MONGOLIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY

Chemical characterization and assessment of genetic diversity in Mongolian seabuckthorn (*Hippophae rhamnoides* L.*)* varieties using RAPD-PCR method

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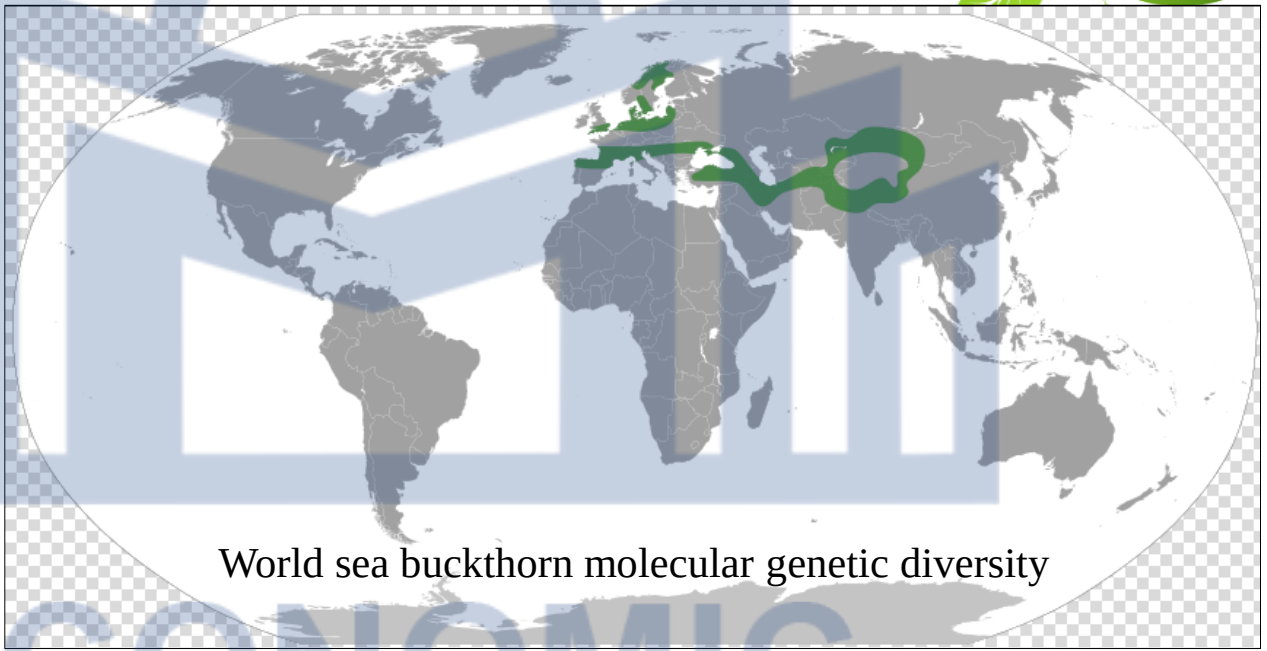
ШУТИС
МОНГОЛ УЛСЫН ШИНЖЛЭХ УХААН ТЕХНОЛОГИЙН ИХ СУРГУУЛЬ
ХҮНС СУДЛАЛЫН ЭРДЭМ ШИНЖИЛГЭЭНИЙ ТӨВ

Characterization and Investigation of Bioactivity Compound of Sea buckthorn (*Hippophae rhamnoides* L.)

- Investigation of Chemical compound
- Investigation of Molecular biology and Genetics
- Investigation of immunology

