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Assessment of genetic diversity of Latvian sea buckthorn (*Hippophae rhamnoides* L.) germplasm using molecular markers

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Abstract

Sea buckthorn (*Hippophae rhamnoides* L.) is becoming an interesting and promising crop in Latvia due to its high content of valuable nutrients and bioactive substances. Increasing horticultural use of sea buckthorn is stimulating also an interest in research and breeding of different sea buckthorn forms for fruit production. Plant material developed from crosses among *H. rhamnoides* ssp. *mongolica*, ssp. *rhamnoides* and ssp. *fluviatilis* was introduced as well as bred in Latvia to acquire locally well adapted and valuable cultivars. Utilization of available sea buckthorn plant material is dependent on the reasonable genetic diversity evaluation and plant material identification. Therefore 36 sea buckthorn accessions grown in Latvia were characterized using previously tested eight microsatellites or simple sequence repeat (SSR) and sixteen random amplified polymorphic DNA (RAPD) markers. Applied markers allowed complete discrimination of the tested sea buckthorn accessions, as well as determination of genetic similarity and relatedness. Some correlation between the cluster analysis of molecular data and the plant material origin and known pedigree was found, which demonstrated the suitability of the utilized markers for the characterization of sea buckthorn germplasm. Higher correspondence with known origin was stated for the set of RAPD markers, which discriminated sea buckthorn accessions according to the place of origin and breeding program. The two applied methods of molecular markers complemented each other and provided genetic information for the Latvian sea buckthorn breeders for development of further hybridization strategy as well as a basic tool for marker assisted selection.

Key words: genetic diversity, germplasm, *Hippophae rhamnoides*, microsatellites, RAPD.

Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) (2n = 24) is a promising fruit crop in Latvia, due to valuable nutrients and bioactive substances including vitamins, fatty acids, free amino acids and elemental components. There were several attempts to introduce sea buckthorn in Latvia. Successful introduction of sea buckthorn was realized in the 1980's using cultivars developed from crosses among *H. rhamnoides* ssp. *mongolica*, ssp. *rhamnoides* and ssp. *fluviatilis*. Initial plant material was acquired from Prof. Tit Trofimov at Moscow State University. Local breeding of sea buckthorn cultivars was performed by Latvian breeders Andrejs Bruvelis and Karlis Blums, who continued to strengthen the adaptation of introduced cultivars by backcrosses with *H. rhamnoides* ssp. *rhamnoides* (Bruvelis, 2003; 2007). Several well adapted cultivars have been developed. Open pollinated elite seedlings selected at the M. A. Lisavenko Scientific Research Institute of Barnaul were also introduced for field evaluation and further utilization in growing and breeding. This germplasm represents a geographically distant group of sea buckthorn in comparison with the previously introduced material. As a result, the sea buckthorn plant material used in Latvia both for breeding and growing has geographically distant origin and high potential genetic variability. Therefore characterization and evaluation is an important and critical activity, which determines the success of further utilization efforts.

The identification of cultivars and assessment of breeding material could be performed on the basis of morphological and agronomic characteristics of plant material. In fruit crops, including sea buckthorn, these traits are associated with several important limiting factors: phenotypic characters are generally influenced by environment and the growth stage of the plant. It results in long and expensive evaluation during the whole vegetative growth period to obtain satisfactory morphological data for genetic diversity and relatedness evaluation. Therefore different molecular markers are very useful to distinguish between accessions and for investigations of genetic diversity or relatedness. Markers such as random amplified polymorphic DNA (RAPD) (Persson, Nybom, 1998; Jeppsson et al., 1999;

Bartish et al., 2000; Ruan et al., 2004; Sheng et al., 2006; Singh et al., 2006; Sun et al., 2006), inter-simple sequence repeats (ISSR) (Tian et al., 2004 a; b), amplified fragment length polymorphism (AFLP) (Ruan, Li, 2005) and microsatellites or simple sequence repeats (SSR) (Wang et al., 2008; Lacis et al., 2014) have been applied in sea buckthorn species genetic diversity studies. The application of different molecular marker methods for characterization of plant material increases the value of the obtained information, but at the same time creates problems with interpretation of results and choice of the most appropriate ones.

The aim of the study was to compare usefulness of two molecular marker types in the characterization of sea buckthorn, and to characterize genetic diversity of sea buckthorn (*Hippophae rhamnoides* L.) grown in Latvia.

