Eurasian Journal of Applied Biotechnology. №.3, 2024
DOI: 10.11134/btp.3.2024.2

UDC 34.15.23 *Original Article*

DNA BARCODING OF *HIPPOPHAE RHAMNOIDES* L. COLLECTED FROM NATURAL AND INTRODUCED POPULATIONS IN KAZAKHSTAN

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ABSTRACT

In this study, we analyzed eight samples of *Hippophae rhamnoides* L. collected in Kazakhstan, along with twenty-five samples of *Hippophae* species and three outgroup species from NCBI GenBank, using ITS sequences. The alignment of the ITS sequences, which was 639 bp in length with outgroup samples and 642 bp without them, revealed polymorphism, with 31 positions (4.85%) being polymorphic among the ingroup samples. The highest number of polymorphic sites was found in ITS1 (13 sites), followed by ITS2 (10 sites) and the 5.8S rRNA region (8 sites). The maximum likelihood (ML) phylogenetic tree delineated four distinct clades within the *Hippophae* species. The *H. rhamnoides* samples from northeastern Kazakhstan clustered with *H. rhamnoides* ssp. *mongolica* from GenBank suggests a close genetic relationship. Introduced samples formed separate subclades and clustered with various subspecies from GenBank. Notably, hybrid peaks were observed in the ITS sequences of introduced plants, which were not present in samples from natural populations. This study underscores the utility of ITS sequences in identifying plants from natural and introduced populations of *H. rhamnoides* and highlights the marker's importance in plant genetic research and biodiversity conservation.

Key words: Kazakhstan, Elaeagnaceae, *Hippophae,* DNA barcoding, Internal transcribed spacer, phylogeny

INTRODUCTION

The genus *Hippophae* L. is a small genus of Elaeagnaceae Juss. comprising accepted seven species [1] and 17 subspecies [2] encompasses a group of deciduous shrubs known for their ecological resilience and medicinal properties [3]. The genus *Hippophae* is found in Northern Europe, Central Europe, Central Asia, and China, and it is likely originating in Central Asia [4-7]. These plants have been revered for their ability to thrive in harsh environments and their multifaceted applications in traditional medicine, agriculture, and environmental management [7, 8]. All species of *Hippophae* are diploid (2n = 24) [4, 5], wind-pollinated, and dioecious [3].

Among the numerous species within the *Hippophae* genus, *Hippophae rhamnoides* L., or sea buckthorn, stands out as particularly noteworthy due to its extensive distribution across Europe and Asia [9, 10] and its rich bioactive composition [11]. *H. rhamnoides* is a flowering shrub or small tree originating from the Eastern Himalayas to the Hengduan Mountains [12] and thrives in desert, semi-desert, and high mountainous ecosystems across Eurasia [12-14]. *H. rhamnoides* is rich in nutrients and bioactive components, many renowned for their positive effects on health [15, 16]. Its berries contain high levels of vitamins C and E, flavonoids, and carotenoids, contributing to its antioxidant and anti-inflammatory properties [17-19]. Beyond its medicinal properties, *H. rhamnoides* plays a crucial ecological role, particularly in stabilizing soils and preventing erosion in coastal and mountainous regions [20, 21].

H. rhamnoides consists of nine subspecies [22]; in Europe, these include subspecies *rhamnoides*, subspecies *fluviatilis*, and subspecies *carpatica*. Across Asia, the subspecies are represented by subspecies *caucasia*, subspecies *turkestanica*, subspecies *mongolica*, subspecies *sinensis*, subspecies *yunnanensis*, and subspecies *wolongensis* [22, 23]. However, the taxonomic classification and relationships among taxa in this genus are still contentious [3, 5, 6, 24, 25]. Recent research efforts have predominantly investigated its biochemical characteristics [18, 19], genetic structure [3, 19, 26], pharmacological properties [17, 27, 28], germplasm resources [29], and phylogeography [30, 31].