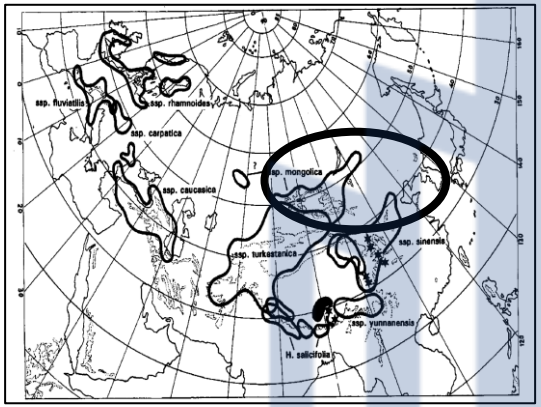
Introduction

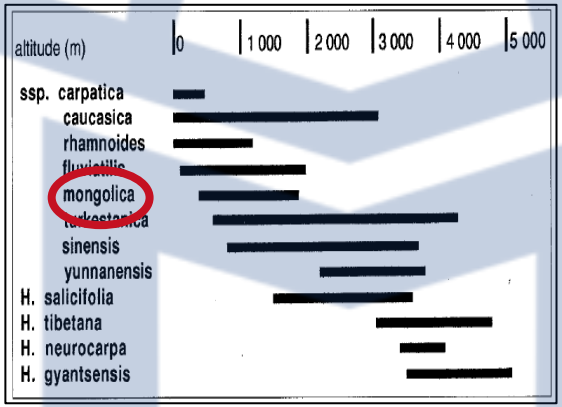


World sea buckthorn molecular genetic diversity

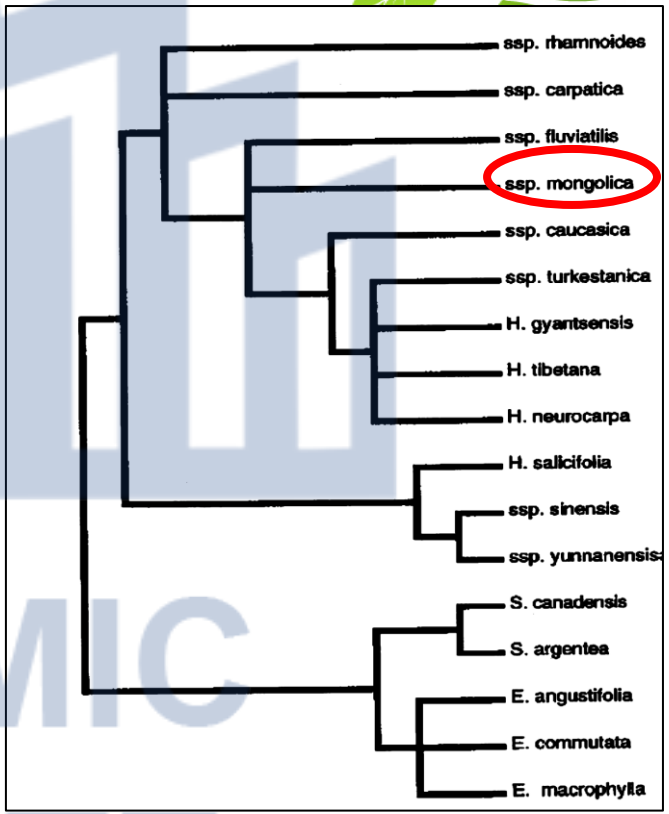
Formal breeding program of sea buckthorn was started in 1933 by Lisavenko Research Institute of Horticulture, Siberia. At present, more than 60 cultivars have been described (Trajkovski and Jeppsson. 1999). To improve local adaptation, the Russian material was crossed with Swedish collections (Jeppsson and Trajkowski. 2003). In addition to H.rhamnoides ssp. mongolica and ssp. Turkestanica of central Asian origin, the European ssp. rhamnoides and carpatica and ssp. caucasica from Minor Asia have been utilized for breeding purposes (Bartish et al. 2003).

Analysis of molecular genetic diversity in Mongolian sea buckthorn





The cultivated varieties from Russia and Mongolia show many promising agronomic traits, such as big fruits, few or no thorns, long fruit stalk, high content of bioactive substances and resistance to dried-shrink disease (some varieties), but show weak adaptability and are slow growing (Ruan and Li. 2005). Many cultivated varieties from Russia and Mongolia have been introduced into China since 1991 to improve local sea buckthorn germplasm for enhancing commercial benefits (Chunjie. 2004 and Jing Tao. 2012).



Hyvonen J and et al, Nord. J. Bot.16 (1), 1996

Purpose

- During the past decades, classical methods to evaluate genetic variation have been complemented by molecular techniques. These are useful for characterizing the genetic diversity among different cultivars or species, for identifying genes of commercial interest and improvement through genetic transformation technology (Gunārs and Kota-dombrovska. 2014).
- In recent years, different molecular markers (RAPD, AFLP, SSR, ISSR, cpDNA, ITS sequences) have been employed for the investigations of cultivars, origin and taxonomic relationships of sea buckthorn (Jeppsson et al. 1999, Sharma. 2010 and Ankit. 2010).
- We report here the application and reliability of RAPD markers to investigate the genetic diversity of *Hippophae rhamnoides L.* cultivars in Mongolia.

Materials and Methods

Extraction of plant genomic DNA

Genomic DNA was isolated from seeds (0.2 to 1 g) using QIAGEN plant genomic DNeasy Plant Mini Kit (Cat. 69106) method. The purity and quantity of extracted DNA were measured with Nanodrop 2000 spectrophotometer in 260 to 280 nm wavelengths. DNA concentration and quality was checked on 1.0% (w/v) agarose gels by electrophoresis using lambda DNA as a reference.

RAPD PCR amplification

The polymerase chain reaction (PCR) conditions for RAPDs were optimized as initial cycle of 94 °C for 5 min, 45 cycles of 94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min with final extension at 72°C for 5 min, before cooling to 4°C. Amplification was carried out in 25 µl volumes containing 2.0 µl of dNTP mix, 0.2 µl of *Taq* DNA polymerase (5 U/µl), 2.0 µl DNA template (20 ng/µl), 2 µl of primer, 2.5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ (25 mM) and 14.8 µl of sterilized distilled water.