Material and methods

Plant material. Thirty six sea buckthorn (*Hippophae rhamnoides* L.) accessions grown in Latvia were used in the investigation carried out in 2011. All sea buckthorn accessions used in the investigation represent different level of inter-subspecies crosses among *H. rhamnoides* ssp. *mongolica*, ssp. *rhamnoides* and ssp. *fluviatilis* (Lacis et al., 2014), and includes cultivars widely grown in Latvia, local selections, introduced breeding material, wild samples or cultivation escapees as well as open pollinated elite seedlings selected at the M. A. Lisavenko Scientific Research Institute of Barnaul, which represents Altai (Siberia, Russia) type of sea buckthorn adapted to continental climate (Table 1).

Table 1. Accessions of the Latvian sea buckthorn collection

No.	Accession name	Country of origin	Accession type	Description of accession
1	'Avgustinka'	Russia	A	MGU, selected sample from Leningrad region o.p.
2	'Botaniczeskaya Lubitelskaya'	Russia	A	MGU, selected sample from Leningrad region o.p.
3	'Edgars'	Latvia	W	unknown origin cultivated male plant
4	H 1.1.	Russia	B	Barnaul, Altai type
5	H 1.10.	Russia	B	Barnaul, Altai type
6	H 1.11.	Russia	B	Barnaul, Altai type
7	H 1.12.	Russia	B	Barnaul, Altai type
8	H 1.13.	Russia	B	Barnaul, Altai type
9	H 1.14.	Russia	B	Barnaul, Altai type
10	H 1.2.	Russia	B	Barnaul, Altai type
11	H 1.3.	Russia	B	Barnaul, Altai type
12	H 1.4.	Russia	B	Barnaul, Altai type
13	H 1.5.	Russia	B	Barnaul, Altai type
14	H 1.6.	Russia	B	Barnaul, Altai type
15	H 1.7.	Russia	B	Barnaul, Altai type
16	H 1.8.	Russia	B	Barnaul, Altai type
17	H 1.9.	Russia	B	Barnaul, Altai type
18	H 2.1.	Russia	B	Barnaul, Altai type
19	H 2.2.	Russia	B	Barnaul, Altai type
20	H 2.3.	Russia	B	Barnaul, Altai type
21	H 3.1.	Russia	B	Barnaul, Altai type
22	H 3.2.	Russia	B	Barnaul, Altai type
23	'Lomonosovskaya'	Russia	A	MGU
24	'Luczistaya'	Russia	A	MGU
25	'Mary'	Latvia	A	'Botaniczeskaya Lubitelskaya' o.p.
26	'Podarok Sadu'	Russia	A	MGU, selected sample from Leningrad region o.p.
27	'Prozracznaya'	Russia	A	MGU
28	'Sjurpriz Pribaltiki'	Russia	A	Mitzcurinsk, selected sample from Kaliningrad region o.p.
29	'Skibes siev'	Latvia	W	unknown origin wild sample
30	'Skibes vir'	Latvia	W	unknown origin wild male sample
31	'Tatjana'	Latvia	A	'Botaniczeskaya Lubitelskaya' o.p.
32	'Trofimovskaya'	Russia	A	MGU
33	Vir 1	Latvia	B	unknown origin cultivated male sample
34	Vir 2	Latvia	B	unknown origin cultivated male sample
35	Vir 3	Latvia	B	unknown origin cultivated male sample
36	Vir 4	Latvia	B	unknown origin cultivated male sample

A – advanced cultivar, B – breeding line, W – wild sample; Barnaul – open pollinated elite seedling selected at the M. A. Lisavenko Scientific Research Institute of Barnaul; MGU – variety originated by T. Trofimov at the Botanical Garden of Moscow State University; Mitzcurinsk – variety originated by V. T. Kondratov at the I. V. Mitzcurin Scientific Research Institute, Mitzcurinsk, o.p. – open pollinated

Isolation of genomic deoxyribonucleic acid (DNA) and polymerase chain reaction (PCR) analysis. Total DNA from young leaves was isolated using a Genomic DNA purification kit ("Fermentas", Lithuania).