In Kazakhstan, the only representative of the *Hippophae* genus is *H. rhamnoides* [32], which is found in the eastern, southeastern, and southern regions [33]. According to Letchamo et al. [22], there are two subspecies of H. rhamnoides in Kazakhstan: *H. rhamnoides* ssp. *turkestanica* and *H. rhamnoides* ssp. *mongolica.* Several studies in Kazakhstan have focused on various aspects of the species, including the current state of its population [34], floristic composition [35], chemical composition [36], and selection assessment [37]. Several reports on the cultivation of *H. rhamnoides* in the southeast region of Kazakhstan [38-40] suggest that this species has been successfully growing in mountain areas since the end of the last century.

There is extensive global research on analyzing *H. rhamnoides* using DNA barcoding, chloroplast genome analysis, and different molecular markers [6, 19, 26]. However, limited research in Kazakhstan utilizes DNA barcoding of *H. rhamnoides*. The authors have a background in DNA barcoding in the genera *Artemisia* [41], *Allium* [42], *Oxytropis* [43], *Agriophyllum* [44], *Ranunculus* [45], and *Salsola* [46], which makes it possible to determine the reliability of the results.

The Internal Transcribed Spacer (ITS) region is known for its high polymorphism and is widely acknowledged as a valuable tool in plant evolutionary research [47, 48]. ITS region comprises ITS1 and ITS2 subregions, 5.8S ribosomal RNA gene [49]. Understanding the evolutionary relationships between species within a genus is critical for reconstructing the genus's phylogeny [50]. The ITS region is highly variable between species, even within the same genus [51], making it particularly effective for species delimitation and resolving

DOI: 10.11134/btp.3.2024.2

taxonomic ambiguities [52]. Numerous studies have utilized the ITS region as a primary DNA barcoding marker in phylogenetic research, owing to its high variability and effectiveness in distinguishing closely related species [53-55].

This study primarily aimed to assess the differences between natural and introduced populations of *H. rhamnoides* from Kazakhstan using the nuclear ribosomal DNA internal transcribed spacer region (ITS).

MATERIALS AND METHODS

Sampling and DNA extraction

Plant leaves of *Hippophae rhamnoides* were collected from natural populations in northeast Kazakhstan (NEK) and individuals introduced to the Mangyshlak Experimental Botanical Garden and the Altay Botanical Garden (Table 1). The introduced specimens at the Mangyshlak Experimental Botanical Garden originated from the Western Karatau, specifically the Kogez Gorge in Western Kazakhstan (44.215778, 52.021500). Additionally, the Altay Botanical Garden samples were originally introduced from the Belkin Mountains in Eastern Kazakhstan (50.33134; 83.54691). The collected leaves were dried in silica gel for subsequent DNA extraction. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol [56]. The extracted DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The quality of the DNA was analyzed through electrophoresis on 1.0% agarose gel.

PCR amplification and sequencing

The internal transcribed spacer (ITS) was amplified, including ITS1, ITS2, and the 5.8S ribosomal RNA gene with the F (5′-AGAAGTCGTAACAAGGTTTCCGTAGG-3′) and R (5′-TCCTCCGCTTATTGATATGC-3′) primers [57]. The amplification was performed in a 20 µl reaction volume containing genomic DNA as a template, buffer, MgCl2, dNTPs, forward and reverse primers, and Taq polymerase. A SimpliAmp Thermal Cycler (Thermo Fisher Scientific, USA) was used for the Polymerase chain reaction (PCR) under the following conditions: initial denaturation at 94°C for 3 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. PCR products were separated on 1.5% agarose gel, and a single distinct band, approximately 650 bp in size, corresponding to the ITS region, was identified and cut out. The extracted bands were then purified using the ULTRAPrep® Agarose Gel Extraction Mini Prep Kit (AHN Biotechnologie GmbH, Nordhausen, Germany). The purification process was conducted according to the manufacturer's instructions. Purified PCR products were sequenced in both forward and reverse directions using BigDye Terminator Cycle Sequencing technology (Applied Biosystems, USA). The sequencing was performed on an ABI 3130 DNA sequencer (Applied Biosystems, Thermo Fisher Scientific, USA).