Analysis of PCR products

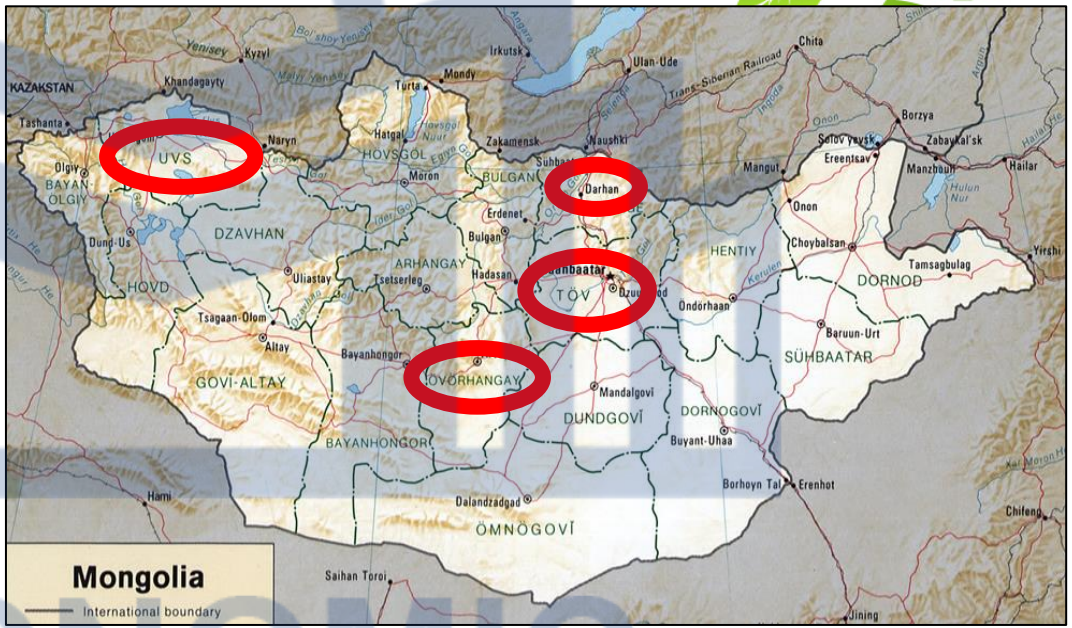
Ten (15 µl) PCR products were mixed with 2 µl of gel loading dye (0.25% bromophenol blue and 40% sucrose) and electrophoresed at 100 volts for 90 min in 1.5% agarose gel in 1× Tris acetate-EDTA (TAE) buffer (40 mM Tris, 40 mM acetic acid Glacial, 1 mM EDTA, pH 8.0) for RAPDs. After electrophoresis the gels were stained with ethidium bromide (0.5µg/ml) for 10 min followed by de-staining in tap water for 30 min. The PCR products were visualized under ultraviolet transilluminator (Bio-Rad, USA) and photographed using the Gel-Documentation Unit (Bio-Rad, USA).

Analysis of RAPD PCR data

Eight primers were initially tested using 28 DNA samples selected from different locations.

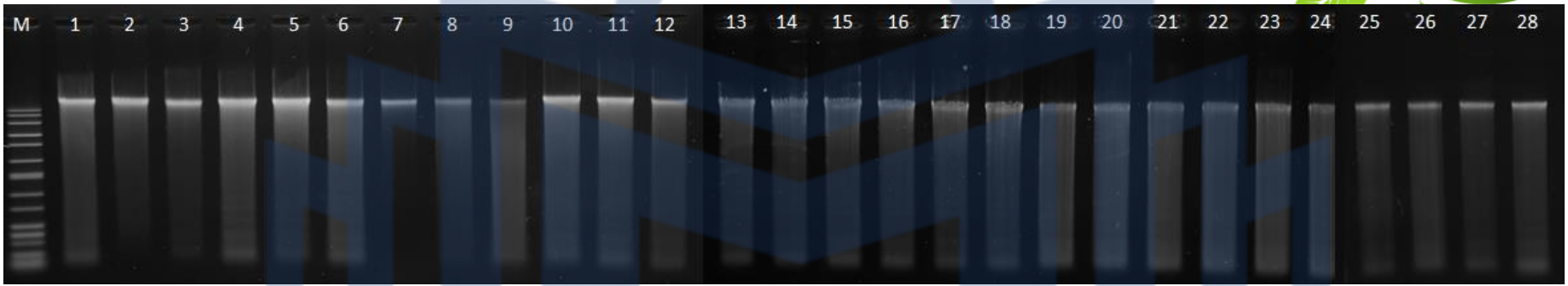
Of these, the four primers # 4, 6, 7 and 8 of all eight (Table 2) that showed clear reproducible banding patterns were chosen for analysis, based on the PCR pattern profiles. The size of amplified DNA fragments ranged from 200 to 6000 bps.

