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Molecular phylogenetic analysis of six *Ribes* L. species from Kazakhstan based on DNA barcodes of nuclear and chloroplast genomes

Ribes L. species are of significant ecological and economic importance. Their berries are rich in functional metabolites, which contribute to both their nutritional value and potential health benefits. However, the genus is taxonomically complex and requires comprehensive studies to resolve its phylogenetic relationships. In this study, we sequenced three genetic regions—the internal transcribed spacer (*ITS*) and the chloroplast genes *matK* and *rbcL* to investigate the phylogeny of *Ribes* species collected in Kazakhstan. There were six species analyzed *Ribes janczewskii* Pojark., *Ribes aureum* Pursh, *Ribes graveolens* Bunge, *Ribes nigrum* L., *Ribes rubrum* L., and *Ribes saxatile* Pall., collected from various regions of Kazakhstan. Phylogenetic trees were constructed using the Maximum Likelihood method implemented in IQ-TREE. The aligned sequence lengths were 686 base pairs (bp) for *ITS*, 750 bp for *matK*, and 498 bp for *rbcL*. Among these, the *ITS* region showed the highest number of polymorphic sites (219), followed by *matK* (195) and *rbcL* (50). Nucleotide diversity (Pi) was also the highest in the *ITS* region (0.0735), nearly double that of *matK* (0.03703), and substantially greater than *rbcL* (0.01378). The nucleotide sequences of *ITS*, *matK*, and *rbcL* obtained from this study have been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under accession numbers PV702933-PV702944, PV730383-PV730394, and PV730395-PV730406, respectively. The newly generated sequence data provide a valuable foundation for future phylogenetic and evolutionary research in *Ribes*.

Keywords: Ribes, Kazakhstan, phylogeny, *ITS*, *rbcL*, *matK*, DNA-barcoding.

Introduction

Ribes L., a genus in the family Grossulariaceae DC., which is primarily distributed across Eurasia, North America, and parts of South America [1]. The genus comprises approximately 200 species of deciduous, perennial shrubs [2]. Commonly referred to as currants and gooseberries, members of *Ribes* are of substantial ecological, economic, and horticultural significance [3]. *Ribes* species are particularly valued for their fruits, which are consumed both fresh and in a variety of processed forms, including jams, wines, juices, and candies. This wide range of uses is attributed to the fruits' high content of functional metabolites, which contribute to their nutritional and commercial appeal [4]. Among the species, *Ribes nigrum* L. (black currant) has been extensively studied for its phytochemical composition [5–7]. These studies have demonstrated that *R. nigrum* berries are particularly rich in phenolic compounds and exhibit significant antioxidant and antimicrobial activities.

In Kazakhstan, the genus *Ribes* is represented by 11 wild species [8], including *Ribes janczewskii* Pojark., which is listed in the Red Book of Kazakhstan [9]. Among these, *Ribes aureum* Pursh, *Ribes graveolens* Bunge, *Ribes nigrum* L., *Ribes rubrum* L., and *Ribes saxatile* Pall. are the most widely distributed. Most of these species are of particular importance due to their high vitamin content and potential use in nutritional and pharmaceutical applications. Several species of *Ribes* in Kazakhstan have been the subject of research involving cryopreservation techniques [10] and molecular genetic studies [11]. In addition, numerous investigations have focused on the biochemical composition and breeding potential of various cultivated *Ribes* varieties, underscoring their value for both conservation and agricultural development [12–14].

Molecular phylogenetic analysis plays a crucial role in resolving complex evolutionary relationships, particularly in plant genera characterized by high levels of morphological convergence. *Ribes* is one such genus with a long history of taxonomic ambiguity and ongoing debate regarding its classification [2]. Despite numerous morphological and molecular studies, the taxonomy of *Ribes* remains controversial. To ad-

dress these challenges, molecular markers from both nuclear and chloroplast genomes have been widely employed in phylogenetic investigations of *Ribes* [15–17]. However, despite these efforts, the molecular taxonomy of the genus is still incomplete and requires further comprehensive studies involving broader sampling.

In this study, we sequenced the internal transcribed spacer (*ITS*) region along with the chloroplast genes *matK* and *rbcL* to evaluate the phylogenetic positions of six *Ribes* species—*Ribes janczewskii*Pojark., *Ribes aureum* Pursh, *Ribes graveolens* Bunge, *Ribes nigrum* L., *Ribes rubrum* L., and *Ribes saxatile* Pall., collected from various regions of Kazakhstan.