Phylogenetic analysis

The obtained nucleotide sequences of ITS were used for phylogenetic analysis. The sequences of *H. rhamnoides* were aligned with those of other *Hippophae* species from the National Center for Biotechnology Information (NCBI) GenBank, along with three outgroup species: *Elaeagnus glabra* (OQ718939), *E. macrophylla* (OQ718944), and *E. moorcroftii* (EF423362). The alignment was performed using BioEdit 7.2 [58] software. The phylogenetic tree was reconstructed using Maximum Likelihood (ML) analysis with TIM3e+G4 as the best nucleotide substitution model determined by the Bayesian Information Criterion (BIC). The phylogenetic analysis was performed using IQ-TREE 2.2.2.6 [59].

RESULTS AND DISCUSSION

In this study, eight samples of *Hippophae rhamnoides* collected in Kazakhstan were analyzed along with the twenty-five samples of *Hippophae* species and three outgroup species from NCBI GenBank using ITS sequences. The alignment of the ITS sequences was 639 bp in length with outgroup samples and 642 bp without them. The alignment of the ITS sequences, which resulted in a length of 639 bp, revealed polymorphism, with 31 positions (4.85%) being polymorphic among the ingroup samples. Similar results with polymorphic positions were found in previous studies [43, 60]. The highest number of polymorphic sites was found in ITS1 (13 sites), followed by ITS2 (10 sites), and the 5.8S rRNA region with 8 variable sites. The high polymorphism in ITS1 has also been

Table 1. Information on collected plant materials

Sample	Collected place and GPS coordinates	Collected year
Hippophae rhamnoides NEK 1	Floodplain of the Irtysh River in the Pavlodar region. 52.032214, 76.966907	2017
Hippophae rhamnoides NEK 2		
Hippophae rhamnoides Mangishlak 1 (intr)	Introduced to the territory of Mangyshlak Experimental Botanical Garden 43.652553, 51.162454	2023
Hippophae rhamnoides Mangishlak 2 (intr)		
Hippophae rhamnoides Ridder 1 (intr)	Introduced to the territory of Altay Botanical Garden 50.329435, 83.548600	2023
Hippophae rhamnoides Ridder 2 (intr)		
Hippophae rhamnoides Ridder 3 (intr)		2023
Hippophae rhamnoides Ridder 4 (intr)		

intr - introduced samples; NEK - northeastern Kazakhstan

DOI: 10.11134/btp.3.2024.2

observed in different plant species [61, 62].

The resulting maximum likelihood (ML) phylogenetic tree delineated four distinct clades within the *Hippophae* species, highlighting the genetic diversity and evolutionary relationships within this genus (Figure 1). The eight samples analyzed in this study formed a large clade with the samples from NCBI GenBank. The *H. rhamnoides* samples collected from the natural population in northeastern Kazakhstan clustered together with samples collected in East Kazakhstan (OR678361) and *H. rhamnoides* ssp. *mongolica* obtained from GenBank, suggesting a close genetic relationship among these species. These results support findings that *H. rhamnoides* ssp. *mongolica* is growing in eastern Kazakhstan [5, 22, 63].

Another subclade clustered *H. rhamnoides* ssp. *turkestanica* and *H. rhamnoides* ssp. *mongolica* samples were obtained from NCBI GenBank and put into one subclade. Notably, the introduced samples analyzed in this study formed separate subclades and clustered together with samples of *H. rhamnoides* ssp. *turkestanica*, *H. rhamnoides* ssp. *mongolica*, *H. rhamnoides* ssp. *rhamnoides*, and *H. rhamnoides* ssp. *caucasica* from GenBank.

The analysis of polymorphic ITS positions was conducted to clarify the polymorphism in the nucleotide sequences from introduced and natural population samples. *H. rhamnoides* subsp. *mongolica* and *H. rhamnoides* subsp. *turkestanica* samples from GenBank were used as a reference. Interestingly, no hybrid peaks were detected in two samples collected from natural populations in Eastern Kazakhstan. The samples from introduced (intr) plants growing within Botanical Gardens in Eastern and Western Kazakhstan formed separated subclades in two neighboring clusters (Figure 1). The nucleotide sequences of ITS in these introduced plants showed hybrid peaks, which were highlighted in Table 2. This result confirmed reports on the presence of hybrid peaks in the introduced plants, which were also presented in other studies [64, 65].

