Genetic diversity analysis of Latvian and Estonian *Saussurea esthonica* populations

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Abstract

Saussurea esthonica is an endangered species included in the Red Data Book of Latvia and the Red Data Book of the Baltic Region. It is found only in Latvia, Estonia and the Leningrad region of Russia. Genetic diversity and differentiation was studied in two Latvian and two Estonian *Saussurea esthonica* populations using a retrotransposon-based inter-primer binding site method. Analysis of molecular variation showed that most of the variation was found within populations; variation between populations was 3 to 5% and among regions 10 to 13%. Analysis did not detect low levels of genetic diversity parameters of the Latvian populations, indicating that they are currently genetically robust.

Key words: genetic diversity; iPBS; *Saussurea esthonica*.
Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; iPBS, inter-primer binding site; ITS, internal transcribed spacer; PCA, principal coordinate analysis.

Introduction

Saussurea esthonica Baer ex Rupr. (Estonian saw-wort), a perennial plant belonging to the Compositae family, is found only in Latvia, Estonia and the Leningrad region of Russia (Ingelög et al. 1993). In Latvia it is protected as endangered species at the national level and consists of only two populations (Andrušaitis 2003). *S. esthonica* is a calcareous species found in rich paludified grasslands, wooded and swampy meadows, and fens (Narits et al. 2000; Ek et al. 2002; Pakalne 2008). Its populations are small, which increases the risk of species extinction due to environmental, reproductive and genetic problems (Luijten et al. 2000; Oostermeijer et al. 2003; Hensen, Wesche 2006).

The genus *Saussurea* is comprised of a large number of species, which occur in Asia, Europe, North America (Lipschitz 1979; Shi et al. 2011). The genus is not well characterised phylogenetically, and it has been suggested that *S. esthonica* can be regarded as a subspecies of *Saussurea alpina* (Narits et al. 2000). However, recent studies of the phylogeny of this genus using ITS region sequencing, suggest that *S. alpina* may not be a monophyletic species (Gailite, Rungis unpublished data). Information about the genetic variability of species is necessary for conservation management and determination of populations in need of protection (Zhuravlev et al. 2010). Genetic diversity is positively correlated with population size and fitness (Reed, Frankham 2003; Šmídová et al. 2011), but in some cases it may be weak or nonexistent (Reed, Frankham 2003).

Morphological, biochemical and molecular markers have been widely used for genetic diversity studies (Chan, Sun 1997; Ellstrand et al. 1999; Nybom 2004). In comparison with morphological markers, molecular markers have the advantages of being stable and detectable at various plant developmental stages, and they are independent of plant growth stage, developmental and environmental effects (Mondini et al. 2009).

Various DNA marker techniques have been used for genetic diversity studies (Agarwal et al. 2008; Mondini et al. 2009; Kalendar 2011). Most of these methods are PCR-based and often utilise species-specific markers. However many species are not well characterised at the genomic level, and there is limited or no sequence data available. Markers based on chloroplast, mitochondrial and ribosomal sequences are widely used for genetic variation studies, but often they are not sufficiently polymorphic to be useful in inter-specific population level studies. One of the methods not requiring prior knowledge of the DNA sequence is the AFLP technique (Vos et al. 1995), which can be used to distinguish closely related individuals and for gene mapping (Mondini et al. 2009). However, this technique can be technically demanding and time consuming. A number of DNA marker techniques based on retrotransposon sequences, both species specific and non-specific, have been developed and utilised for determining genetic diversity (Agarwal et al. 2008; Kalendar 2011). Retrotransposons are ubiquitous mobile genetic elements, which have been found in all genomes studied to date

and show insertional polymorphism within and between species (Kumar, Bennetzen 1999). One recently described retrotransposon-based molecular marker technique is the inter-primer binding site (iPBS) method, which is based on conserved retrotransposon primer binding site sequences (Kalendar et al. 2010). This method detects polymorphism for all plant species without the need for prior sequence data, and therefore it is useful for species with underdeveloped marker systems (Kalendar et al. 2010). It also detects polymorphism of a multitude of insertion sites (Schulman 2007). Previously, genetic diversity and differentiation of the two Latvian *S. esthonica* populations were investigated using the AFLP and iPBS methods (Gailite et al. 2010). The results indicated that the iPBS method is useful for genetic diversity studies in *S. esthonica* and detects higher differentiation between populations than the AFLP method.

The aim of this study was to analyse the genetic diversity and population differentiation of two Latvian and two Estonian *S. esthonica* populations using two variations of the iPBS DNA marker technique.

