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GENETIC DIVERSITY STUDIES OF LATVIAN *VACCINIUM MYRTILLUS* L. POPULATIONS FOR *IN SITU* CONSERVATION

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ABSTRACT

Plants and berries of bilberries (*Vaccinium myrtillus* L.) are traditionally used in many nations as a local medicine as well as edible plants. They are an important feed source for wild animals and birds. *In situ* conservation is an important component for the conservation of crop wild relatives (CWR) and wild harvested plants (WHP). Research on population structure and genetic diversity is important and is required for the development and implementation of *in situ* conservation strategies as well as being useful for ecosystem services management. The aim of this study was to test EST-SSR markers for bilberry genotyping and determine genetic diversity in different forest types – *Vacciniosa, Myrtillosa, Hylocomiosa* as well as compare populations from various regions of Latvia. Our results indicated that there was a small genetic differentiation between bilberries grown in different forest types (0-2%); most of the variation was found within individuals. Analysing populations in different regions of Latvia, 5% of the genetic variation was found among populations. Analysis using the STRUCTURE software package showed that there were no isolated populations or distinct groups. There was a positive correlation between geographic and genetic distances, indicating that the analysed populations differentiation can be explained by isolation-by-distance, without additional dispersal barriers.

Key words: *Vaccinium myrtillus*, *genetic diversity, in situ conservation.*

INTRODUCTION

Vaccinium myrtillus L. (bilberries) are wild plants traditionally used as a local medicine as well as edible plants. It is a woody dwarf shrub (5-90 cm high) typical in the northern hemisphere (Nestby et al., 2010) and can form clonal colonies or patches up to 15 metres in diameter (Ritchie, 1956).

In situ conservation has become increasingly recognised as an important component of conservation strategies. Plant species conserved *in situ* are an essential source for breeding and development of new varieties (Zoratti et al., 2015). Systematic research and conservation activities for crop wild relatives (CWR) and wild harvested plants (WHP) have been initiated in many European countries. Population and genetic diversity studies are important sources of information for the development and implementation of conservation strategies. The main CWR and WHP plant groups in Latvia are forage grasses, aromatic and medicinal plants, and forest fruits and berries, therefore molecular studies of these plants could play an important role in the development of conservation strategies as well as providing information for ecosystem services management.

Latvia is located in the temperate climatic zone with a range of habitats with differing ecological parameters. The amount of rainfall and the depth of snow cover on the highlands is higher, especially on the western slopes. Over the whole territory of Latvia, in the direction of west to east there is a decreasing influence of the Atlantic Ocean and the Baltic Sea, and an increase of climatic continentality, which determines the grouping of Latvian nature districts into regions (Kavacs, 1995). According to FAO data (2015) 54% of Latvia is covered by forests and bilberries are widespread within forests. There are dry site type forests, forests on wet mineral soils, forests on wet peaty soils, forests on drained mineral soils and forests or former bogs on drained peaty soils in Latvia (Zālītis, Jansons, 2013). *V. myrtillus* reaches its maximum development in pine-dominated sites (Ritchie, 1956), therefore all samples for DNA analysis were collected from pine forests.

The majority of molecular studies, including the use of EST-SSR markers, have been done on species of the section *Cyanococcus* (Boches et al., 2005; Rowland et al., 2003). The species endemic to Latvia belong to other section (Nestby et al., 2010), therefore the available DNA markers need to be tested and adapted for use in *V. myrtillus*. In addition, there are only a few studies on genetic diversity of bilberries, including studies with using inter-simple sequence (ISSR) markers (Zoratti et al., 2015). Clonal structure of bilberry was studied with RAPD and AFLP markers (Albert et al., 2003; Albert et al., 2004), mating system and genetic structure – with isozymes (Jacquemart et al., 1994).

The aim of this study was to test EST-SSR markers for bilberry genotyping and determine genetic diversity in different forest types, in different locations.

MATERIALS AND METHODS

During the growing season, bilberry leaves were collected in three types of dry site type forests (*Vacciniosa, Myrtillosa, Hylocomiosa*) in seven locations of Latvia (Fig. 1).