Notably, only four hybrid peaks were recorded for samples from the Mangyshlak Experimental Botanical Garden, suggesting that the introduction of these samples from Eastern Kazakhstan happened recently (Table 2). The hybrid peaks at position 500 were recorded for all introduced plants in Eastern and Western Botanical Gardens (Table 2), suggesting that this SNP is a suitable indicator for introduced plants. Since most ITS polymorphic sites showed the hybrid status in introduced plants, the phylogenetic positions of these samples in Figure 1 were shifted towards the group harboring different subspecies.

Thus, in this study of samples from natural and introduced populations of *H. rhamnoides,* we used nucleotide sequences of ITS, which is a powerful tool in the DNA barcoding of plants, offering high resolution for species identification [66, 67] and phylogenetic studies [68, 69]. The widespread representation of ITS in genetic databases makes it a valuable resource for researchers in taxonomy [70, 71]. As molecular

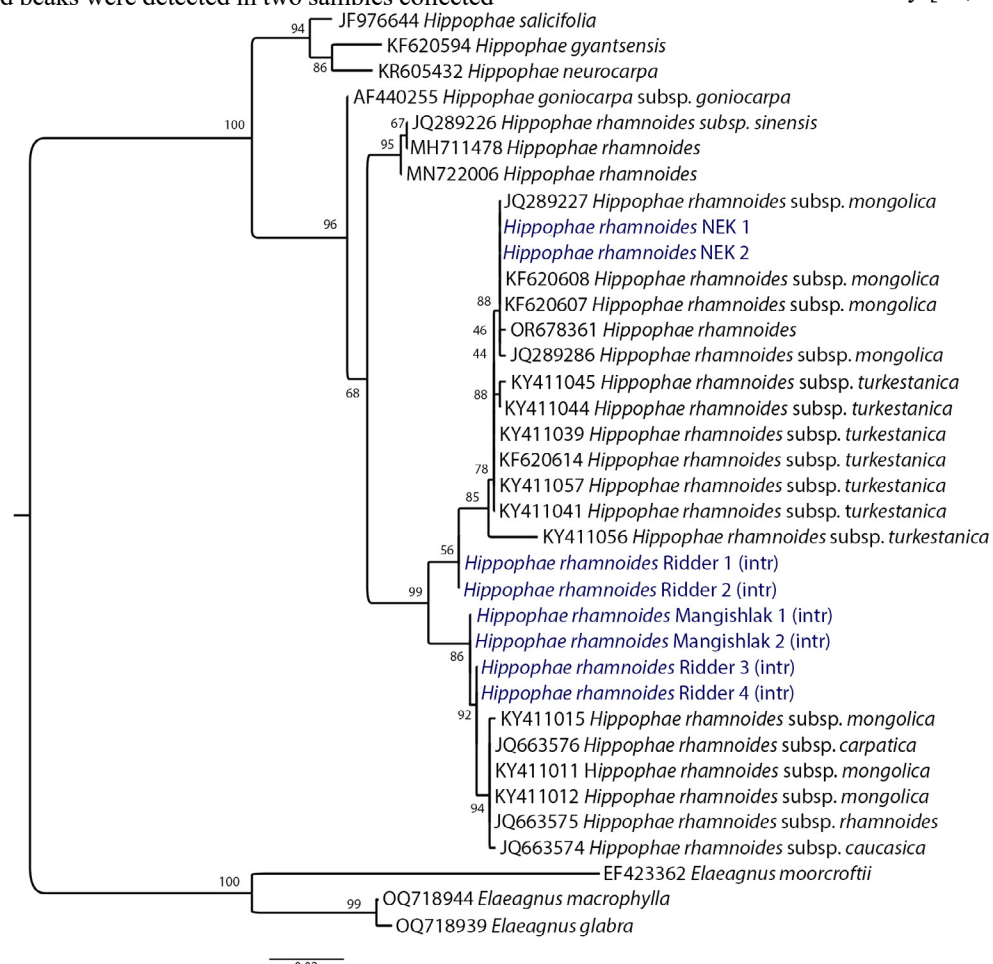

Figure 1. Maximum Likelihood phylogenetic tree of *Hippophae* and outgroup species based on nucleotide sequences of ITS. The numbers at the branch nodes represent ML bootstrap value. The species examined in this study are indicated in blue. NEK – samples collected in northeast Kazakhstan. int - introduced samples.