Simple sequence repeat (SSR) analysis. PCR reactions were performed in a 20 µL reaction with 25 ng DNA, 2 mM each primer, 200 mM of each nucleotide, 1.5 mM $MgCl_2$ and 0.5 U REDTaq® DNA polymerase ("Sigma", USA) per reaction, in the Eppendorf Mastercycler ep Gradient thermal cycler ("Eppendorf", Germany) for 40 cycles with denaturation at 95°C for 30 s, annealing at 48, 50, 51 ºC for 30 s and extension at 72°C for 30 s, with a final extension step of 10 min at 72°C (Wang et al., 2008). For SSR markers PCR products were first checked on 1% agarose gels in 1 × TAE (Tris-acetate-EDTA) buffer and visualized by staining with ethidium bromide to test for the presence of PCR products. The same PCR products were subsequently analyzed on a genetic analyzer ABI PRISM® 3100 ("Applied Biosystems", USA) and genotyped using software *GeneMapper® v4.0* ("Applied Biosystems", USA).

Random amplified polymorphic DNA (*RAPD) analysis.* RAPD profiles were generated by using 16 previously selected highly polymorphic 10-mers (Table 2). PCR reactions were performed in 20 µL final volume containing 15 ng of DNA, 2 mM of 10-mer, 200 mM of each nucleotide, 1.5 mM $MgCl_2$ and 0.5 U REDTaq® DNA polymerase ("Sigma", USA). Amplifications were carried out in gradient Eppendorf Mastercycler ep Gradient ("Eppendorf", Germany) thermal cycler under the following conditions: 94°C for 3 min, followed by 45 cycles at 94°C for 30 s, 43°C for 1 min, and 72°C for 1 min. At the end an additional 7 min at 72°C was added. The amplification products were separated by electrophoresis in 2% agarose gels in 1 × TAE buffer, visualized by staining with ethidium bromide, and documented under UV light with digital photography. Amplifications and scorings were repeated twice.

Table 2. Genotyping results for the Latvian sea buckthorn accessions using random amplified polymorphic DNA (RAPD) markers

RAPD primer	Sequence	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands	Size range, bp
OPA-02	TGCCGAGCTG	3	2	66.6	270–780
OPA-03	AGTCAGCCAC	14	14	100	150–1050
OPA-08	GTGACGTAGG	10	10	100	220–1150
OPA-11	CAATCGCCGT	15	15	100	190–1400
OPA-18	AGGTGACCGT	9	9	100	180–1100
OPB-07	GGTGACGCAG	18	18	100	170–1400
OPB-09	TGGGGGACTC	10	10	100	170–1500
OPB-17	AGGGAACGAG	10	10	100	180–1100
OPD-02	GGACCCAACC	5	4	80	300–680
OPD-03	GTCGCCGTCA	16	16	100	200–1050
OPD-05	TGAGCGGACA	7	7	100	180–620
OPD-08	GTGTGCCCCA	23	23	100	150–1400
OPD-11	AGCGCCATTG	15	15	100	190–1050
OPD-13	GGGGTGACGA	4	4	100	350–610
OPD-18	GAGAGCCAAC	16	16	100	120–1150
OPD-20	ACCCGGTCAC	20	20	100	170–1450

Data analysis. To ensure the comparison of two molecular marker types SSR and RAPD data were registered as 1 for the presence of a particular band and 0 for its absence and were transformed into the binary matrix. Marker characteristics were computed using the computer program *GENALEX 6.1* (Peakall, Smouse, 2006). Nei and Li/Dice similarity index calculations (Nei, Li, 1979) and UPGMA (unweighted pair group method with arithmetic mean) clustering was performed using *FreeTree* (Hampl et al., 2001) and presented using *MEGA 6* (Tamura et al., 2013).