#	Sample name	Genotype	Region	Location /Company/
1	HrL.1	Chuyskaya	Western region	Uvs province /Uvs MULS-RI/
2	HrL.2	Solnechnaya		
3	HrL.3	Vitaminnaya	Central region	Tuv province /Tovkhon LLC/
4	HrL.4	Chuyskaya		
5	HrL.5	Bayangol	Northern region	Darkhan-Uul province /PSATRI/
6	HrL.6	Prevoshodnaya		
7	HrL.7	Chuyskaya	Central region	Tuv province /Polyvit LLC/
8	HrL.8	Vilican		
9	HrL.9	Altaiskya	Western region	Uvs province /Uvs Khuns LLC/
10	HrL.10	Elisabetta		
11	HrL.11	Inya	Central region	Tuv province /Khaan Jims LLC/
12	HrL.12	Chuyskaya		
13	HrL.13	Elisabetta		
14	HrL.14	Altaiskya	Central region	Uvurkhangai province /Kharkhorin LLC/
15	HrL.15	Inya		
16	HrL.16	Elisabetta		
17	HrL.17	Chechek		
18	HrL.18	Chuyskaya		
19	HrL.19	Rubin		
20	HrL.20	Vilikan	Western region	Uvs province /Khet zakh LLC/
21	HrL.21	Lyubimaya		
22	HrL.22	Chuyskaya		
23	HrL.23	Solnechnaya я		
24	HrL.24	Prevoshodnaya		
25	HrL.25	Inya		
26	HrL.26	Lyubimaya	Western region	Uvs province /Uvs MULS-RI/
27	HrL.27	Elisabetta		Uvs province /Khet zakh LLC/
28	HrL.28	Minus		Uvs province /Uvs MULS-RI/



We collected 28 samples to belong 13 genotypes of *H. rhamnoides* L. from eight locations from three distinct geographic regions that include Western, Northern and Central region in Mongolia. Collections were made during the months of September to November, 2014

Results



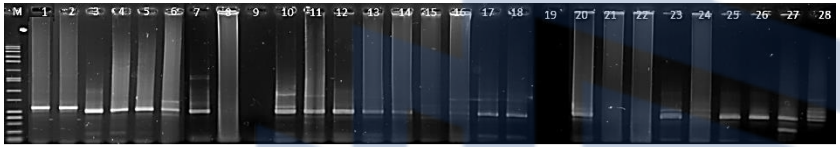
Genomic DNA from 13 *Hippophae rhamnoides L* cultivars. Sea buckthorn (*Hippophae rhamnoides* L.) plant material random amplified polymorphic DNA (RAPD) data were used in the estimation of genetic diversity and relatedness of Mongolian sea buckthorn cultivars.

Oligo name	Sequence	Nucleotides	Annealing temperature
1	GGTGCTCCGT	10 mer	37°C - 45°C
2	GTCGTCGTCT	10 mer	37°C - 45°C
3	AATGGCGCAG	10 mer	37°C - 45°C
4	AGGGCCGTCT	10 mer	37°C - 45°C
5	GTCCACACGG	10 mer	37°C - 45°C
6	TGGGTCCCTC	10 mer	37°C - 45°C
7	GTGAGGCGTC	10 mer	37°C - 45°C
8	GTGGGCTGAC	10 mer	37°C - 45°C

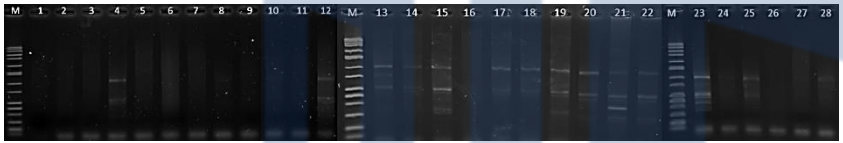
Overall comparison among cultivars across the eight primers revealed the power of RAPD in distinguishing among cultivars grown in the same location. These markers can be used in subsequent experiments to detect molecular markers for genes with genetic diversity.

