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ARTICLE

Assessment of genetic diversity in sea buckthorn (*Hippophae rhamnoides* L.) accessions from Mongolia based on RAPD markers

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Abstract: *Hippophae rhamnoides* L., *which belongs to the Elaegnaceae family, is one of the medically and environmentally valuable berry crops with its high nutritious and bioactive compounds. Despite its high demand in the food, medicinal and agricultural industries, this species has been less studied molecularly. In view of this, an effort has been made in the present study to characterize 24 accessions of H. rhamnoides collected from different geographical regions of Mongolia through random amplified polymorphic DNA (RAPD) markers. A total of 10 RAPD primers were used in the present study for their ability to produce clear, scorable amplicons. The RAPD analysis totally generated 87 bands, of which 84 (96.34%) were polymorphic, pointing to a high degree of genetic variation. The similarity coefficient ranged from 0.4-1 with the mean of 0.78. The UPGMA dendrogram was generated using these data grouped accessions into two main clusters. Cluster analysis reflected a relatively close relationship between accessions grown at the same or neighbouring areas. Thus, our data could be informative for further selection and management of germplasm collections and crossing strategies for sea buckthorn.*

Keywords: *Hippophae rhamnoides* L.*; genetic distance; RAPD; UPGMA dendrogram; Dice similarity coefficient;*

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) is a dioecious and multipurpose plant, belonging to the *Elaegnaceae* [1] family. Sea buckthorn is known for its tolerance of growing extreme conditions, such as temperatures ranging from -40 to 40^{0}C, droughts, and high altitudes [2]. These diverse growing conditions are essential for its genetic diversity.

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According to the latest classification, four subspecies of *H. rhamnoides* L. are distributed across Asia (subsp. *mongolica, turkestanica, sinensis* and *yunnanesis*), in Europe (subs. *rhamnoides, fluviatilis* and *carpatica*) and in Asia Minor (subsp. *caucasica*) [3], [4]. This wind-pollinated woody plant is widely distributed across Asia and Europe with distribution ranging from seashores to mountainous terrain [5]. Mongolia has a broad range of natural production of sea buckthorn covering 29,000 km^2 (data only available for Uvs aimag - province), China's natural production range covers more than 10,000 km^2, while former USSR covers 472 km^2 [6]–[8].

This plant attracts very high medicinal interest due to its therapeutic potential and a range of pharmacological applications, such as for cardiovascular diseases, cancer, obesity, diabetes, ulcers, inflammations, immune system diseases, burn wounds, and radiation damage [9], [10].

Various important bioactive substances have been reported for *H. rhamnoides* L. plant including vitamins, mineral elements, alkaloids, flavonoids, carotenoids, phytosterols, tannins, triterpenoids, phospholipids, catechins, coumarin, fatty acids and unsaturated fatty acids [11]–[13].

In the last few decades, sea buckthorn has been admired not only for its medicinal properties, but also for its ecological and commercial value. It is traditionally processed into juice, jam, alcoholic drinks, oil, and cosmetic products [6]. It too has a high potential for preventing soil erosion and soil water loss, contributing to ecological stability [8].

There are various methods for assessing the genetic diversity of the plant species. It can be analyzed either using morphological methods or molecular markers. However, phenotypic characters are affected by environmental factors, with some limited usage. Molecular markers have proven to be a powerful tool for assessing genetic variation and interpreting genetic relationship within and among the species [14]. Random amplified polymorphic DNA (RAPD) has been extensively used for determining genetic relationship among germplasm collection, genetic diversity of *H. rhamnoides* L.[15]–[17].

As per our knowledge and literature survey, there is a lack of information on the molecular characterization of *H. rhamnoides* L. plant in Mongolia. And so the present study was carried out with different accessions of *H. rhamnoides* L. collected from various geographical regions of Mongolia using RAPD markers. Our data provides the scientific basis for future selection and management of germplasm.

MATERIALS AND METHODS

Plant material

A total of 24 *H. rhamnoides* L. samples (seeds) were collected from different geographical regions of Mongolia. Samples were collected between 2015 and 2019 from Uvs, Khovd, Zavkhan, Bayan-Olgii, and Selenge aimags. Seeds were frozen by liquid nitrogen and stored at -80^0C until genomic DNA extraction.