Experimental

Collection of the plant leaves and DNA isolation

Plant samples were collected from the southeastern, western, and eastern regions of Kazakhstan by Ivaschenko A., Imanbayeva A., and Sumbembayev A., respectively. Detailed information on the collection sites is provided in Table 1. The collected plant materials were dried using silica gel and subsequently used for DNA extraction. Genomic DNA was isolated following the cetyltrimethylammonium bromide (CTAB) protocol [18]. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and DNA quality was assessed by electrophoresis on a 1.0 % agarose gel.

Table 1

Collected sites of the studied seven *Ribes* species from Kazakhstan

Species	Collected site	Collected by
Ribes nigrum L.	Western Altai, Lineisky ridge	Sumbembayev A.
Ribes rubrum L.	Western Altai, Kholzun ridge	Sumbembayev A.
Ribes saxatile Pall.	East Kazakhstan region, Kokpekty district, Eastern Kalba ridge	Sumbembayev A.
Ribes graveolens Bunge	East Kazakhstan region, Ridder district, Koksu ridge	Sumbembayev A.
Ribes janczewskii Pojark.	Almaty region, upper reaches of Turgen	Ivaschenko A.
Ribes aureum Pursh	Mangistau region, Mangyshlak, Western Karatau, Kogez gorge	Imanbayeva A.

PCR amplification and sequencing

DNA barcoding markers, including *ITS*, *matK*, and *rbcL*, were employed for phylogenetic analysis. PCR amplification was carried out in a 20 μL reaction mixture comprising genomic DNA as the template, buffer solution, $MgCl_2$, dNTPs, forward and reverse primers, and Taq DNA polymerase. The amplifications were performed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, USA). PCR conditions and the nucleotide sequences of the primers used were applied as described in White et al. (1990) [19] for *ITS*, Kress & Erickson (2007) [20] for *matK* and *rbcL*. Following amplification, PCR products were separated by electrophoresis on a 1.5 % agarose gel. Target bands were excised from the gel and purified using the ULTRAPrep® Agarose Gel Extraction Mini-Prep Kit (AHN Biotechnologie GmbH, Nordhausen, Germany), following the manufacturer's protocol. The purified PCR products were then sequenced in forward and reverse directions using BigDye™ Terminator Cycle Sequencing chemistry (Applied Biosystems, USA). Sequencing was carried out on an ABI 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA). Two samples from each species were sequenced and included in the analysis.

Phylogenetic analysis

For the phylogenetic analysis, nucleotide sequences of *ITS*, *matK*, and *rbcL* markers were utilized. Analyses were conducted using both individual and concatenated sequence datasets. A total of 25 samples were included, comprising 12*Ribes* samples (representing six species) collected from Kazakhstan, 11*Ribes* sequences retrieved from NCBI GenBank, and two additional outgroup sequences (*Rosa laevigata* and *Rhamnus grubovii*). Sequence alignment was performed using MEGA X [21]. Phylogenetic trees were reconstructed using the Maximum Likelihood (ML) approach. The optimal nucleotide substitution models were selected based on the Bayesian Information Criterion (BIC), with the following models: TIM+F+I+R2for ITS, TPM3u+F for *matK*, and TPM2 for *rbcL* nucleotide sequences. ML analysis was conducted using IQ-TREE v2.2.2.6 [22]. The subgenus and section names were given according to Schultheis & Donoghue (2004) [16]. Schultheis & Donoghue (2004) proposed a molecular phylogenetic classification system for the genus *Ribes*, based on data from nuclear and chloroplast DNA markers [16].

Results and Discussion

In the present study, three datasets were employed for phylogenetic analysis: (1) nucleotide sequences of the internal transcribed spacer (*ITS*) region, (2) nucleotide sequences of the *matK*gene, and (3) nucleotide sequences of the *rbcL* gene. The nucleotide sequences of *ITS*, *matK*, and *rbcL* obtained in this study have been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under accession numbers PV702933-PV702944, PV730383-PV730394, and PV730395-PV730406, respectively. Phylogenetic trees for each dataset were constructed using the Maximum Likelihood (ML) method. A total of 23 *Ribes* samples were included in the analysis, comprising 12 samples representing 6 species collected in this study, along with sequences obtained from the NCBI GenBank. Additionally, two species—*Rosa laevigata* and *Rhamnus grubovii* were used as outgroups to root the phylogenetic trees. Detailed information on all samples is provided in Table 2.