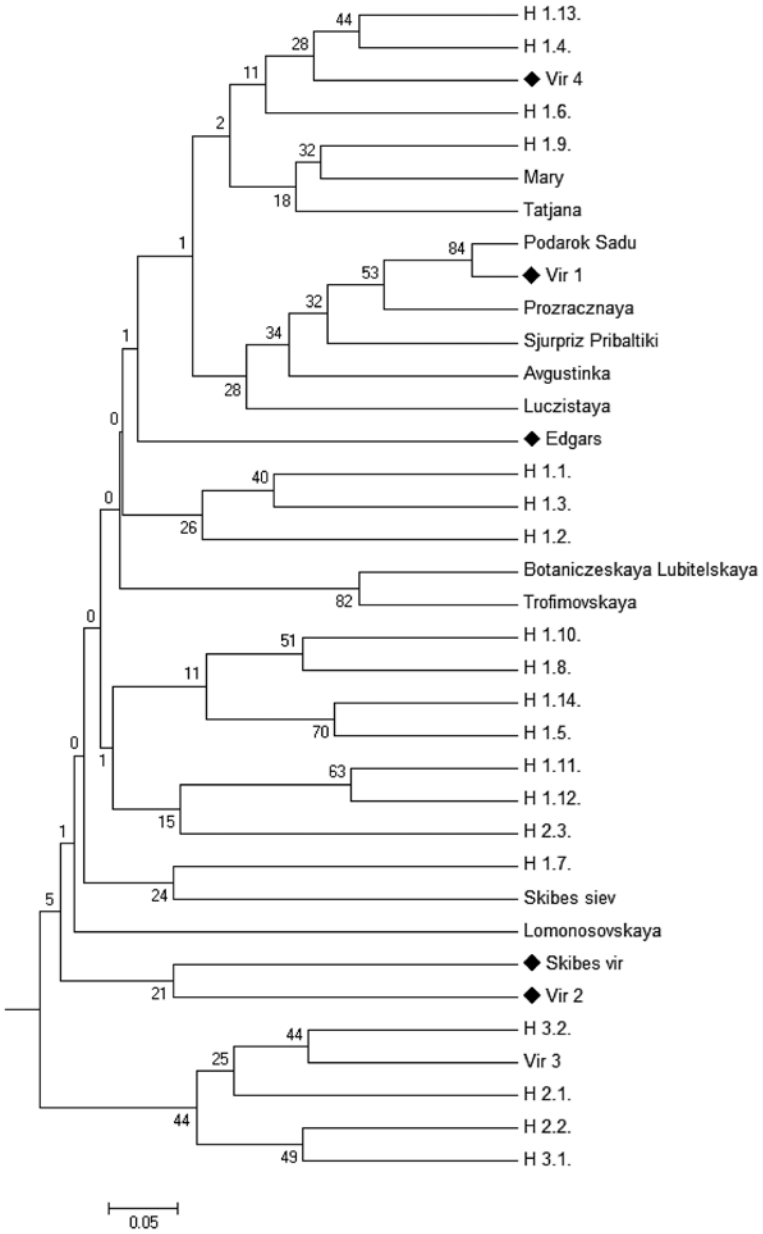
Results and discussion

Both molecular marker types used provided unique genotypes for all analyzed sea buckthorn accessions ensuring their identification. It was possible due to high level of marker polymorphism and subsequent discrimination power. The level of polymorphism for eight previously selected SSR markers (Lacis et al., 2014) was high, with the number of alleles identified ranging from 4 (*Hr08*) to 22 (*Hr04*) (average 10.25) (Table 3).

The number of effective alleles ranged from 2.653 (*Hr05*) to 8.945 (*Hr04*). Observed heterozygocity

Table 3. Genotyping results for the Latvian sea buckthorn accessions using eight microsatellite (SSR) loci

	Loci							
	Hr01	*Hr02*	*Hr03*	*Hr04*	*Hr05*	*Hr06*	*Hr07*	*Hr08*
Number of alleles	14	10	10	22	6	5	11	4
Number of effective alleles	7.043	5.684	5.153	8.945	2.653	3.713	4.853	2.723
Information index	2.171	1.932	1.870	2.643	1.235	1.425	1.861	1.103
Observed heterozygosity	0.889	0.861	0.972	0.815	0.861	0.889	0.781	0.833
Expected heterozygosity	0.858	0.824	0.806	0.888	0.623	0.731	0.794	0.633
Unbiased expected heterozygosity	0.870	0.836	0.817	0.905	0.632	0.741	0.807	0.642
Fixation index	−0.036	−0.045	−0.206	0.083	−0.382	−0.216	0.016	−0.317