Table 1. Details of H. rhamnoides accession collected from different regions of Mongolia for genetic diversity analysis

S. no	Accession name	Place of collection	Accession type	Date
1	Aldarkhaan	Zavkhan aimag, Aldarkhaan soum	W	2015
2	Chuiskaya	Uvurkhangai aimag, Kharkhorin soum	C	2019
3	Zavkhan Tes	Zavkhan aimag, Tes soum	W	2019
4	Khar but 8	Zavkhan aimag, Dorvoljin soum	W	2019
5	Khar but 4	Zavkhan aimag, Dorvoljin soum	W	2019
6	Khar but 2	Zavkhan aimag, Dorvoljin soum	W	2019
7	Jirmiin burgas	Zavkhan aimag, Dorvoljin soum	W	2019
8	Khovd Erdene buren	Khovd aimag, Erdene buren soum	W	2019
9	Bokhmoron	Uvs aimag, Bokhmoron soum	W	2015

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10	Uvs Tes Toormt	Uvs aimag, Tes soum	W	2015
11	Aldarkhaan Borkh 2	Zavkhan aimag, Aldarkhaan soum	W	2019
12	Khovd Bulgan	Khovd aimag, Bulgan soum	W	2019
13	Selenge Buduunii guur	Selenge aimag, Zuunburen soum	W	2015
14	Uvs Turgen	Uvs aimag, Turgen soum	W	2015
15	Uvs Tes 1	Uvs aimag, Tes soum 1	W	2014
16	Uvs Tes 2	Uvs aimag, Tes soum 2	W	2014
17	Khovd Bulgan	Khovd aimag, Bulgan soum	W	2019
18	Selenge wangiin tohoi	Selenge aimag, zuunburen soum	W	2015
19	Khar but	Zavkhan aimag, Dorvoljin soum	W	2015
20	Khovd Bulgan 2015	Khovd aimag, Bulgan soum	W	2015
21	Aldarkhaan 4	Zavkhan aimag, Aldarkhaan soum	W	2019
22	Zavkhan Tes 2015	Zavkhan aimag, Tes soum	W	2015
23	Khovd Bulgan 2	Khovd aimag, Bulgan soum	W	2019
24	Bayan-Olgii	Bayan-Olgii aimag, Altantsogts soum	W	2019

C-cultivar, W-wild sample

DNA extraction

DNA was extracted from each seed sample. Samples were placed in a mortar with liquid nitrogen and ground to fine powder and transferred to a 1.5 ml microcentrifuge tube. 1 ml extraction buffer (250 mM NaCl, 25 mM EDTA, 0.5% SDS, 200 mM Tris-HCl pH 8.0) was added into the tube. The mix was incubated at 65°C for 1 hr 30 min, vortexed every 30 min. 60 mg Polyvinylpyrrolidone (PVP), 500 µl 7.5 M ammonium acetate were added, and incubated on ice for 30 min. The tube was centrifuged at 13000 rpm for 10 min at 4°C, supernatant was transferred into a clear tube. Same amount of isopropanol was added and incubated in -20°C for 30 min. The mix was centrifuged in 13000 rpm in 4°C for 20 min, and supernatant was removed. The resulting DNA pellet was washed with 1 ml of 70% ethanol, and then resuspended in RNAse/DNAse free water [18]. The purity and concentration of DNA samples were assessed by Nanodrop 2000/2000c and 1% agarose gel electrophoresis.

RAPD analysis

RAPD profiles were generated by using 10 polymorphic 10-mers (Table2). PCR amplification reagents were carried out in 20 µl final volume of reaction mixture containing Dream Taq buffer (2 mM $MgCl_2$), 0.2 mM of dNTP, 0.2 µM of primer (Macrogen), 1.5 U DreamTaq DNA Polymerase (Thermo Fisher Scientific), and 50 ng of genomic DNA. A PCR thermocycler (Applied Biosystems 2720) was used with the following program: an initial step of 3 min at 94°C 3 min, followed by 45 cycles of 45 sec at 94°C, 45 sec at 36°C, and 1 min 30 sec at 72°C, and a final extension step of 7 min at 72°C. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV-transillumination after ethidium bromide staining.

