific) and Sanger sequencing (BigDye® Terminator chemistry). Visualization and analysis of the data were conducted using SeqMan 6.1 software.

Sample identification was based on the analysis of the primary nucleotide sequences compared against the GenBank database (http://www.ncbi.nlm.nih.gov) using multiple sequence alignment in MEGA 11 [19]. The evolutionary divergence between nucleotide sequences of the plant species studied was calculated based on the ITS sequences from all eight populations of the Betula L. genus using MEGA 11 software.

Results

In our study, DNA was extracted from herbarium samples of *Betula* collected at the Altai Botanical Garden. The ITS marker used for barcoding demonstrated high reproducibility of results. It is noteworthy that the success of obtaining DNA sequences varied among taxa. Generally, the success of DNA extraction and subsequent amplification was dependent on the quality of the herbarium material and the chosen plant part. The sizes of the amplified regions varied according to the primers used and matched the expected values: the ITS2 fragment sizes ranged from 423 to 670 base pairs (Figure 2).

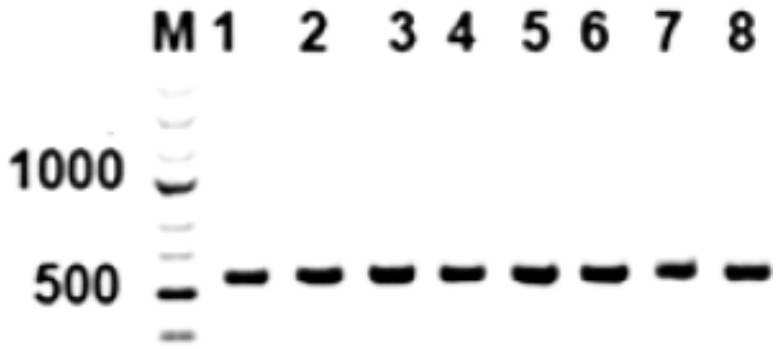


Figure 2. Results of DNA Amplification for Herbarium Samples of the Betula Genus Using ITS Region Primers

The sequencing results included a chromatogram for each sample and a file with its interpretation. The ITS nucleotide sequences for the *Betula* genus ranged from 402 to 638 base pairs. Figure 3 displays the alignment of nucleotide sequences for *Betula* L. samples, with differences in sequences highlighted by boxes, indicating the presence of polymorphic regions. For instance, variations in *Betula glandulosa* and *Betula microphylla* suggest that they belong to the same section, *Apterocaryon.*

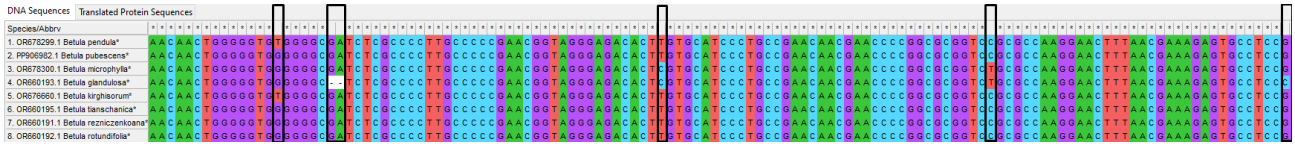


Figure 3. Fragment of the multiple alignment of ITS sequences for *Betula L.* samples using MEGA X software

The variability in the length and nucleotide composition of the ITS marker reflects the genetic diversity within the *Betula* genus. These results are consistent with similar studies and confirm the complex evolutionary history of the genus [20-23].

The average nucleotide composition for the ITS marker is as follows: T = 18.9%, C = 30.9%, A = 20.2%, and G = 29.8%. The highest AT content (40.6%) and the lowest GC content (59.5%) were observed in the *B. kirghisorum* population, while the lowest AT content (38.4%) and the highest GC content (61.6%) were found in the *B. tianschanica* population (Table 4). Nucleotide frequencies calculated according to the Tamura-Nei model [19] for the ITS marker are: 19.87% (A), 19.14% (T/U), 31.10% (C), and 29.90% (G).