Table 2

The list of samples used in the study

Species	NCBI accession numbers		
	ITS	***matK***	***rbcL***
Ribes aciculare	AY138050.1	PQ348655.1	PQ337383.1
Ribes americanum	AF426375.1	MK520528.1	HQ590238.1
Ribes aureum	PQ443797.1	OQ847549.1	PQ337465.1
Ribes cynosbati	AY138051.1	MK520529.1	HQ590239.1
Ribes glaciale	MH710923.1	MW382543.1	MW382720.1
Ribes graveolens	MZ366410.1	MZ361533.1	MZ361434.1
Ribes himalense	MH711381.1	MH659873.1	JF944130.1
Ribes lacustre	AF426366.1	KX677769.1	HQ590240.1
Ribes nigrum	AF426374.1	HE967476.1	PQ337447.1
Ribes stenocarpum	AY138056.1	MW382557.1	MW382733.1
Ribes janczewskii	PP464115.1	PP708896.1	PP493207.1
Ribes nigrum KZ 1	PV702933.1	PV730383.1	PV730395.1
Ribes nigrum KZ 2	PV702934.1	PV730384.1	PV730396.1
Ribes rubrum KZ 1	PV702935.1	PV730385.1	PV730397.1
Ribes rubrum KZ 2	PV702936.1	PV730386.1	PV730398.1
Ribes saxatile KZ 1	PV702937.1	PV730387.1	PV730399.1
Ribes saxatile KZ 2	PV702938.1	PV730388.1	PV730400.1
Ribes graveolens KZ 1	PV702939.1	PV730389.1	PV730401.1
Ribes graveolens KZ 2	PV702940.1	PV730390.1	PV730402.1
Ribes janczewskii KZ 1	PV702941.1	PV730391.1	PV730403.1
Ribes janczewskii KZ 2	PV702942.1	PV730392.1	PV730404.1
Ribes aureum KZ 1	PV702943.1	PV730393.1	PV730405.1
Ribes aureum KZ 2	PV702944.1	PV730394.1	PV730406.1
Rosa laevigata	FJ416663.2	MH552372.1	GU363797.1
Rhamnus grubovii	KR083248.1	MZ361530.1	MZ361431.1

The Maximum Likelihood (ML) phylogenetic tree constructed from ITS nucleotide sequences divided the *Ribes* species into three primary clades, corresponding to the two major subgenera: *Ribes* and *Grossularia*. The samples of *Ribes aureum* analyzed in this study clustered together with *R. aureum* reference sequences from the NCBI GenBank, confirming their species identity. Similarly, the samples of *Ribes graveolens*, *Ribes nigrum*, and *Ribes janczewskii* formed a distinct subclade along with corresponding sequences of these species from GenBank, indicating strong phylogenetic coherence. *Ribes saxatile* samples grouped with *Ribes glaciale* from GenBank, suggesting a close genetic relationship between these taxa, indi-

cating that the *Ribes saxatile* might belong to the section *Berisia*. Additionally, *Ribes rubrum* samples from this study clustered with *Ribes himalense* sequences, indicating shared ancestry, which is consistent with their placement in the same subgenus (subg. *Ribes*) and section (sect. *Ribes*) according to the sectional classification proposed by Schultheis & Donoghue (2004). Species from the subgenus *Grossularia*—namely *Ribes aciculare*, *Ribes cynosbati*, and *Ribes stenocarpum*formed a well-supported clade together with *Ribes lacustre* (classified under the subgenus *Ribes*) from GenBank. This grouping suggests a complex evolutionary relationship between members of the two subgenera (Fig. 1). The section and subgenus names of the analyzed species were assigned according to Schultheis and Donoghue (2004) [16].