◆ – sea buckthorn male plants

Figure 1. Genetic relatedness of Latvian sea buckthorn accessions estimated using microsatellite (SSR) molecular markers based on Nei and Li/Dice similarity index and UPGMA (unweighted pair group method with arithmetic mean) clustering

also was high, ranging from 0.781 (*Hr07*) to 0.972 (*Hr03*). The fixation index was negative for all loci except *Hr04* (0.083) and *Hr07* (0.016). The probability of identity for these eight tested markers was 7.56E-10, which is the probability that two samples with an identical genotype are in fact unrelated individuals. The length of alleles (in base pairs) was widely distributed for some loci, in particular *Hr04* (146–294 bp), *Hr07* (168–306 bp) and *Hr08* (129–225 bp). High level of polymorphism and good discrimination power was stated also for RAPD markers. The number of bands amplified by each RAPD primer varied from 3 (OPA-02) to 23 (OPD-08), with an average of 12.2 bands per primer (Table 2). Of the total 195 bands, 193 (98.97%) were polymorphic, with 12.1 polymorphic bands per primer on average. The size of the amplified fragments ranged from 120 to 1500 bp, but most were from 300 to 1100 bp. The set of RAPD markers used showed much higher level of polymorphism than those used in other investigations with comparable number of markers (Sun et al., 2006).

The high number of detected alleles could be explained by the origin of the tested plant material, which combines accessions of probably diverse genetic background, including cultivars of geographically distant origin (e.g., sea buckthorn accessions collected or developed from the European *Hippophae rhamnoides* material as well as accessions originated from the Altai region (Siberia) and reciprocal hybrids between these sea buckthorn groups). The same statements as well as the fact, that sea buckthorn is an out-crossing species, could explain the high level of heterozygocity found in this study. The hybrid background could be the reason for large size range of some SSR and RAPD markers. The fixation index of SSR markers was negative for all loci except *Hr04* and *Hr07*, indicating an excess of heterozygotes. However, as these samples were not from natural populations, the deviation from Hardy-Weinberg equilibrium could not be determined.

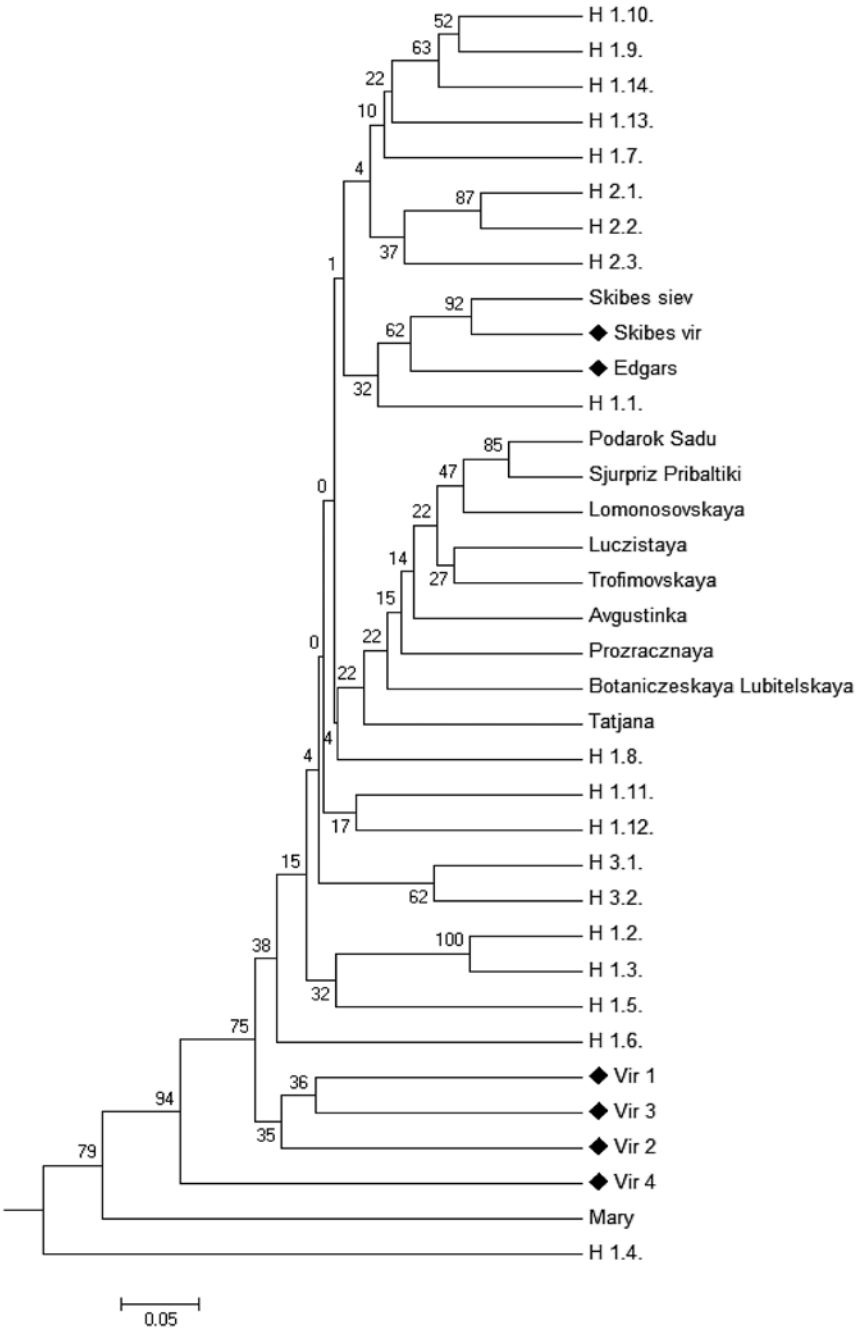
SSR, RAPD and combined SSR-RAPD data were used in the estimation of genetic diversity and relatedness of Latvian sea buckthorn cultivars. Sea buckthorn accessions showed high genetic diversity. In 99.4% and 93.4% of the cases, for SSR and RAPD markers respectively, genetic distances among accessions had values over 0.30 and for both marker types there were no cases with genetic distance value under 0.01. In the case of SSR markers the average pair-wise value of Nei and Li/Dice similarity indices (Nei, Li, 1979) among sea buckthorn accessions was 0.3998, range 0.0667 to 0.9333. Higher similarity index values were found for RAPD marker data: 0.6194 in average, range 0.2118 to 0.9048. The highest similarity value using SSR marker data was found between accessions 'Podarok Sadu' and Vir 1 (distance 0.9333), whereas the lowest – between accessions 'Avgustinka' and H 3.1. (0.0667). In the case of RAPD data the highest genetic similarity value was found between cultivars 'Podarok Sadu' and 'Sjurpriz Pribaltiki' (distance 0.9048), and the lowest – between hybrids H 1.2. and H 1.4. (0.2118). The combined SSR-RAPD data showed closer accordance with RAPD data – showing the same variety pair for maximal similarity: 'Podarok Sadu' – 'Sjurpriz Pribaltiki' (distance 0.8590) whereas variety pair for minimal similarity was different – H 2.2. and H 1.4. (0.2478).