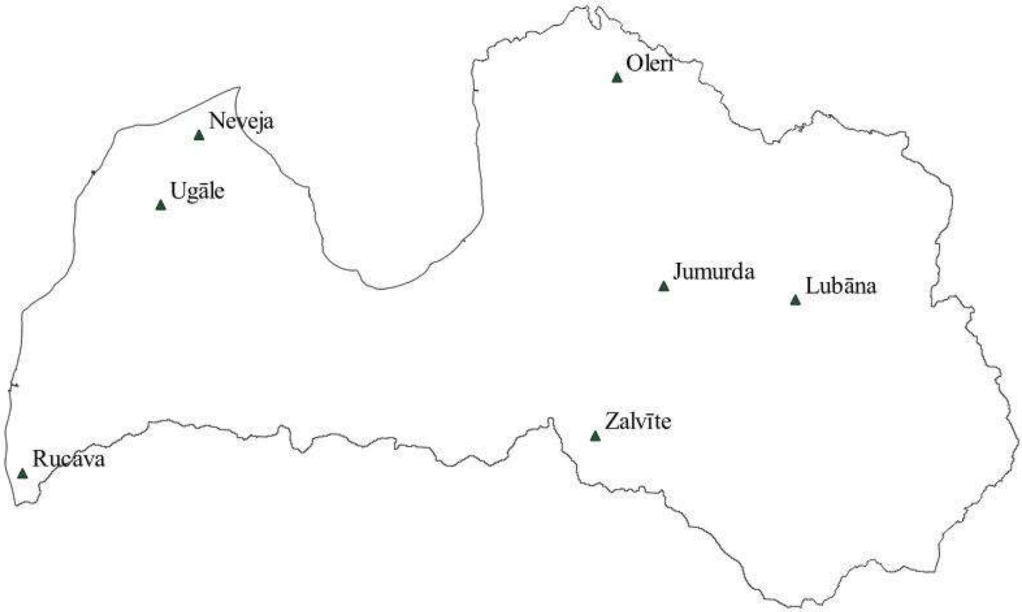

Fig.1. Locations of collected samples.

In each location, three forest types (sites) were analysed. In each site, at least 24 stems with leaves were collected. The distance between collected samples was approximately 15 metres. DNA was extracted using a modified CTAB method (Doyle, Doyle, 1990). From 18 tested EST-SSR markers (Boches et al., 2005), analyses were performed with eight markers – Na741, CA236, CA421, CA112, CA483, NA 961, VCC_K4, VCC_J5 (Table 1) labelled with one of three fluorophores (6-FAM, HEX or TAMRA). PCR reactions were performed in a volume of 10 µl containing approximately 50ng DNA, 1µl HOT FIREPol® 10x Buffer B2 (Solis BioDyne), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM forward and reverse primers. PCR was carried out in a thermocycler (Eppendorf Mastercycler epgradient): initial denaturation at 95 °C for 20 min, followed by 30 cycles at 94°C for 30 sec, annealing temperature of the primer pair (Table 1) for 45 sec, 1 min 72°C and a final extension at 72°C for 10 min.

Table 1. Markers utilised for analyses.

Locus	Annealing temperature, ºC	Allele size range (bp)	Number of alleles
Na741	58	310-320	5
CA236F	60	231-247	2
CA421F	60	183-247	23
CA112F	58	170-190	4
CA483F	58	312-340	8
NA 961	60	174-198	6
VCC_K4	60	201-265	13
VCC_J5	54	266-330	29

The PCR fragments were visualised on an Applied Biosystems ABI Prism 3100xl Genetic Analyser. Genotyping was performed using GeneMapper 4.0. (Applied Biosystems). Genotype data were analysed with programs GenAlEx 6.501. (Peakall, Smouse, 2012), Micro-Checker 2.2 (Van Oosterhout et al., 2004), STRUCTURE 2.3.4. (Pritchard et al., 2000).

RESULTS AND DISCUSSION

Of the seven analysed markers, two (VCC J5 and NA 961) had high fixation indices (0.66 and 0.76 respectively), indicating inbreeding or the presence of null alleles. The presence of null alleles can be expected due to the use of cross-species SSR markers. The EST-SSR markers (NA 741, NA 961, CA 421F and CA 483F) were reported to be successfully used for bilberry genotyping (Dahlǿ, 2011), in contrast to our results with marker NA961 showing increased $F_{is,}$ Therefore the use of these two markers (VCC_J5 and NA 961) for analysis of Latvian bilberry populations should be further assessed.

The number of alleles detected by the utilised markers ranged from 2 (CA 236F) to 29 (VCC_J5) (Table 1). The effective number of alleles varied from 2.384 to 3.381. The mean number of private alleles, unique to one population is rather small - 0.25 to 0.75. No locally common alleles present in less than 25% of populations were found, and locally common alleles present in less than 50% of populations was also low within each population (1.375-1.625). The mean Information index (I) in each population, which is equivalent to the Shannon-Weaver index, varied between 0.984 to 1.208 (Table 2).