Table 2. The nucleotide sequences and melting temperatures of the ten selected primers

Primer	Primer sequence (5'-3')	Melting temp. (°C)
OPA-07	GAAACGGGTG	32.0
OPA-08	GTGACGTAGG	32.0
OPD-03	GTCGCCGTCA	34.0
OPD-16	AGGGCGTAAG	32.0
OPA-02	TGCCGAGCTG	34.0
OPA-11	CAATCGCCGT	32.0
OPC-15	GACGGATCAG	32.0
OPD-05	TGAGCGGACA	32.0
OPD-15	CATCCGTGCT	32.0
OPD-18	GAGAGCCAAC	32.0

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Data analysis

In this study, RAPD bands were scored as present (1) and absent (0) and a binary matrix was computed; only clear and distinct bands were included in the analysis. Genetic distance and similarity were determined by Dice's coefficient of similarity. Dice's coefficient of similarity presented as percentage was calculated pairwise comparisons between individual samples according the formula:

$$Dice\ (P_i\ , P_j)D \equiv \frac{2 \times n_{ij}}{n_i + n_j}$$

where:

n_{ij} is common band number between samples P_i and P_j

n_i band number for sample P_j

Based on this similarity coefficient, the genetic distance was calculated and an UPGMA dendrogram was constructed. In order to construct the dendrogram, primers were chosen containing at least one monomorphic band and most polymorphic band pattern.

RESULTS AND DISCUSSION

RAPD marker analysis

Known for thier reliability and simplicity, the RAPD markers were used. A genetic diversity study among the 24 accessions of *H. rhamnoides* L. was carried out using a total of 10 random decamer oligonucleotide primers to investigate the ability of amplification of genomic DNA. From among them, 8 primers (OPA-07; OPA-08; OPD-03; OPD-16; OPA-02; OPA-11; OPD-15; OPD-18) that successfully produced clear amplicons, were selected for our further study (Fig.1; Table 3). According to RAPD analysis, out of total of 87 bands 84 (96.34%) bands were polymorphic, whereas 3 (3.66%) were monomorphic. The number of bands per primer ranged from 4 to 16. On an average, 10.87 bands per primer were seen. Taken together, our data showed that the highest polymorphism was detected by OPA-11 primer, while the lowest polymorphism was detected by the OPD-03 primer. Previously, various studies have explored the genetic relationship and diversity of sea buckthorn either in the same species or different species. Jeppsson et al. reported that native sea buckthorn populations of Northern Europe from the Netherlands to Sweden revealed 85% variation [19]. Bartish et al. revealed that after RAPD analysis on 55 *H. rhamnoides* L. cultivars and accession of gene bank in Sweden showed 86.3% polymorphism, which is a sign of high diversity [20].

Table 3. Number of amplification products generated with random oligonucleotide primers for 24 accessions

Primer	Total number of RAPD products per primer	Total number of polymorphic RAPD products	Sequence of primers
OPA-07	12	12	5'-GAAACGGGTG-3'
OPA-08	10	10	5'-GTGACGTAGG-3'
OPD-03	4	4	5'-GTCGCCGTCA-3'
OPD-16	9	8	5'-AGGGCGTAAG-3'
OPA-02	11	9	5'-TGCCGAGCTG-3'
OPA-11	16	16	5'-CAATCGCCGT-3'
OPD-15	13	13	5'-CATCCGTGCT-3'
OPD-18	12	12	5'-GAGAGCCAAC-3'
Total	87	84	
Total %		96.34	