Nei and Li/Dice similarity matrices acquired from SSR and RAPD molecular marker sets as well as combination of these were used in UPGMA clustering (Figs 1–3). Analysis using SSR data did not identify separated variety groups according to origin of plant material (Fig. 1), probably due to the hybrid nature of the analyzed material. However, several groups of closely related accessions could be marked out. Common grouping was observed for the widely grown cultivars, which have a common place of origin ('Avgustinka', 'Luczistaya', 'Podarok Sadu', 'Prozrachnaya', 'Sjurpriz Pribaltiki'). Close relatedness based on the SSR data was found also for cultivars 'Mary' and 'Tatjana', which are open pollinated seedlings of 'Botaniczeskaya Lubitelskaya'.

RAPD amplification data showed strict separation of sea buckthorn accessions into groups according to geographical origin: cultivars from Moscow State University and their offspring and groups of hybrids from Altai (designed with "H", Fig. 2). The exceptions were cv. 'Mary', which was located distantly from the parental cv. 'Botaniczeskaya Lubitelskaya' and sibling 'Tatjana', as well as H 1.4., H 1.6. and H 1.8., which were located distantly from other "H" hybrids. Distant compact group was for male accessions of sea buckthorn (Fig. 2).

As exceptions, we could mention male cultivars 'Edgars' and 'Skibes vir', which showed close relatedness with H 1.1. accession as well as variety 'Skibes siev'. Accessions 'Skibes vir' and 'Skibes siev' are semi wild or wild growing accessions, collected at the same place, and showed close relatedness also by SSR marker data. Specific bands for male plants were not detected, but combination of presence or absence of particular RAPD bands ensured distant male variety grouping, which was not observed by SSR marker data analysis. This information could be used in the further studies to find out additional gender specific molecular markers for sea buckthorn. There is RAPD-based gender-specific marker already developed in sea buckthorn F1 populations of some particular crosses (Persson, Nybom, 1998). Unfortunately, this marker did not amplify gender specific bands for the tested plant material and was not included in this study. In general, despite serious RAPD marker drawbacks (Sefc et al., 2001), they ensured better characterization of sea buckthorn plant material according to geographical origin and gender.