DOI: 10.11134/btp.3.2024.2

Table 2. Identified polymorphic positions in ITS sequences of *Hippophae* species

	Number of polymorphic sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	ITS		ITS 1											5.8S		ITS 2					
	Positions	23	38	39	40	119	124	154	170	180	209	234	248	257	443	495	500	532	537	563	565
Samples	KY411044 *H. rhamnoides* subsp. *turkestanica*	G	A	G	C	G	A	C	T	A	G	T	T	T	C	A	C	T	C	T	T
	KY411045 *H. rhamnoides* subsp. *turkestanica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	KY411056 *H. rhamnoides* subsp. *turkestanica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.
	KY411057 *H. rhamnoides* subsp. *turkestanica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	KF620607 *H. rhamnoides* subsp. *mongolica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	KF620608 *H. rhamnoides* subsp. *mongolica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	JQ289286 *H. rhamnoides* subsp. *mongolica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	JQ289227 *H. rhamnoides* subsp. *mongolica*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H. rhamnoides NEK 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H. rhamnoides NEK 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	H. rhamnoides Mangishlak 1 (introduced)	R	G	T	T	T	T	M	C	C	T	C	C	C	.	C	Y	A	Y	C	G
	H. rhamnoides Mangishlak 2 (introduced)	R	G	T	T	T	T	M	C	C	T	C	C	C	.	C	Y	A	Y	C	G
	H. rhamnoides Ridder 1 (introduced)	.	G	K	Y	K	W	.	Y	M	K	Y	Y	Y	Y	M	Y	W	.	Y	K
	H. rhamnoides Ridder 2 (introduced)	.	G	K	Y	K	W	.	Y	M	K	Y	Y	Y	Y	M	Y	W	.	Y	K
	H. rhamnoides Ridder 3 (introduced)	R	G	T	T	K	W	M	Y	M	K	Y	Y	Y	T	M	Y	W	Y	Y	K
	H. rhamnoides Ridder 4 (introduced)	R	G	T	T	K	W	M	Y	M	K	Y	Y	Y	T	M	Y	W	Y	Y	K

DOI: 10.11134/btp.3.2024.2

techniques advance, the ITS marker will continue to play a crucial role in plant genetic research, enhancing our comprehension and conservation of plant biodiversity [72]. Particularly, the results of this study indicated that ITS nucleotide sequences can effectively identify plants in natural and introduced populations of *H. rhamnoides*.

CONCLUSION

This study demonstrated the effectiveness of ITS nucleotide sequences in identifying and analyzing genetic diversity among natural and introduced populations of *Hippophae rhamnoides* in Kazakhstan. The ITS sequences revealed significant polymorphism, with 13 and 10 polymorphic sites in ITS1 and ITS2, respectively. The maximum likelihood phylogenetic tree highlighted distinct clades, reflecting the genus's genetic diversity and evolutionary relationships. Introduced samples showed unique hybrid peaks, distinguishing them from natural population samples. These findings support the continued use of ITS as a powerful tool in DNA barcoding, phylogenetic studies, and conservation efforts. As molecular techniques evolve, the ITS marker will remain crucial for enhancing our understanding and preservation of plant biodiversity, proving its value in taxonomy and genetic research.