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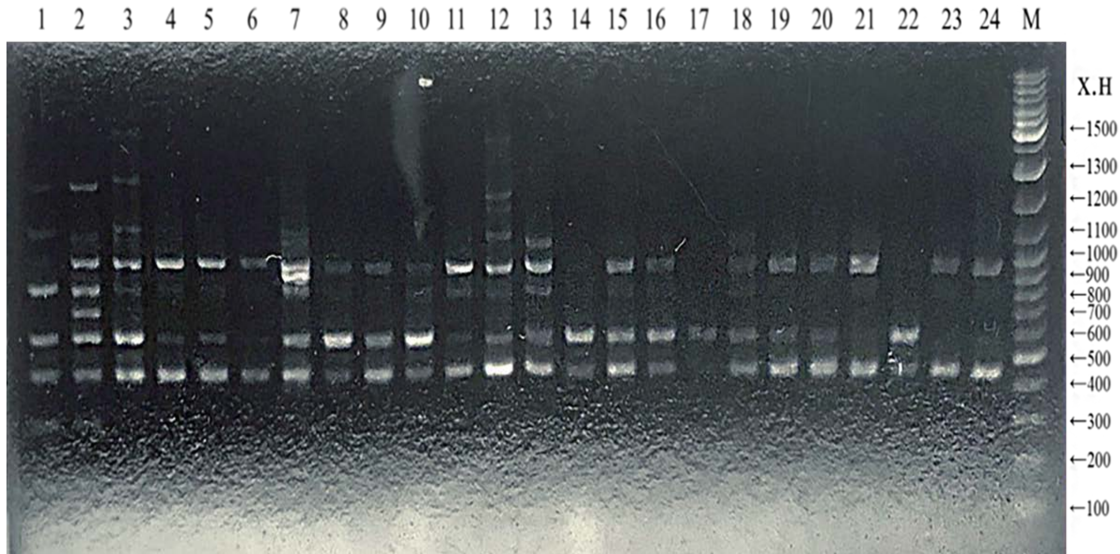

Figure 1. RAPD patterns amplified with primer OPA-08 H. rhamnoides. M indicates the size standard (Generuler 1kb Plus DNA Ladder, Thermo Fisher) Plus DNA Ladder, Thermo Fisher)

RAPD-based cluster analysis

Based on RAPD analysis, OPA-02 primer showed high degree of polymorphism and contained at least one monomorphic band, and so OPA-02 was chosen for further analysis (Figure 2). The Dice similarity coefficient was computed and the obtained values per OPA-02 primer were compared. Matrix analysis of RAPD data revealed that Dice's similarity coefficient is in the range of 0.4-1, with a mean of 0.78 (Table 4). This reflected the presence of a high genetic variability among accessions under study.

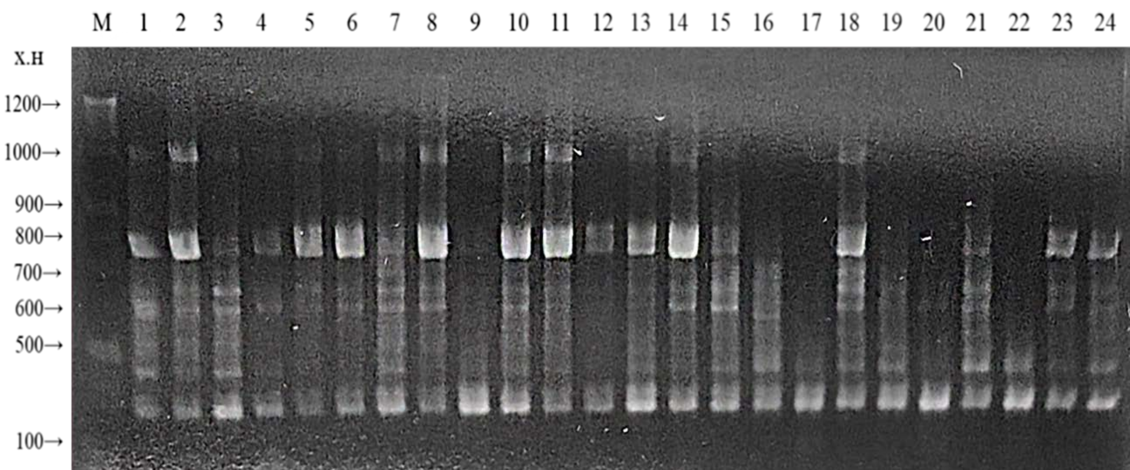

Figure 2. RAPD patterns amplified with primer OPA-02 H. rhamnoides

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Table 4. Dice's similarity coefficient based on RAPD analysis in 24 H. rhamnoides accessions according primer OPA-02 (%)