RAPD - PCR

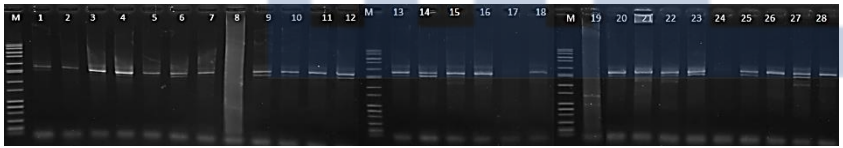
1. OPN-20



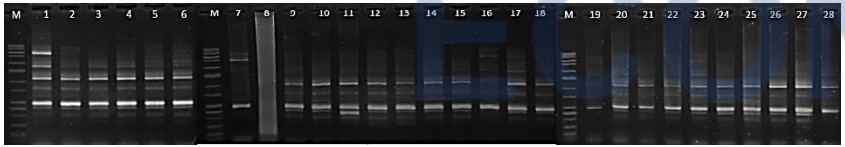
2. OPAC-2



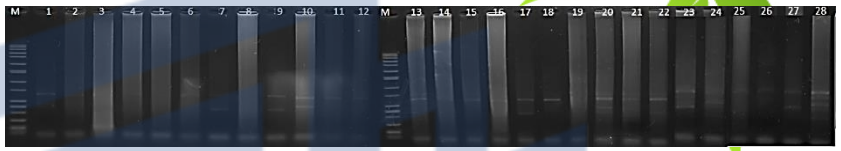
3. OPH-15



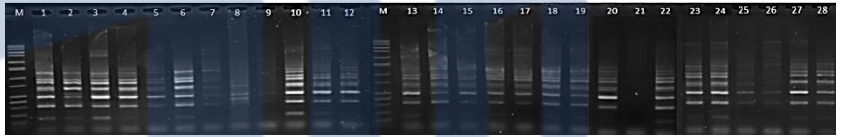
4. OPG-10



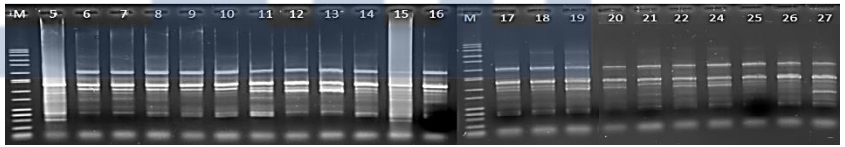
5. OPB-8



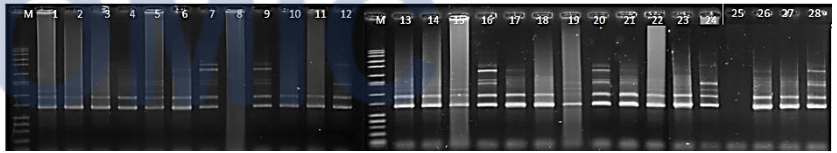
6. OPU-14



7. OPC-2



8. OPP-6



RAPD profile of the 28 samples of 13 cultivars amplified with four RAPD primers of all eight different primers. M:1Kb ladder marker, Samples 1 to 28.

Jaccard index

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
A2	1	1	1	1	0	1	1	1	1	1	1	0	0	0
A3	1	1	1	1	0	1	1	1	1	1	1	0	1	1
A4	1	1	1	1	1	1	1	1	1	1	1	1	0	0
A5	0	1	1	1	1	1	1	1	0	1	1	1	0	0
A6	1	1	0	1	0	1	1	1	1	1	1	0	0	0
A7	0	1	0	1	1	0	0	0	0	0	1	1	0	1
A8	0	1	1	1	0	1	1	1	0	0	0	0	0	0
A9	0	0	0	1	0	0	1	1	0	0	0	0	0	0
A10	1	1	1	1	1	1	1	1	1	1	0	1	0	0
A11	1	1	1	1	1	1	1	1	1	1	0	1	0	1
A12	0	1	1	1	1	1	1	1	1	1	0	1	0	0
A13	1	1	1	0	1	1	1	1	1	1	0	0	0	0
A14	1	1	1	0	1	1	1	1	1	1	0	0	0	0
A15	1	1	1	0	1	1	1	1	1	1	0	0	0	0
A16	1	1	1	0	1	1	1	1	1	1	0	0	1	1
A17	0	1	1	0	1	1	1	1	0	1	0	0	1	1
A18	1	1	1	0	1	1	1	1	1	1	0	0	0	0
A19	1	1	1	0	1	1	1	1	1	1	0	1	0	0
A20	0	1	1	0	1	1	1	1	1	1	0	1	0	0
A21	0	1	1	1	0	1	0	0	0	0	0	0	1	1
A22	1	1	1	0	1	1	1	1	1	1	0	1	0	0
A23	1	1	1	1	1	1	1	1	1	1	0	0	0	1
A24	1	1	1	1	1	1	1	1	1	1	1	0	1	1
A25	0	1	1	1	1	1	1	1	1	0	0	1	0	0
A26	0	1	1	1	0	1	0	0	0	0	0	0	1	1
A27	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A28	1	1	1	1	1	1	1	1	1	1	1	0	0	0

The Jaccard index is used to quantify the similarity between two datasets. The Jaccard index takes on a value between 0 and 1. An index of 1 means that the two dataset are identical, and an index of 0 indicates that the datasets have no common elements. The Jaccard index is defined by the following formula:

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|} = \frac{TP}{TP + FP + FN}$$

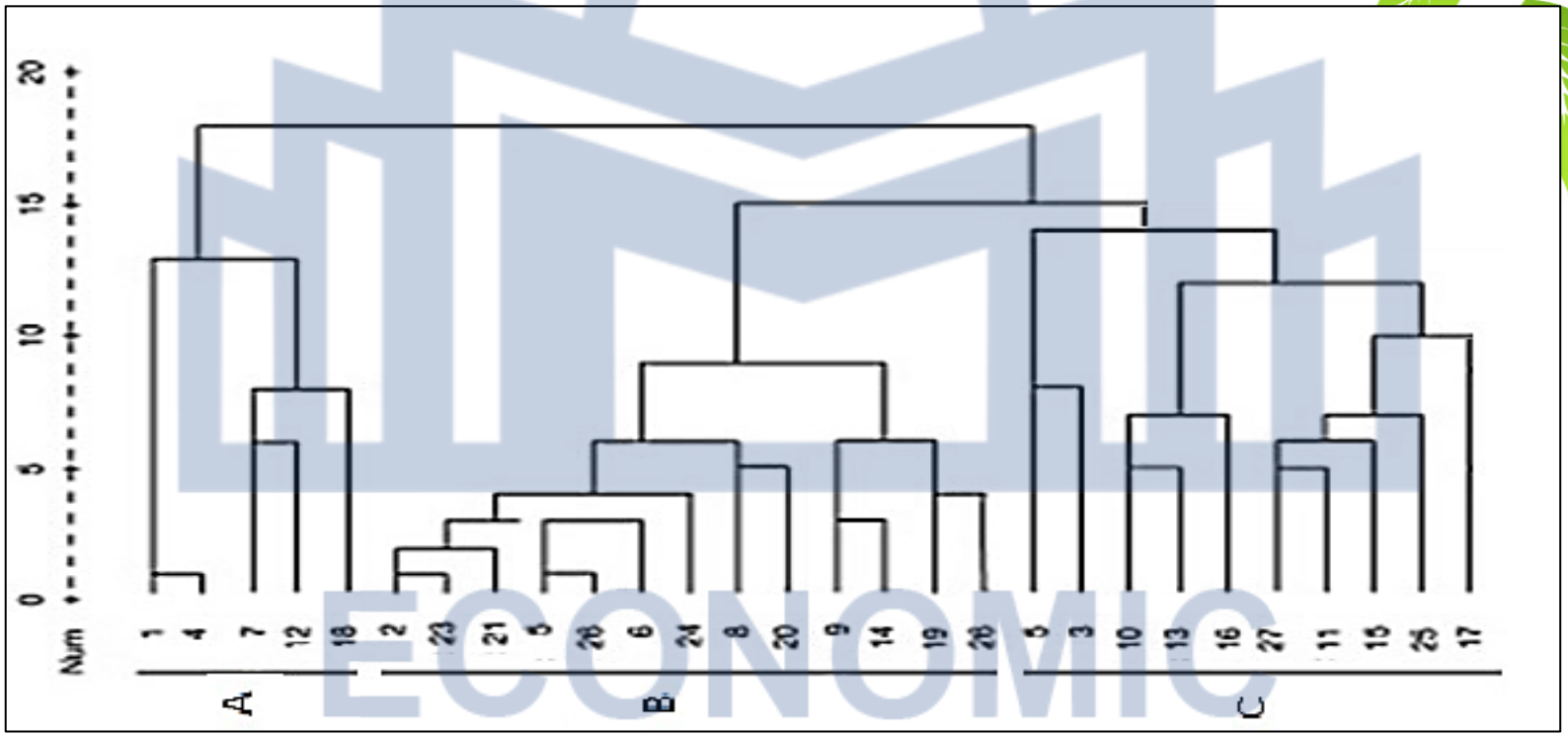
This is simply the number of unique elements common to both sets divided by the total number of unique elements in both sets.