Materials and methods

Plant material and DNA extraction

Samples from two *Saussurea esthonica* populations in Latvia (localities of Pope and Apšuciems) and two populations in Estonia (localities of Pärnu-Jaagupi and Kalevi) were collected in July 2008 and 2009. To evaluate population size generative plants in all populations were counted in 2009. In total leaves from 53 randomly chosen vegetative plants in Latvia and 51 plants in Estonia were collected. DNA was extracted from fresh leaves using a protocol based on the Fermentas genomic DNA purification kit, previously described by Gailīte et al. (2010).

PCR amplification and fragment analysis

Four PBS primers were used for genetic analysis: 2001, 2076, 2081, and 2083 (Kalendar et al. 2010). The iPBS marker analysis was performed using two methods – one utilising ethidium bromide staining and the other utilising fluorescently labelled PBS primers.

Reactions with non-labelled PBS markers were performed with 100 ng DNA in a 25 μL PCR mixture containing 1x *Dream Taq* buffer, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 1 μM primer, 1 U *Dream Taq* polymerase, 0.04 U *Pfu* polymerase. PCR amplification was carried out in a thermocycler under the following conditions: initial denaturation at 94 °C for 4 min followed by 38 cycles of 94 °C for 20 sec, 50 °C for 1 min, and 68 °C for 1 min and then terminated by 72 °C for 5 min. Amplified products were separated on 1.7% agarose gel with 0.2 μg mL^{-1} ethidium bromide in TAE buffer. Gel electrophoresis was performed at 50 V for 15 h and images were captured by using the Alpha DigiDoc digital system.

Reactions with HEX and 6-FAM dye-labelled PBS primers were performed with 100 ng DNA in a 20 μL reaction mixture containing 1x *Dream Taq* buffer, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 1 μM primer, 0.6 U *Dream Taq* polymerase, and 0.15 U *Pfu* polymerase. The PCR thermocycler protocol was comprised of initial denaturation at 95 °C for 3 min followed by 38 cycles of 95 °C for 30 sec, 50 °C for 40 sec, and 68 °C for 1 min and then ended by 72 °C for 10 min. Amplified products were separated using an ABI PRISM 3130xl Genetic Analyser and genotyped using GeneMapper v4.0.

For both methods a binary data matrix was constructed. Results were analysed using GenAlEx 6 (Peakall, Smouse 2006). Genetic diversity parameters such as fragment number, frequency and percentage of polymorphic loci were calculated, as well as the expected heterozygosity (assuming Hardy-Weinberg equilibrium). Nei's standard genetic distance was calculated between individuals and populations and principal coordinate analysis (PCA) performed on the genetic distances between individuals. The division of genetic diversity within populations, among populations and among regions (Latvia and Estonia) was calculated using analysis of molecular variance (AMOVA).

Results

The number of generative plants in each population differed. A larger number were found in the Latvian populations, with 52 generative individuals found in the Pope population and more than 80 in the Apšuciems population. In the Estonian populations, 17 generative individuals were found in the Pärnu-Jaagupi population and 31 in the Kalevi population.

The iPBS genotyping using four unlabelled primers (2001, 2076, 2081, and 2083) produced a total of 51 fragments. The lowest number of fragments was detected in the Kalevi population (41), and the highest number in the Apšuciems population (50) (Table 1). The majority of genotyped fragments had a frequency over 5%. The mean expected heterozygosity ranged from 0.219 (Kalevi) to 0.276 (Apšuciems). The only unique fragment was detected in the Apšuciems population. As previously reported by Kalendar et al. (2010) the iPBS method produces on average 15 to 50 bands from 100 to 5,000 bp in length that can be detected on agarose gels.

Utilising the same iPBS primers when they were fluorescently labelled, a total of 365 fragments were visualised on an ABI PRISM 3130xl Genetic Analyser. This method was more sensitive in comparison with the first and predominantly smaller fragments (from 100 to 1000 bp) were visualised. The number of fragments genotyped in each population ranged from 130 (Kalevi) to 226 (Pope). The number and proportion of fragments with frequency below 5% was also considerably higher than that identified with the unlabelled primers. Accordingly, the number of population-specific fragments was also higher – 50 unique fragments in the Apšuciems population, 48 unique

Table 1. Genetic variability of parameters of *Saussurea esthonica* in different populations. Method 1 – calculated from iPBS detected with ethidium bromide staining. Method 2 – calculated from iPBS with fluorescently labelled primers.