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	-																							
2	1	-																						
3	0.95	0.95	-																					
4	0.88	0.88	0.82	-																				
5	0.88	0.88	0.82	1	-																			
6	0.88	0.88	0.82	1	1	-																		
7	0.89	0.89	0.95	0.88	0.88	0.88	-																	
8	0.94	0.94	0.89	0.93	0.93	0.93	0.94	-																
9	0.5	0.5	0.46	0.4	0.4	0.4	0.5	0.55	-															
10	0.94	0.94	0.89	0.93	0.93	0.93	0.94	1	0.55	-														
11	0.94	0.94	0.89	0.93	0.93	0.93	0.94	1	0.55	1	-													
12	0.8	0.8	0.75	0.92	0.92	0.92	0.8	0.86	0.44	0.86	0.86	-												
13	0.94	0.94	0.89	0.93	0.93	0.93	0.94	1	0.55	1	1	0.86	-											
14	0.94	0.94	0.89	0.93	0.93	0.93	0.94	1	0.55	1	1	0.86	1	-										
15	0.94	0.94	0.89	0.93	0.93	0.93	0.94	1	0.55	1	1	0.86	1	1	-									
16	0.71	0.71	0.67	0.67	0.67	0.67	0.71	0.77	0.75	0.77	0.77	0.73	0.77	0.77	0.77	-								
17	0.5	0.5	0.46	0.4	0.4	0.4	0.5	0.55	1	0.55	0.55	0.44	0.55	0.55	0.55	0.75	-							
18	0.9	0.9	0.95	0.78	0.78	0.78	0.9	0.84	0.43	0.84	0.84	0.71	0.84	0.84	0.84	0.63	0.43	-						
19	0.67	0.67	0.63	0.62	0.62	0.62	0.67	0.71	0.67	0.71	0.71	0.67	0.71	0.71	0.71	0.91	0.67	0.71	-					
20	0.71	0.71	0.67	0.67	0.67	0.67	0.71	0.77	0.75	0.77	0.77	0.73	0.77	0.77	0.77	1	0.75	0.63	0.91	-				
21	0.82	0.82	0.78	0.67	0.67	0.67	0.71	0.75	0.55	0.75	0.75	0.71	0.75	0.75	0.75	0.77	0.55	0.84	0.86	0.77	-			
22	0.5	0.5	0.46	0.4	0.4	0.4	0.5	0.55	1	0.55	0.55	0.44	0.55	0.55	0.55	0.75	1	0.43	0.67	0.75	0.55	-		
23	0.88	0.88	0.82	0.86	0.86	0.86	0.88	0.93	0.6	0.93	0.93	0.92	0.93	0.93	0.93	0.83	0.6	0.78	0.77	0.83	0.8	0.6	-	
24	0.88	0.88	0.82	0.86	0.86	0.86	0.88	0.93	0.6	0.93	0.93	0.92	0.93	0.93	0.93	0.83	0.6	0.78	0.77	0.83	0.8	0.6	1	-

Furthermore, UPGMA grouping reveals that the sea buckthorn germplasm that we have studied can be divided into two distinct clusters (Figure 3). The first cluster was larger and comprised of 17 accessions (1, 2, 3, 18, 4, 5, 6, 8, 10, 11, 13, 14, 15, 7, 12, 23, 24). The second cluster was smaller comprising of 7 accessions (9, 16, 17, 19, 20, 21, and 22). The estimated genetic distance between these two clusters is 0.14. The first cluster contains eight subgroups. The first subgroup consists of two accessions - wild and cultivated (1 and 2), which were the two most closely related accessions. This means that wild accession could be cultivated in Kharkhorin. The second subgroup includes 1, 2, 3, with 1 and 3 being geographically close. Also, 4, 5, 6 grouped in same subgroup, which were closely related and three of them have same geographical origin, respectively Khar But, Zavkhan aimag, Durvuljin Soum. 8, 10, 11, 13, 14, 15 accessions make another subgroup and are closely related. Next subgroup includes three accessions (12, 23, 24), two of them are from the same geographical area, and one of them is geographically nearby. Some studies suggest that genetic diversity within populations can vary due to altitudinal gradients [21].

The second cluster is grouped into four subgroups. First subgroup consists of closely related two accessions (16 and 20), with these two 19 is added, making the next subgroup. Interestingly, accessions 9, 17, 22 showed close relatedness, which are growing in different geographical areas. This indicates to the possibility that they could be genetically similar. Accession 21 showed the highest genetic distance from other accessions.