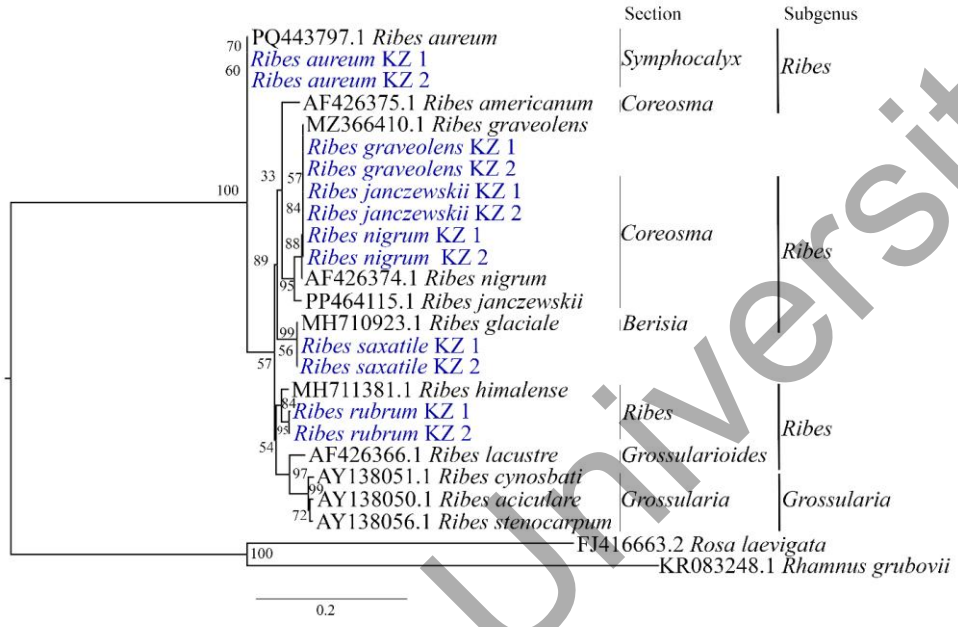

Figure 1. Maximum Likelihood phylogenetic tree based on *ITS* sequences reconstructed using 23 ingroup and 2 outgroup samples. The numbers at the branch nodes represent ML bootstrap value. The species analyzed in this study are highlighted in blue.

The ML phylogenetic trees reconstructed from the nucleotide sequences of the *matK* (Fig. 2) and *rbcL* (Fig. 3) genes exhibited similar topologies, supporting congruent evolutionary relationships among the *Ribes* species. Samples of *Ribes aureum* and *Ribes rubrum* obtained in this study clustered together with *Ribes americanum*, *Ribes himalense*, and *Ribes aureum* reference sequences from GenBank, indicating close genetic relationships among these taxa. Likewise, samples of *Ribes graveolens*, *Ribes nigrum*, and *Ribes janczewskii* from this study formed a clade with corresponding GenBank sequences of the same species. Additionally, the samples of *Ribes saxatile* from this study grouped with *Ribes glaciale* and *Ribes aciculare* from GenBank, suggesting a potential phylogenetic affinity among these species.