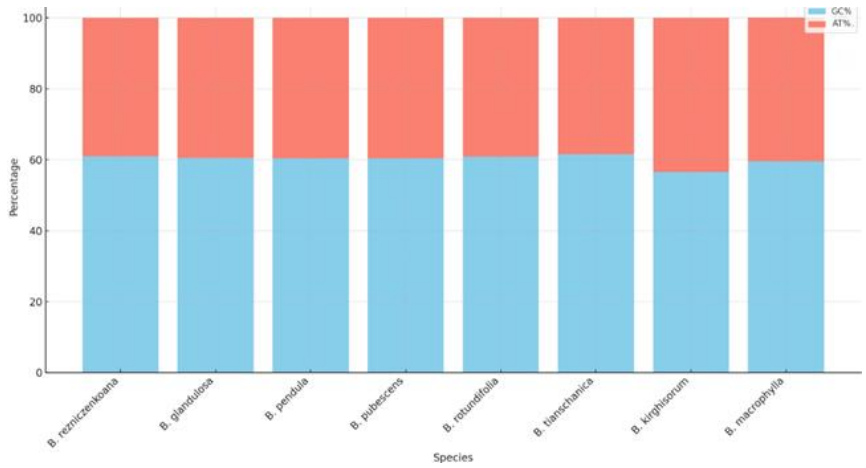


Figure 4. AT% and GC% by Betula sp for ITS

The analysis of nucleotide substitutions reveals a predominance of transition substitutions over transversions. This suggests their relative conservativeness and significance for evolutionary studies within the genus *Betula*. Transitions (substitutions between purines or pyrimidines) are less disruptive to the structural integrity of DNA than transversions (substitutions between a purine and a pyrimidine).

Table 4

Evaluation of Maximum Likelihood Estimates of Nucleotide Substitution Patterns for ITS

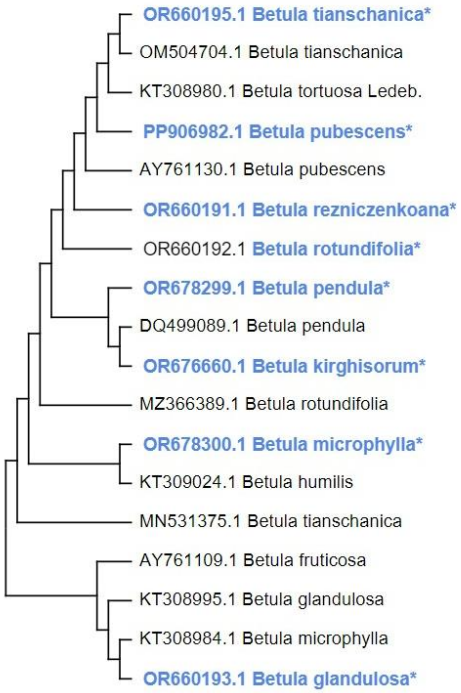
	A	T	C	G
A	-	4,764	7,56	12,07
T	5,00	-	18,99	7,29
C	5,00	11,67	-	7,29
G	8,28	4,64	7,56	-

Note: Frequencies of different transition substitutions are shown in bold, while frequencies of transversion substitutions are shown in italics

Transition substitutions between purines (A↔G) and pyrimidines (C↔T) are detailed in Table 4. The frequency of C↔T substitutions is 18.99%, indicating a high probability of this type of substitution. In contrast, transversion substitutions between purines and pyrimidines show lower frequencies, with A↔C and A↔T substitutions at 5, and G↔C and G↔T substitutions varying at 7.56 and 7.29, respectively.

The use of DNA barcoding successfully identified species in all cases, with consensus marker sequences aligning 98-100% with known sequences. Consequently, we have deposited eight sequences in GenBank: OR660193 *(B. glandulosa),* OR660156 *(B. pendula),* OR821848 *(B. pubescens)*, OR660192 *(B. rotundifolia),* OR660191 *(B. rezniczenkoana),* OR660195 *(Betula tianschanica),* OR660176 *(B. microphylla),* and OR660092 *(B. kirghisorum).* This ensures open access to the data and supports the transparency of the research.