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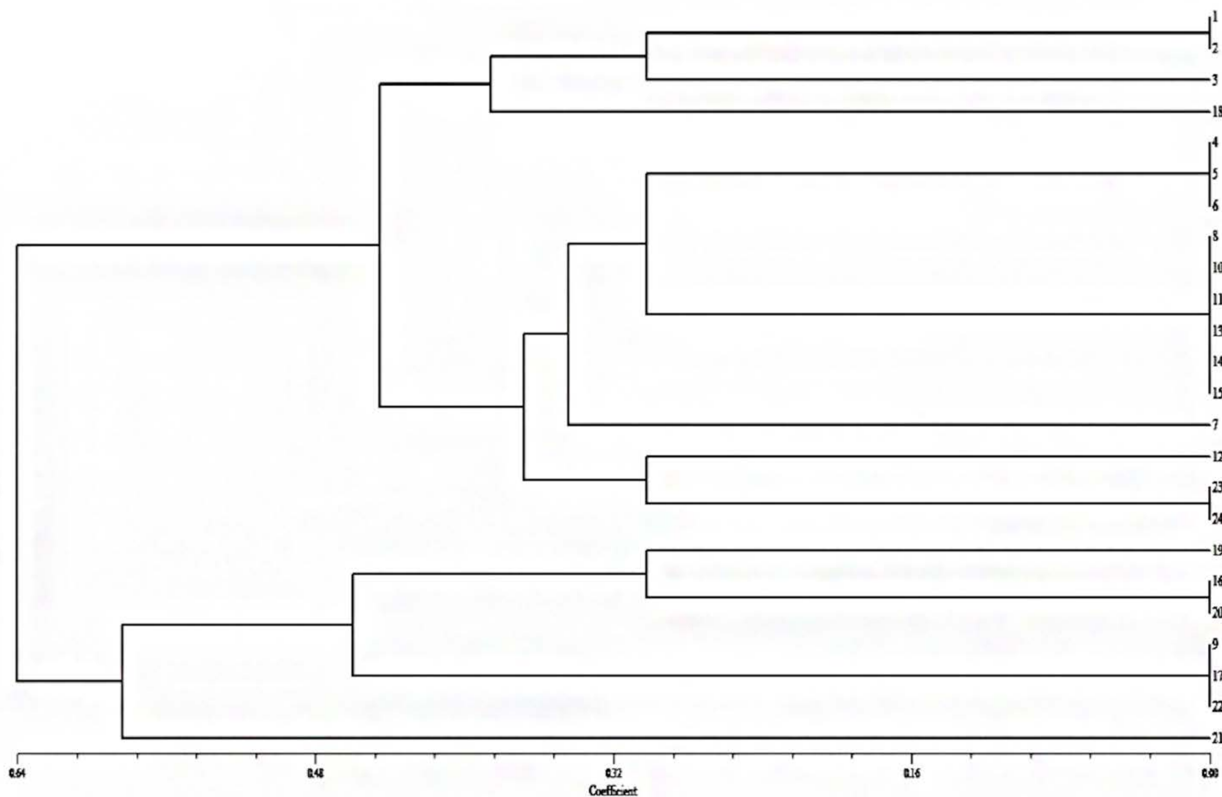

Figure 3. UPGMA dendrogram estimating the genetic distance among 24 different sea buckthorn populations from Mongolia based on RAPD markers

To date, there are limited studies on the genetic relationship and diversity of sea buckthorn of Mongolia. Our result demonstrates the utility of morphological and RAPD-PCR markers for detecting genetic relationship between the accessions of H. rhamnoides growing in different sites in Mongolia. The uniqueness of certain bands indicates that a RAPD analysis approach of *H. rhamnoides* is well suited. Resulting information can be used as guidelines for improved germplasm collection and breeding.

CONCLUSIONS

On the basis of findings of the present study, it can be concluded that genetic variability in the studied accessions of *H. rhamnoides* L. is high, the accessions of which were collected from different geographical regions. Moreover, selected sets of RAPD markers ensured high level of polymorphism and further can be applied in different *H. rhamnoides* L. subspecies.

Our results suggest that RAPD markers, which were used in our research, can also be applied to access the genetic variability of selected accessions of *H. rhamnoides* L.. Thus, above results can be the basis for future research to understand genetic variability of Mongolian *H. rhamnoides* L..

Our results suggest that RAPD markers could be used for more precise evaluation of genetic diversity and internal relatedness of sea buckthorn plant material.

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