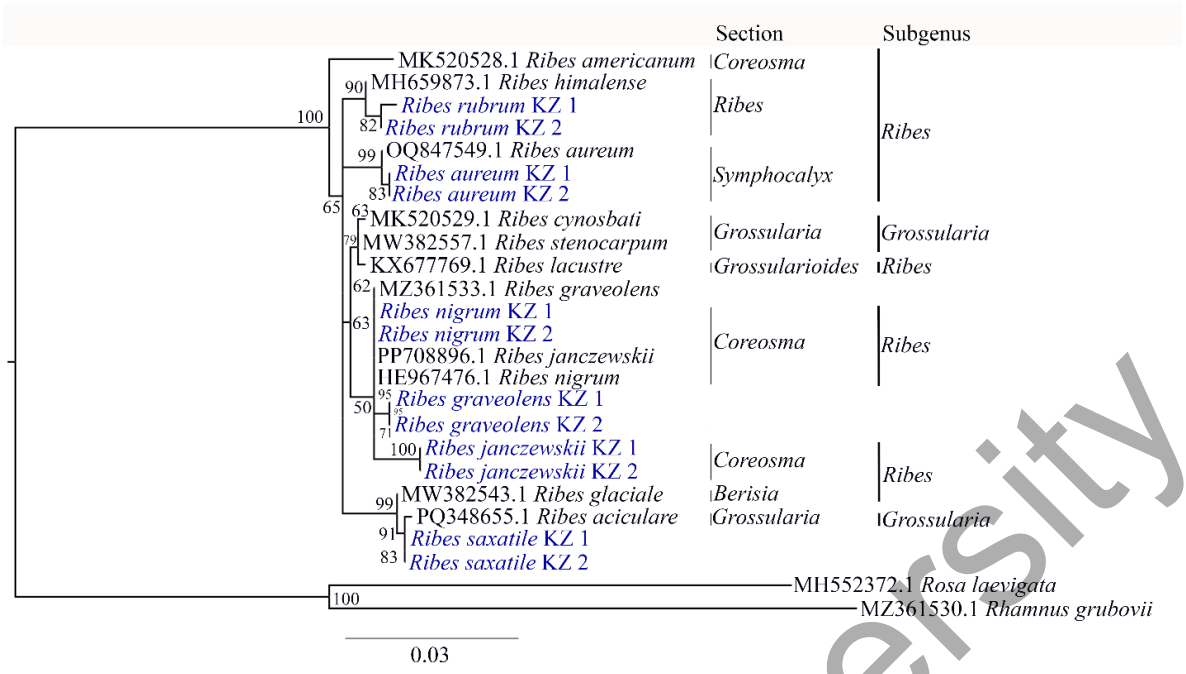

Figure 2. Maximum Likelihood phylogenetic tree based on *matK* sequences reconstructed using 23 ingroup and 2 outgroup samples. The numbers at the branch nodes represent ML bootstrap value. The species analyzed in this study are highlighted in blue.

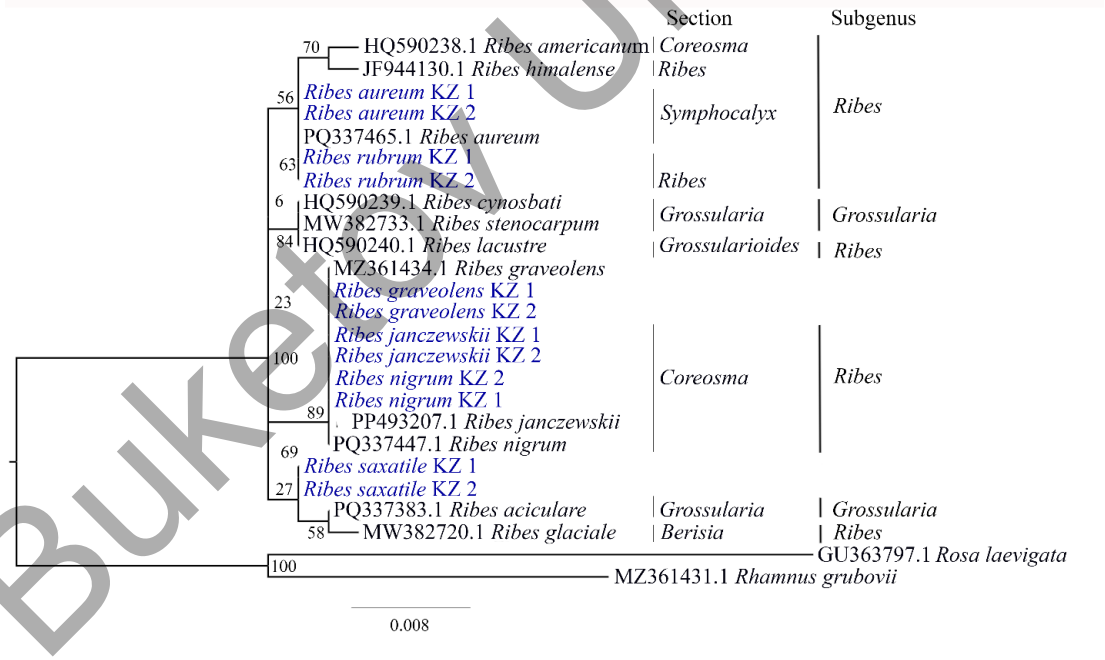

Figure 3. Maximum Likelihood phylogenetic tree based on *rbcL* sequences reconstructed using 23 ingroup and 2 outgroup samples. The numbers at the branch nodes represent ML bootstrap value. The species analyzed in this study are highlighted in blue.