The importance of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA for elucidating the informative nature of ITS sequences in assessing the phylogenetic relationships within the Betula genus was demonstrated by Chen et al. [16]. A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method, with reference sequences downloaded from the NCBI database for the Betulaceae family. This tree revealed diversity in the clustering of *Betula* species. Additionally, *B. tortuosa, B. himilis,* and *B. fruticosa* from the Altai region of Kazakhstan were included in the analysis (sequence data for these species were obtained from GenBank).



Note: indicates sequences downloaded from GenBank

Figure 4. Phylogenetic tree of Kazakhstani Betula species constructed using the Neighbor-Joining method based on ITS sequencing results

Discussion

According to the classification by Ashburner K and McAllister, all analyzed samples belong to the subgenus *Betula* [13, 24]. The use of the ITS marker allowed the construction of a phylogenetic tree and the identification of 2 clades. The species B. tianschanica, B. pubescens, B. pendula, and B. glandulosa were grouped in one clade along with their reference sequences.

The first clade is characterized by the fact that *Betula* pubescens Ehrh. often forms hybrids with *B. pendula* Roth, *B. rezniczenkoana* (Litv.) Schischk., and *B. microphylla* Bunge, which complicates their diagnosis [2]. *B. rezniczenkoana* (Litv.) Schischk. is a hybrid resulting from the cross between *B. pendula* Roth and *B. microphylla* Bunge [25]. Other sources also indicate hybridization between *B. pendula* Roth and *B. tianschanica* Rupr [26].

Betula rotundifolia grouped into a clade with its reference sequence from the NCBI database, but not in immediate proximity, which may be related to difficulties in species identification and hybridization with *B. pendula* Roth (B. × pseudomiddendorffii V.Vassil.). For the *Betula* kirghisorum sample, as with *B. rezniczenkoana*, there are no reference sequences in the NCBI database, making these species less studied compared to the other analyzed samples.

In the second clade of the *Apterocaryon* section, *B. glandulosa* was placed close to its reference sequence. Interestingly, *B. microphylla* was included in the*Betula* section, although it is classified as part of the *Apterocaryon* section. This discrepancy may be due to the high polymorphism of *B. microphylla* [27]. Its significant genetic plasticity is attributed to the diverse habitat conditions and is reflected in a wide range of morphological variability used in birch diagnoses. When growing alongside other birch species (*B. pendula)*, it undergoes hybridization, producing various intermediate forms. It is also notable that *B. rotundifolia* and *B. microphylla* each had only one sequence in the international NCBI database, which limited the comparative analysis possibilities.

Nevertheless, our results align with previous studies by Järvinen et al. [17] and Fazekas et al. [28] and confirm that DNA barcoding using the ITS marker can successfully identify the *Betula* genus but lacks sufficient resolution for species differentiation [24]. The absence of reference sequences in the GenBank database for some species, such as *B. rezniczenkoana* and *B. kirghisorum,* highlights the need for further research and database expansion to improve the accuracy of phylogenetic analyses.

Our findings emphasize the importance of exploring and utilizing additional DNA regions as supplementary barcoding markers to resolve species-level identification in *Betula*. Future research should focus on investigating other potential barcoding regions and their applicability for differentiating *Betula* species and related taxa.

Conclusion

The results obtained expand our understanding of the genetic architecture and evolutionary relationships within the *Betula* L. species. Future integration of multi-marker approaches and enhanced sampling efforts will refine classification and provide a deeper understanding of the evolutionary dynamics of this taxonomic group. Such efforts are crucial for developing effective conservation strategies and sustainable management of *Betula* species in the face of ecological challenges.

Author Contributions

A.T. and **S.M. –** design of the study, collection, analysis, and interpretation of the results. **O.K.** – supervisor of the study, critically revising its content.

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Conflicts of Interest

The authors declare no conflicts of interest.

Compliance with ethical standards

This article does not contain a description of studies performed by the authors involving people or using animals as objects.