FUNDING

This research has been funded by the Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No. BR21882166)

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УДК 34.15.23

ДНК БАРКОДИРОВАНИЕ ПРИРОДНОЙ И ИНТРОДУЦИРОВАННЫХ ПОПУЛЯЦИЙ *HIPPOPHAE RHAMNOIDES* L., СОБРАННЫХ В КАЗАХСТАНЕ

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АННОТАЦИЯ

В данном исследовании проанализировано восемь образцов природных и интродуцированных популяций вида *Hippophae rhamnoides* L., собранных в Казахстане, а также двадцать пять образцов *Hippophae* и три вида аутгруппы из NCBI GenBank с использованием нуклеотидных последовательностей ITS. В результате выравнивания последовательностей ITS, длина которых составила 639 п.н. с аутгруппами и 642 п.н. без них, выявлена 31 полиморфная позиция (4,85%). Наибольшее количество полиморфных сайтов было обнаружено в ITS1 (13 сайтов), за ним следуют ITS2 (10 сайтов) и регион 5.8S рРНК (8 сайтов). В филогенетическом древе, построенном с использованием метода maximum likelihood (ML), прослеживаются четыре четко различимые клады внутри рода *Hippophae*. Образцы *H. rhamnoides* из северо-восточного Казахстана сгруппировались с *H. rhamnoides* ssp. *mongolica* из GenBank, что свидетельствует об их близком генетическом родстве. Интродуцированные образцы образовали отдельные субклады и кластеризовались с различными образцами подвидов *H. rhamnoides* из GenBank. Примечательно, что в последовательностях ITS интродуцированных растений наблюдались гибридные пики, которые отсутствовали в образцах из природных популяций. Данное исследование подтверждает эффективность использования ITS маркеров для идентификации растений из природных и интродуцированных популяций *H. rhamnoides,* широко применяемых в генетических исследованиях растений для изучения и сохранения биоразнообразия.

Ключевые слова: Казахстан, Elaeagnaceae, *Hippophae*, ДНК баркодирование, внутренний транскрибируемый спейсер, филогения.

ӘОЖ 34.15.23

ҚАЗАҚСТАНДА ЖИНАҒАН *HIPPOPHAE RHAMNIDES* L. ТАБИҒИ ЖӘНЕ ИНТРОДУЦИЯЛАНҒАН ПОПУЛЯЦИЯЛАРЫН ДНҚ БАРКОДТАУ

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ТҮЙІН

Бұл зерттеуде Қазақстанда жиналған *Hippophae rhamnoides* L. түрінің табиғи және интродукцияланған популяцияларының сегіз үлгісі, сондай-ақ NCBI GenBank базасынан алынған *Hippophae* туысының жиырма бес үлгісі ITS нуклеотидтер тізбегі негізінде талданды. Ұзындығы 639 н.ж. сыртқы топтармен және 642 н.ж. сыртқы топсыз болған ITS тізбегін талдау нәтижесінде, 31 (4,85%) полиморфтық позиция анықталды. Полиморфты сайттардың ең көп саны ITS1 (13 сайт), одан кейін ITS2 (10 сайт) және 5,8S rRNA аймағында (8 сайт) табылды. Maximum likelihood (ML) әдісі арқылы құрастырылған филогенетикалық шежіре *Hippophae* туысы ішінде анық ажыратылатын төрт кластерді көрсетті. Қазақстанның солтүстік-шығысындағы *H. rhamnoides* үлгілері GenBank-тан алынған *H. rhamnoides* ssp. *mongolica* үлгілерімен топтастырылды, бұл олардың жақын генетикалық байланысын көрсетеді. Интродукцияланған үлгілер әртүрлі қосалқы кластерлерді құрады және GenBank-тен *H. rhamnoides* түрінің түр астындағы үлгілерміне топталды. Табиғи популяциялардан алынған үлгілерде айқындалмаған гибридті аймақтар интродукцияланған өсімдіктердің ITS тізбегінде байқалды. Бұл жұмыс *H. rhamnoides* табиғи және интродукцияланған популяцияларынан өсімдіктерді анықтау үшін, биологиялық алуантүрлілікті зерттеу және сақтау үшін өсімдіктердің генетикалық зерттеулерінде кеңінен қолданылатын, ITS маркерін пайдаланудың тиімділігін растайды.

Кілт сөздер: Қазақстан, Elaeagnaceae, *Hippophae*, ДНК баркодтау, ішкі транскрипцияланған спейсер, филогения.