Summary statistics for the aligned sequences of the *ITS*, *matK*, and *rbcL* are presented in Table 3. The aligned sequence lengths were 686 base pairs (bp) for *ITS*, 750 bp for *matK*, and 498 bp for *rbcL*. Among these, the *ITS* region exhibited the highest number of variable (polymorphic) sites (219), followed by *matK* (195) and *rbcL* (50), indicating that *ITS* was the most informative marker for detecting sequence variation in the studied *Ribes* species. A comparable number of polymorphic sites in the *ITS* region has also been reported in *Asclepias* species [23]. The number of observed haplotypes was highest for *matK* (17), slightly exceed-

ing *ITS* (16), while *rbcL* displayed a lower haplotype count (10). Haplotype (gene) diversity (Hd) was high across all markers, with *matK* showing the greatest diversity (0.953), followed by *ITS* (0.917) and *rbcL* (0.833).

Nucleotide diversity (Pi), a measure of average pairwise sequence divergence, was highest in the *ITS* region (0.0735), nearly double that of *matK* (0.03703), and significantly higher than *rbcL* (0.01378). Similarly high Pi values exceeding 0.07 have been reported for chloroplast DNA markers in certain orchid species, such as *Caularthronbicornutum* and *Myrmecophilathomsoniana* [24], suggesting that elevated nucleotide diversity is not restricted to nuclear regions in all taxa. These results confirm that the *ITS* region provided the relatively greatest resolution for assessing variability within *Ribes*, whereas *rbcL* was the most conserved marker among the three.

Table 3

Summary of sequence characteristics and genetic diversity parameters for *ITS*, *matK*, and *rbcL* in analyzed *Ribes* species

	ITS	***matK***	***rbcL***
Aligned length	686	750	498
Variable (polymorphic) sites	219	195	50
Number of Haplotypes, h	16	17	10
Haplotype (gene) diversity, Hd	0,917	0,953	0,833
Nucleotide diversity, Pi	0,0735	0,03703	0,01378

The nucleotide sequences of the *ITS* are widely utilized in resolving phylogenetic relationships across diverse plant taxa [25–27]. The high informativeness of *ITS* has also been demonstrated in several previous studies. For example, its effectiveness in species-level discrimination has been reported in families such as Cactaceae [28], Orchidaceae [29], as well as Fabaceae and Poaceae [30]. The phylogenetic analyses conducted in this study demonstrated that the *ITS* nucleotide sequences exhibited the highest level of polymorphism among the markers tested, indicating that *ITS* is potentially more informative for resolving phylogenetic relationships among *Ribes* species than the chloroplast markers *matK* and *rbcL*. The newly generated nucleotide sequence data represent a valuable resource for future phylogenetic and evolutionary studies in *Ribes*. These findings might contribute to a better understanding of species relationships within the genus and provide a foundation for future taxonomic revisions.

Conclusions

In this study, nucleotide sequences of the nuclear internal transcribed spacer (*ITS*) and the chloroplast genes *matK* and *rbcL* were generated and utilized for phylogenetic analysis of *Ribes* species. The results indicate that the *ITS* region is the most informative among the three markers, offering the highest resolution for detecting genetic variability within the genus. In contrast, *rbcL* was found to be the most conserved marker, providing limited phylogenetic differentiation. These results underscore the effectiveness of the *ITS* region in resolving intrageneric relationships in *Ribes*. The newly obtained sequences enrich the molecular dataset for the genus and provide a foundation for future phylogenetic and evolutionary studies. However, further studies, including a broader sampling of *Ribes* species, are necessary to achieve a more comprehensive and robust understanding of the genus taxonomy.

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Author Contributions

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tion, resources; **Imanbayeva A.A.** — Investigation, Resources, Funding acquisition; **Turuspekov Y.K.** — Conceptualization, Data curation, Writing — review and editing, project administration.