Similarity matrix calculated with Jaccard coefficient by UPGMA method

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25	A26	A27	A28
A1	1	0.909	0.769	0.917	0.75	0.818	0.308	0.545	0.273	0.833	0.769	0.75	0.818	0.818	0.818	0.692	0.538	0.818	0.75	0.667	0.308	0.75	0.833	0.846	0.667	0.308	0.786	1
A2		1	0.833	0.833	0.667	0.9	0.231	0.6	0.3	0.75	0.692	0.667	0.727	0.727	0.727	0.615	0.462	0.727	0.667	0.583	0.333	0.667	0.75	0.769	0.583	0.333	0.714	0.909
A3			1	0.714	0.571	0.75	0.286	0.5	0.25	0.643	0.714	0.571	0.615	0.615	0.615	0.769	0.615	0.615	0.571	0.5	0.5	0.571	0.769	0.923	0.5	0.5	0.857	0.769
A4				1	0.833	0.75	0.385	0.5	0.25	0.917	0.846	0.833	0.75	0.75	0.75	0.643	0.5	0.75	0.833	0.75	0.286	0.833	0.769	0.786	0.75	0.286	0.857	0.917
A5					1	0.583	0.455	0.6	0.3	0.75	0.692	0.818	0.583	0.583	0.583	0.5	0.583	0.583	0.667	0.727	0.333	0.667	0.615	0.643	0.727	0.333	0.714	0.75
A6						1	0.25	0.5	0.333	0.667	0.615	0.583	0.636	0.636	0.636	0.538	0.385	0.636	0.583	0.5	0.25	0.583	0.667	0.692	0.5	0.25	0.643	0.818
A7							1	0.2	0.125	0.308	0.385	0.333	0.154	0.154	0.154	0.214	0.25	0.154	0.231	0.25	0.333	0.231	0.308	0.357	0.364	0.333	0.429	0.308
A8								1	0.5	0.545	0.5	0.6	0.5	0.5	0.5	0.417	0.5	0.5	0.455	0.5	0.5	0.455	0.545	0.462	0.667	0.5	0.429	0.545
A9									1	0.273	0.25	0.3	0.2	0.2	0.2	0.167	0.2	0.2	0.182	0.2	0.125	0.182	0.273	0.231	0.333	0.125	0.214	0.273
A10										1	0.917	0.909	0.818	0.818	0.818	0.692	0.538	0.818	0.909	0.818	0.308	0.909	0.833	0.714	0.818	0.308	0.786	0.833
A11											1	0.833	0.75	0.75	0.75	0.769	0.615	0.75	0.833	0.75	0.385	0.833	0.917	0.786	0.75	0.385	0.857	0.769
A12												1	0.727	0.727	0.727	0.615	0.583	0.727	0.818	0.9	0.333	0.818	0.75	0.643	0.9	0.333	0.714	0.75
A13													1	1	1	0.818	0.636	1	0.9	0.8	0.25	0.9	0.818	0.692	0.636	0.25	0.643	0.818
A14														1	1	0.818	0.636	1	0.9	0.8	0.25	0.9	0.818	0.692	0.636	0.25	0.643	0.818
A15															1	0.818	0.636	1	0.9	0.8	0.25	0.9	0.818	0.692	0.636	0.25	0.643	0.818
A16																1	0.818	0.818	0.75	0.667	0.417	0.75	0.833	0.846	0.538	0.417	0.786	0.692
A17																	1	0.636	0.583	0.636	0.5	0.583	0.667	0.692	0.5	0.5	0.643	0.538
A18																		1	0.9	0.8	0.25	0.9	0.818	0.692	0.636	0.25	0.643	0.818
A19																			1	0.9	0.231	1	0.75	0.643	0.727	0.231	0.714	0.75
A20																				1	0.25	0.9	0.667	0.571	0.8	0.25	0.643	0.667
A21																					1	0.231	0.417	0.462	0.364	1	0.429	0.308
A22																						1	0.75	0.643	0.727	0.231	0.714	0.75
A23																							1	0.846	0.667	0.417	0.786	0.833
A24																								1	0.571	0.462	0.929	0.846
A25																									1	0.364	0.643	0.667
A26																										1	0.429	0.308
A27																											1	0.786
A28																												1

Results of DNA amplification using RAPD PCR, alleles were scored as present (1) or absent (0). The Bi-variate data matrix was designed to estimate further genetic diversity (GD) among genotypes through UPGMA (Un-weighted Pair Group of Arithmetic Means) procedure described by Nei & Li (1979).

Dendrogram



Dendrogram of 28 sea buckthorn (*Hippophae rhamnoides* L.) samples using 8 randomly polymorphic DNA markers.

- Maximum genetic diversity (GD = 95%) was found among # 1 & 4 and 7 & 12 and showed minimum genetic diversity (GD = 65%) was found among # 1 & 15 and 1 & 17 while 28 sample comparisons in *Hippophae rhamnoides L* cultivars.
- Those genotypes were distributed in three major groups A, B, and C. Group B was the largest group comprised of 15 subgroups with 28 samples. Group A and C had 5 and 8 subgroups with 28 samples.
- It is recommended that these genotypes can be used by breeders in order to obtain genetically diverse segregating populations with genetic diversity.