	Pärnu-Jaagupi		Kalevi		Apšuciems		Pope	
	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2	Method 1	Method 2
No. of fragments	43	152	41	130	50	222	47	226
No. of fragments (freq. ≥ 5%)	41	127	37	87	49	145	47	151
No. of unique fragments	0	28	0	22	1	50	0	48
Mean expected heterozygosity	0.231	0.051	0.219	0.041	0.279	0.078	0.236	0.077
Polymorphic loci (%)	76.4	41.64	72.55	35.62	78.43	60.82	68.63	61.92

fragments in the Pope population, 28 – in Pärnu-Jaagupi and 22 – in Kalevi.

Most of the genetic variation was found within populations (Fig. 1). The genetic differentiation between populations was low (3 to 5%), and the differentiation between regions (Latvia and Estonia) was relatively higher (10 to 13%). This was reflected in the PCA ordination (Fig. 2), where a clear differentiation was seen between the Latvian and Estonian populations. The Nei's genetic distance between the Latvian populations was 0.062 and 0.003 with the unlabelled and labelled iPBS primers respectively, while between the Estonian populations the genetic distances were 0.041 and 0.002 respectively. The difference in genetic distance using the two visualisation techniques is a consequence of the different numbers of fragments detected, the Nei genetic distance between the Estonian populations was lower than between the Latvian populations. Our previous results showed that iPBS markers were able to detect genetic diversity within *S. esthonica* and this method was more sensitive to genetic variations among populations than was the AFLP method (Gailīte at al. 2010).

The number of fragments detected using the two iPBS techniques (ethidium bromide staining and fluorescently labelled primers) differed, with the fluorescently labelled primers detecting a much larger number of fragments.

Correspondingly, the proportion of fragments with low frequency and proportion of unique alleles was higher, and the expected heterozygosity estimates were lower. However, the tendencies were similar, with lower genetic polymorphism detected in the Estonian populations in comparison with the Latvian populations.

Discussion

Most of the genetic variation was found within populations. Šmídová et al. (2011) concluded that high genetic diversity within *Ligularia sibirica* populations showed that this species are not yet threatened by genetic factors.

The iPBS marker analysis showed a clear differentiation of the Latvian and Estonian populations. The differentiation of populations within Latvia and Estonia was less pronounced. The number of fragments detected with the unlabelled PBS primers was smaller than with the labelled primers, which was expected due to the increased sensitivity of the analysis using the fluorescently labelled primers. This was also reflected by the higher proportion of fragments with lower frequency detected using the labelled primers, as well as the difference in expected heterozygosity estimates obtained using the two different detection methods. However, both tecniques indicated the same general differences between the populations, with a lower
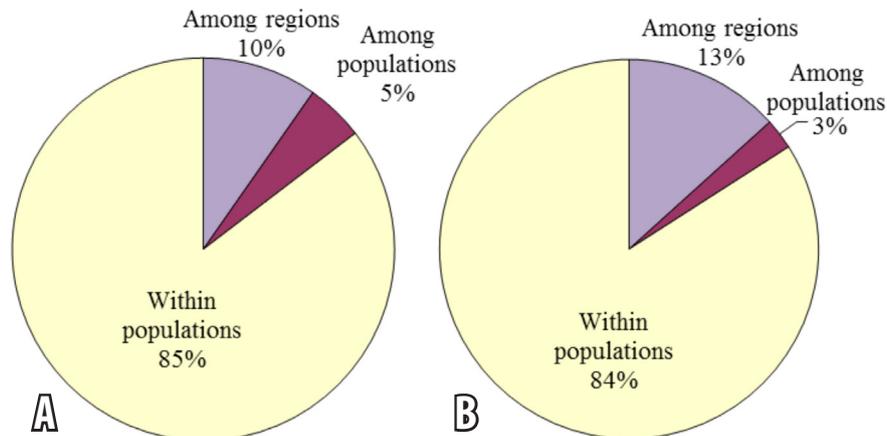
Fig. 1. Percentage of molecular variance of *Saussurea esthonica* based on iPBS analysis using ethidium bromide staining (A) and fluorescently labelled primers (B) (p < 0.001).
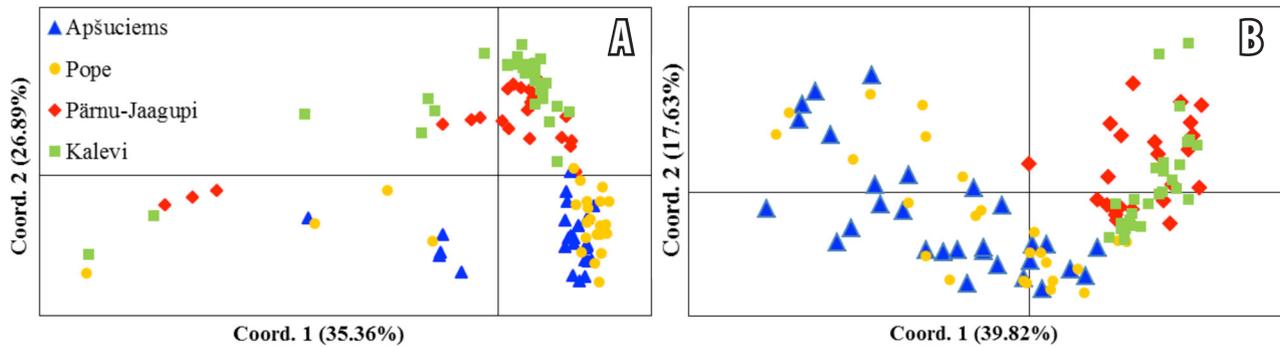
Fig. 2. Principal coordinate analysis (PCA) based on iPBS analysis of *Saussurea esthonica* using ethidium bromide staining (A) and fluorescently labelled primers (B).