The total characterization of Latvian sea buckthorn plant material was performed by combined

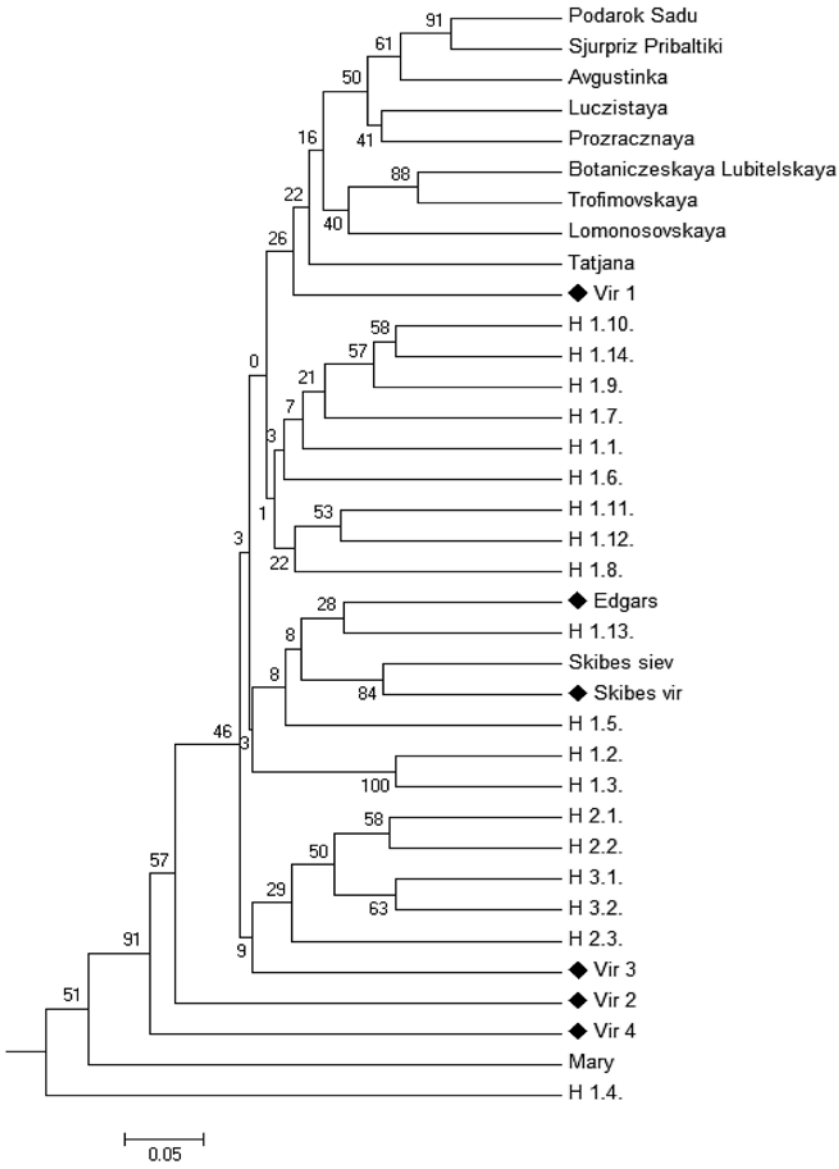


◆ – sea buckthorn male plants

Figure 2. Genetic relatedness of Latvian sea buckthorn accessions estimated using random amplified polymorphic DNA (RAPD) molecular markers based on Nei and Li/Dice similarity index and UPGMA (unweighted pair group method with arithmetic mean) clustering

RAPD and SSR data set. The relatedness of sea buckthorn plant material according to UPGMA clustering for total data set was similar to the distribution using RAPD amplification data (Fig. 3).