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Қазақстандық алты *Ribes* L. түрлерінің ядролық және хлоропласт геномдарының ДНҚ-баркодтары негізінде молекулалық-филогенетикалық талдауы

Ribes L. туысы түрлері айтарлықтай экологиялық және экономикалық маңыздылыққа ие. Олардың жидектері функционалды метаболиттерге бай, бұл олардың тағамдық құндылығына да, денсаулыққа пайдалы қасиеттеріне де ықпал етеді. Алайда, бұл туыс оның филогенетикалық байланыстарын нақтылау үшін кешенді зерттеулерді қажет ететін таксономиялық күрделі топ. Зерттеуде Қазақстан аумағында жиналған *Ribes* түрлерінің филогенетикасын зерттеу мақсатында үш генетикалық аймақ — ішкі транскрипцияланатын спейсер (*ITS*) және *matK*мен*rbcL* хлоропласт гендері секвенирленді. Қазақстанның әр аймақтарында жиналған алты түр талданды: *Ribes janczewskii* Pojark., *Ribes aureum* Pursh, *Ribes graveolens* Bunge, *Ribes nigrum* L., *Ribes rubrum* L. және*Ribes saxatile* Pall. Филогенетикалық дендрограммалар IQ-TREE бағдарламасында Maximum Likelihood әдісімен жүзеге асырылды. Түзетілген тізбектердің ұзындығы *ITS* үшін 686 нуклеотидтік жұбы (н.ж.), *matK* үшін 750 н.ж. және *rbcL* үшін 498 н.ж. болды. Олардың ішінде полиморфты аймақтардың ең көп саны *ITS* аймағында (219), одан кейін *matK* (195) және *rbcL* (50) нуклеотидтік тізбектерінде анықталды. Нуклеотидтік алуантүрлілік (Pi) те*ITS* нуклеотидтік тізбегінде ең жоғары (0,0735) болды, *matK* (0,03703) мен *rbcL* (0,01378) нуклеотидтік тізбектерінен айтарлықтай жоғары екендігі айқындалды. Алынған *ITS*, *matK*, және*rbcL* нуклеотидтік тізбектері сәйкесінше PV702933-PV702944, PV730383-PV730394 және PV730395-PV730406 тіркеу нөмірлерімен Ұлттық биотехнологиялық ақпарат орталығының (NCBI) GenBank деректер базасына жүктелді. Жаңа нуклеотидтік тізбектер *Ribes* туысының болашақ филогенетикалық және эволюциялық зерттеулері үшін құнды негіз болып табылады.

Кілт сөздер: Ribes, Қазақстан, филогения, *ITS*, *rbcL*, *matK*, ДНҚ-баркодтау.

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Молекулярно-филогенетический анализ шести видов *Ribes* L. из Казахстана на основе ДНК-баркодов ядерного и хлоропластного геномов

Виды рода *Ribes* L. обладают значительной экологической и экономической важностью. Их ягоды богаты функциональными метаболитами, которые способствуют как их пищевой ценности, так и потенциальным полезным свойствам для здоровья. Однако данный род представляет собой таксономически сложную группу, требующую комплексных исследований для уточнения его филогенетических взаимосвязей. В настоящем исследовании были просеквенированы три генетических региона — внутренний транскрибируемый спейсер (*ITS*) и хлоропластные гены *matK* и *rbcL* — с целью изучения

филогенетики видов *Ribes*, собранных на территории Казахстана. Было проанализировано шесть видов: *Ribes janczewskii* Pojark., *Ribes aureum* Pursh, *Ribes graveolens* Bunge, *Ribes nigrum* L., *Ribes rubrum* L. и *Ribes saxatile* Pall., собранных в различных регионах Казахстана. Филогенетические деревья были построены методом максимального правдоподобия (Maximum Likelihood) в программе IQ-TREE. Длина выровненных последовательностей составила 686 пар нуклеотидов (п.н.) для *ITS*, 750 п.н. для *matK* и 498 п.н. для *rbcL*. Среди них наибольшее число полиморфных участков было выявлено в регионе *ITS* (219), за ним следовали *matK* (195) и *rbcL* (50). Нуклеотидное разнообразие (Pi) также оказалось наивысшим в *ITS* (0,0735), почти в два раза превышая значение для *matK* (0,03703) и значительно превосходя *rbcL* (0,01378). Полученные нуклеотидные последовательности *ITS*, *matK*, и *rbcL* были депонированы в базу данных GenBank Национального центра биотехнологической информации (NCBI) под регистрационными номерами PV702933-PV702944, PV730383-PV730394 и PV730395-PV730406 соответственно. Новые нуклеотидные последовательности представляют ценную основу для будущих филогенетических и эволюционных исследований рода *Ribes*.

Ключевые слова: Ribes, Казахстан, филогения, *ITS*, *rbcL*, *matK*, ДНК-баркодирование.

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