number of fragments and lower expected heterozygosity in the Estonian populations.

Oostermeijer et al. (2003) concluded that in small populations of perennial plants it is possible that genetic variation is not correlated with population size. It might be possible that high numbers of flowering plants help to maintain high levels of heterozygosity and to stabilize population fitness and that the loss of genetic variation results mainly from genetic drift (Oostermeijer et al. 2003). This difference is also reflected in the number of flowering plants in our studied populations, with a lower number detected in the Estonian populations in comparison to the Latvian populations. The number of generative individuals found in the Latvian populations was higher than in Estonia, in both Estonian populations more inflorescences per capitulum were found (Gailite et al., unpublished data). Nevertheless, plant fitness parameters, such as number of flowering stems and florets per capitulum are important, since small populations mainly consist of flowering plants and heterozygosity is higher in flowering plants than in vegetative (Luijten et al. 2000). The population with the lowest number of generative individuals was observed in the Pärnu-Jaagupi population, but heterozygosity and number of polymorphic loci was higher in that population than in the Kalevi population. The reason for this discrepancy partly might be due to the different habitat of the Pärnu-Jaagupi population. This population is found in a forested area, and this may account for some physiological differences affecting the number of generative individuals found in this population, or alternatively, the individuals may have been more difficult to discover due to the more overgrown terrain. Habitat characteristics, in addition with the effective population size, are significant for successful seed production, which is dependent on the mating system, the number of flowering plants, the presence of pollinators (if required) and ovule production (Oostermeijer et al. 1998).

The genetic diversity indicators in the Estonian populations were lower than in the Latvian populations. Based on population genetic theory, correlation between population size, fitness and heterozygosity can be expected

(Reed, Frankham 2003), which indicates the ability of individuals to adapt to varying conditions for survival and reproduction. The effective population size is dependent on the number of vegetative and generative individuals. The reasons for the lower number of generative individuals found in the Estonian populations is not known, but may be due to eco-physiological factors that influence the flowering of this species, or to genetic differences between the populations. The higher numbers of flowering individuals and the increased levels of genetic diversity within the Latvian populations indicate that they are genetically robust, and do not show signs of a genetic bottleneck. The mating patterns and systems of this species have not been studied, and the extent of gene flow between populations has not been determined. In addition, the iPBS markers utilised in this study are dominant, and therefore heterozygosity cannot be assessed directly. Furthermore, there are no DNA marker techniques available for *S. esthonica* that are co-dominant. However, based on these results, it seems that the Latvian populations have a relatively high level of genetic diversity, and that they are not genetically differentiated, indicating that they have common provenance. The Latvian populations are isolated, as they are separated by ~95 km. Therefore, it seems unlikely that there is gene flow between these populations. The genetic distances between the Estonian populations were lower than between the Latvian populations, even though the geographic distance between the Estonia populations was larger. This might be due to higher gene flow between the Estonian populations via intermediate populations, given that *S. esthonica* populations are found throughout Estonia. However, monitoring of the Latvian populations should be maintained, in order to identify any decrease in genetic diversity that may indicate a threat to the long-term survival of these endangered populations of *S. esthonica*. For plant conservation purposes, environmental, genetic and demographic factors must be considered, as they are interrelated.

In conclusion, most of the genetic variation was found within populations of *S. esthonica*. Latvian and Estonian *S. esthonica* populations are genetically differentiated,

but the populations within each country are more closely related. The two iPBS genotyping techniques had differing sensitivity, leading to differences in the number of genotyped fragments. However, the comparison of genetic parameters between populations showed similar results for both genotyping techniques. Measures of genetic diversity – unique fragments, expected heterozygosity and polymorphic loci were higher in the Latvian populations of S. esthonica, indicating that currently these populations are genetically robust.

Acknowledgements

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