Table 2. Mean allelic patterns across populations.

Population	Zalvīte	Neveja	Rucava	Oleri	Lubāna	Ugāle	Jumurda
Na	7.750	7.500	6.875	6.750	6.250	7.875	6.750
Na Freq. >= 5%	3.375	3.000	2.875	3.375	3.250	3.375	3.250
Ne	3.381	2.678	2.786	2.730	2.384	3.055	2.903
I	1.208	1.044	1.058	1.077	0.984	1.155	1.119
No. Private Alleles	0.250	0.750	0.125	0.250	0.000	0.375	0.250
No. locally common alleles (<=25% of pops)	0.000	0.000	0.000	0.000	0.000	0.000	0.000
No. locally common alleles (<=50% of pops)	1.375	1.250	1.500	1.375	1.625	1.500	1.375
He	0.558	0.479	0.501	0.513	0.470	0.524	0.526

Na – number of alleles; Ne – effective number of alleles; I – Information index; He - expected heterozygosity.

Each location was analysed separately. Analysis of molecular variation (AMOVA) indicated only a small difference (0-2%) between populations growing in different forest types. Analysis of each forest type in different locations, indicated that most of the variation was found within individuals (63-76%) and among individuals (24-36%). As *V. myrtillus* is a long-lived species with a mixed breeding system (Jacquemart et al., 1994) it is clear that most of genetic variation was found within individuals. Similar results were obtained previously, using RAPD markers to investigate population in differing habitats (Alberts et al., 2004), where genotypic diversity indicators did not vary significantly between habitats.

AMOVA indicated that 5% of molecular variance was found between populations in different locations in Latvia, 31% of the variation was found among individuals and 64% within individuals. STRUCTURE analysis did not identify any population substructure or isolated populations.

Comparison of pairwise genetic and geographic distance matrices show that there is a positive and significant ($p<0.001$) correlation between geographic and genetic distances (Fig. 2).

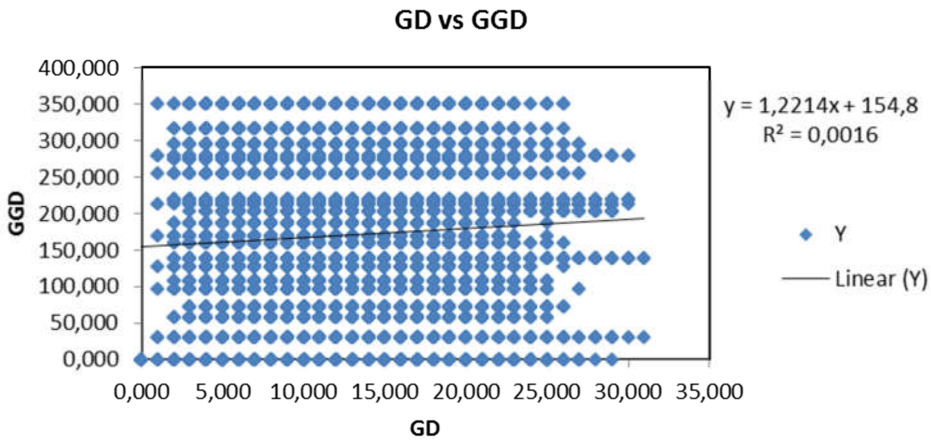

Fig. 2. Correlation between genetic (GD) and geographic (GGD) distances.

A similar significant correlation between geographic and genetic distances, indicating isolation by distance, was previously reported in Norwegian, Finnish, Icelandic and German genotypes (Zoratti et al., 2015).

Genetic diversity is essential for evolution and adaptation over long time (Sgrò et al., 2010). These results indicated that the Latvian bilberry populations were not highly differentiated, and the small number of unique alleles in each population were rare, low frequency alleles.

CONCLUSIONS

Our results indicate that genetic differentiation of Latvian bilberry populations is not influenced by forest type. The utilised markers did not identify genetically unique populations. The genetic differentiation of bilberry populations growing in different regions of Latvia is most likely a result of isolation by distance.

Therefore, the correlation between genetic and geographic distances should be taken into account when developing an *in situ* conservation strategy for bilberries and other forest berry species in Latvia.

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