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Қазақстан Алтайындағы *Betula* түрлерінің ДНҚ штрих-кодтауында ITS маркерлерінің тиімділігін бағалау

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Аңдатпа. Бұл зерттеуде Қазақстан Алтайының флорасында өсетін сегіз қайың ағаш түрін (*Betula* glandulosa, *B. pendula, B. pubescens, B. rotundifolia, B. rezniczenkoana, B. tianschanica, B. microphylla* және *B. kirghisorum*) идентификациялау мен дифференциациялау үшін ITS (ішкі транскрибтелетін ДНҚ спайсері) штрих-код маркерін қолданудың тиімділігі зерттелген. ДНҚ-ны бөлу және гербарий үлгілерін секвенирлеу арқылы жүргізілген зерттеу арқылы генетикалық әртүрлілікті бағалауға және осы түрлер арасындағы филогенетикалық байланысты нақтылауға бағытталған, олардың көпшілігінің морфологиялық белгілері ұқсас болып келеді, бұл дәстүрлі таксономиялық жіктелуге қиындықтар туғызуы мүмкін. Нәтижелер көрсеткендей, ITS маркерлері зерттелген түрлер арасында анық генетикалық дифференциацияны қамтамасыз етеді, тіпті Betula туысының жақын туыстас түрлерін ажыратуда олардың тиімділігін растайды. Қосылған көрші әдісі (NJ әдісі) екі негізгі кластердің бар екенін анықтады, бұл Apterocaryon және *Betula* филогенетикалық топтары – туыстағы белгілі классификацияларға сәйкес келеді. Зерттеу сондай-ақ *B. rezniczenkoana* және *B. kirghisorum* түрлерінің ITS тізбегінің мәліметтерін алғаш рет ұсынады, бұл NCBI дерекқорында жаңа генетикалық ақпарат қосуға мүмкіндік береді. Алынған нәтижелер экологиялық мәселелерді ескере отырып, Қазақстан Алтайындағы жергілікті қайың ағашы түрлерінің биоәртүрлілігін сақтау үшін маңызды болып табылады. Сенімді молекулярлық маркерлерді ұсына отырып, бұл зерттеу түрлердің таралуын, генетикалық әртүрлілікті зерттеу және биологиялық тұрғыдан алуан түрлі әрі экологиялық тұрғыдан сезімтал аймақта сақтау шараларын жоспарлау бойынша жұмыстарды қолдайды.

Түйін сөздер: Қайың түрлері (*Betula* L.), ITS маркерлері, ДНҚ штрих-кодтауы, эволюциялық байланыстар, генетикалық өзгергіштік

Оценка эффективности ITS-маркеров в ДНК-штрихкодировании видов *Betula* в Казахском Алтае

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Аннотация. В данном исследовании изучается эффективность использования маркера штрих-кода ITS (внутренний транскрибируемый спейсер ДНК) для идентификации и дифференциации восьми видов березы, произрастающих во флоре Казахского Алтая: *Betula glandulosa, B. pendula, B. pubescens, B. rotundifolia, B. rezniczenkoana, B. tianschanica, B. microphylla* и *B. kirghisorum*. Путем выделения ДНК и секвенирования гербарных образцов исследование направлено на оценку генетического разнообразия и уточнение филогенетических связей между этими видами, многие из которых имеют сходные морфологические признаки, что может затруднить их традиционную

таксономическую классификацию. Результаты показали, что маркеры ITS обеспечивают четкую генетическую дифференциацию между изучаемыми видами, подтверждая их эффективность в различении даже близкородственных видов рода *Betula*. Анализ методом объединения соседей (NJ method) выявил наличие двух основных кластеров, которые согласуются с известными филогенетическими группами Apterocaryon и *Betula* – установленными классификациями внутри рода. Кроме того, в исследовании впервые представлены данные последовательности ITS для видов *B. rezniczenkoana* и *B. kirghisorum,* что добавляет новую генетическую информацию в базу данных NCBI. Полученные результаты имеют важное значение для сохранения биоразнообразия, учитывая экологические проблемы, с которыми сталкиваются местные виды берез Казахского Алтая. Предоставляя надежные молекулярные маркеры, это исследование поддерживает дальнейшие работы по изучению распространения видов, генетического разнообразия и планирования мер по сохранению в этом биологически разнообразном и экологически чувствительном регионе.

Ключевые слова: виды березы (*Betula* L.), ITS-маркеры, штрих-кодирование ДНК, эволюционные взаимоотношения, генетическая изменчивость

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