Information gathered by SSR markers had only complementary role to ensure more detailed plant material characterization and ensure repeatability and transformability of data (Fig. 3). The number of SSR and RAPD markers used (8 and 16, respectively) could be a reason for better characterization of Latvian sea buckthorn plant material by RAPD marker genotyping that is not typical of dominant ones. In this case selected RAPD markers ensured better genome coverage of tested plant material and thereby higher distinction ability. The SSR markers used have been developed in *H. rhamnoides* ssp. *sinensis* (Wang et al., 2008), which is not represented in tested sea buckthorn germplasm. Although they showed good applicability and high level of polymorphism, sequence specificity of SSR markers can influence their grouping ability.



◆ – sea buckthorn male plants

Figure 3. Genetic relatedness of Latvian sea buckthorn accessions estimated using microsatellite (SSR) and random amplified polymorphic DNA (RAPD) molecular markers based on Nei and Li/Dice similarity index and UPGMA (unweighted pair group method with arithmetic mean) clustering

Conclusions

1. Sea buckthorn (*Hippophae rhamnoides* L.) plant material grown in Latvia has high genetic diversity, as it was confirmed by microsatellite (SSR) and random amplified polymorphic DNA (RAPD) marker data as well as a combination of both.

2. Selected sets of SSR and RAPD markers ensured high level of polymorphism and are suitable for application in different *H. rhamnoides* subspecies, as well as in crosses among *H. rhamnoides* ssp. *mongolica*, ssp. *rhamnoides* and ssp. *fluviatilis*.

3. More adequate grouping of sea buckthorn accessions according to geographical origin and gender was for RAPD markers.

4. Comprehensive sea buckthorn plant material evaluation should be performed using both marker types, because SSRs are species specific markers, which ensure higher repeatability and transformability of data.

5. Combination of data from both marker types could ensure more precise evaluation of genetic diversity and internal relatedness of sea buckthorn plant material.

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Dygliuotojo šaltalankio (*Hippophae rhamnoides* L.) latviškos pradinės selekcinės medžiagos genetinės įvairovės įvertinimas naudojant molekulinius žymeklius

G. Lācis, I. Kota-Dombrovska

Latvijos valstybinis vaisių auginimo institutas

Santrauka

Dėl didelės vertingų maistingų ir biologiškai aktyvių medžiagų koncentracijos dygliuotasis šaltalankis (*Hippophae rhamnoides* L.) Latvijoje tampa įdomiu ir perspektyviu augalu. Didėjantis jo panaudojimas sodininkystėje ir daržininkystėje mokslininkus bei selekcininkus skatina kurti įvairias šaltalankio formas, skirtas auginti vaisiams. Siekiant gauti gerai prisitaikiusias ir vertingas veisles augalinė medžiaga, gauta sukryžminus *H. rhamnoides* ssp. *mongolica*, ssp. *rhamnoides* ir ssp. *fluviatilis*, buvo introdukuota ir sukurta Latvijoje. Turimos šaltalankio augalinės medžiagos panaudojimas priklauso nuo tinkamo genetinės įvairovės įvertinimo ir tėvinės augalinės medžiagos identifikavimo. Šiuo tikslu buvo apibūdinti 36 šaltalankio genotipai, naudojant anksčiau ištirtus 8 miksrosatelitinius arba paprastųjų pasikartojančių sekų (PPS) ir 16 atsitiktinai amplifikuotų polimorfinių DNR (AAPD) žymeklių. Panaudoti žymekliai leido atskirti tirtus šaltalankio genotipus ir nustatyti jų genetinį panašumą bei giminingumą. Nustatyta koreliacija tarp klasterinės molekulinių žymeklių analizės duomenų ir augalinės medžiagos kilmės; tai parodė panaudotų žymeklių tinkamumą apibūdinant šaltalankio pradinę selekcinę medžiagą. Didesnis atitikimas su žinoma kilme nustatytas naudojant AAPD žymeklius, kurie šaltalankio genotipus suskirstė pagal kilmės vietą ir selekcinę programą. Šie du taikyti molekulinių žymeklių metodai papildė vienas kitą ir suteikė genetinės informacijos Latvijos šaltalankio selekcininkams, kurie ją panaudos kurdami tolesnę hibridizacijos/kryžminimo strategiją, be to, padės vykdyti žymekliais paremtą selekciją.

Reikšminiai žodžiai: AAPD, genetinė įvairovė, *Hippophae rhamnoides*, mikrosatelitai, pradinė selekcinė medžiaga.