DISCUSSION

- Diverse banding pattern (amplification profile) was obtained for different genotypes using same RAPD primers, which indicated presence of sufficient magnitude of genetic diversity in sea buckthorn genotypes (Rajwant et al. 2011).
- Procedures of “Un-weighted Pair Group of Arithmetic Means (UPGMA) described by Nie & Li (1979) was used to estimate genetic distance among genotypes.
- The data obtained was also used to find out phylogenetic relationships through Dendrogram (cluster) analysis (Anindita et al. 2009). Twenty eight samples of sea buckthorn were grouped in 3 groups A, B and C which comprised of 5, 18, and 8 genotypes, respectively.
- Results can be used for breeding purposes to obtain more potent and diverse populations of sea buckthorn. It was also concluded that further more studies can be carried out using variety of molecular markers that is RFLP, AFLP and SSR primers for better understanding of existing genetic diversity in sea buckthorn genotypes grown in Mongolia.

References

1. Anindita. K., David. D. M and Arvind. K. M. 2007 (June 2009). Molecular diversity of *Frankia* from root nodules of *Hippophae salicifolia* D. Don found in Sikkim. Indian J. Microbiol. 49:196–200
2. Asad. H. S., Syed. D. A., Ishtiaque. K., Farhat. B., Lutful. H and Stephen. R. P. 2009. Evaluation of phylogenetic relationship among Sea buckthorn (*hippophae rhamnoides. L* spp. *Turkestanica*) wild ecotypes from Pakistan using amplified fragment length polymorphism (AFLP). *Pak. J. Bot*., 41(5): 2419-2426
3. Bartish I. V. and Jeppsson N. 2003. Application of molecular markers to study the systematics, phylogeny, biogeography, genetic diversity and population genetics of *Hippophae*L. In: Singh V. (Ed.): Seabuckthorn (*Hippophae*L.) – a multipurpose wonder plant. Vol I. Botany, Harvesting and Processing Technologies. Indus Publishing Company, New Delhi, 64–71
4. Chunjie. T., Yidong. L., Suhua. S., Peng. N., Jiakuan. C and Yang. Z. 2003. Genetic diversity of sea buckthorn (*Hippophae rhamnoides*) populations in northeastern and northwestern China as revealed by ISSR markers. New Forests 27: 229–237
5. Dong-Rui. Jia., Richard. J. A., Teng-Liang. L., Kang-Shan. M., Igor V. B and Jian-Quan L. 2012. Out of the Qinghai–Tibet Plateau: evidence for the origin and dispersal of Eurasian temperate plants from a phylogeographic study of *Hippophae rhamnoides* (Elaeagnaceae). New Phytologist. 194: 1123–1133
6. Hyvonen. J., 1996. On phylogeny of Hippophae (*Elaeagnaceae*). Copenhagen. ISSN 0107-055X. Nord. J. Bot. 16: 51-62.
7. Jain. A., Ghangal. R., Grover. A., Raghuvanshi. S and Sharma. P. C. 2010. Development of EST-based new SSR markers in sea buckthorn. Physiol Mol Biol Plants. 16(4):375-8
8. Jeppsson. N., Bartish. I.V and Persson. H. A. 1999. DNA analysis as a tool in sea buckthorn breeding. Reprinted from: Perspectives on new crops and new uses. J. Janick (ed.), ASHS Press, Alexandria, VA
9. Jeppsson N. and Trajkovski V. 2003. Research and development of sea buckthorn in Sweden. In: Singh V. (Ed.) Seabuckthorn (*Hippophae*L.) – a multipurpose wonder plant. Vol I. Botany, Harvesting and Processing Technologies. Indus Publishing Company, New Delhi, 494–498
10. Lācis Gunārs and Kota-dombrovska Irita. 2014. Assessment of genetic diversity of Latvian sea buckthorn (*Hippophae rhamnoides* L.) germplasm using molecular markers. Zemdirbyste-Agriculture, vol. 101, No. 3 (2014), 333–340
11. Rajwant. K. K., Rohtas. S., Manoj. K. Rai., Gyan. P. M., Sharbati. R. S., Dhawan. A. K. 2011. Biotechnological interventions in sea buckthorn (*Hippophae rhamnoides* L.): current status and future prospects. Trees 25:559–575
12. Ruan, C. and D. Li. 2005. AFLP fingerprinting analysis of some cultivated varieties of seabuckthorn (*Hippophae rhamnoides L.*). J. Genet., 84(3), 311-316
13. Sharma. A., Zinta. G., Rana S and Shirkot. P. 2010. Molecular identification of sex in *Hippophae rhamnoides* L. using isozyme and RAPD markers. For. Stud. China. 12(2): 62–66
14. Tao. J., Chen. M., Zong. S. X and Luo Y. Q. 2012. Genetic structure in the sea buckthorn carpenter moth (*Holcocerus hippophaecolus*) in China: the role of outbreak events, geographical and host factors. Journal. Pone .0030544
15. Trajkovski, V. and Jeppsson, N. 1999. Domestication of sea buckthorn. Botanica Lithuanica. Suppl. 2:37–46

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