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PRELIMINARY STUDIES ON THE GENETIC DIVERSITY OF AN ENDEMIC AND ENDANGERED SPECIES *SAUSSUREA ESTHONICA* BAER EX RUPR. IN LATVIA

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Saussurea esthonica Baer ex Rupr. is a perennial species included in the Red Data Book of Latvia, Red Data Book of the Baltic Region and EU Habitats Directive Annex II. It is important to maintain genetic diversity within and between populations for conservation of this species in Latvia. There are two known populations of *S. esthonica* in Latvia – in the protected wetlands in the vicinity of Apšuciems and Pope.

The aim of this study is to evaluate genetic diversity between and within populations to understand the threats to the future existence of these populations. The genetic diversity and differentiation of these populations were investigated using retrotransposon and AFLP markers.

Keywords *Saussurea esthonica*, genetic diversity, retrotransposons, AFLP

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INTRODUCTION

Saussurea esthonica Baer ex Rupr. is a perennial species included in the Red Data Book of Latvia, Red Data Book of the Baltic Region and EU Habitats Directive Annex II. The genus *Saussurea* was first described in 1810 (Lipschitz, 1979). The genus is represented in Europe by three to nine or more species, depending on the species concept used (Narits et al., 2000). According to Ingelog et al. (1993) *S. esthonica* is a neoendemic of the Baltic Sea region, and originated from wetlands of the late glacial period. Between 1930 and 1990, this species was considered to be extinct in Latvia. However, in 1991, a Latvian population first described in 1884 was rediscovered, along with another new population (Andrušaitis, 2003). There are two known populations of *S. esthonica* in Latvia – in the protected wetlands in the vicinity of Apšuciems and Pope.

Genetic diversity is not well understood for many species inhabiting isolated, fragmented systems. In general, genetic diversity is expected to decrease in small and isolated populations as a consequence of processes such as genetic drift, and inbreeding (Šingliarova et al. 2008). For diversity analysis DNA marker methods have been widely used. There are no species specific markers for genus *Saussurea.* Therefore it was decided to investigate the genetic diversity of these populations using retrotransposon and AFLP markers. Both analyses are based on PCR techniques and do not need any prior sequence information of the species under study (anonymous markers).

Retrotransposons are found in the genomes of many eukaryotic organisms, and can amplify and transpose themselves to a new genomic location via a RNA intermediate. This transposition induces genetic polymorphism that can be detected by PCR with primers based on retrotransposon sequences. Retrotransposon marker analyses are technically quite simple but due to the only recent development of universal primers for them, have not been utilised on a large scale. In this study non-specific retrotransposon primers were used, which were designed using conserved retrotransposon primer binding site sequences (Kalendar et al. in press). The Amplified Fragment Length Polymorphism (AFLP) method is a well known anonymous marker method. It detects polymorphism at restriction enzyme sites by amplification of a subset of all the fragments generated by a given enzyme pair in the genome using PCR between ligated adapters. AFLP detects variations at one or only a few nucleotide positions in comparison to retrotransposons, which offer the advantage of detecting a multitude of insertion sites (Schulman, 2007).

The present study was aimed at establishing DNA marker protocols for this species as well as determining the extent of genetic diversity within and among *S. esthonica* populations known in Latvia. We wanted to compare the two DNA marker techniques to determine the most suitable method. The retrotransposon method is technically straightforward; however it has not been widely utilized as it was very recently developed. The AFLP technique, while technically more demanding, has widely been applied to a wide range of species for genetic diversity studies. Knowledge of genetic variation is fundamental to designing strategies for conservation, since the primary goal of conservation is to preserve the prevalent spectrum of genetic diversity and thus the evolutionary potential (Holsinger, Gottlieb 1991).

MATERIAL AND METHODS

Plant material and DNA extraction

Leaves from 29 randomly chosen plants of Apšuciems population and 24 randomly chosen plants of Pope population were used.

DNA was extracted from fresh leaves by using the protocol based on Fermentas Genomic DNA purification kit: plant material was ground in a Ball Mill MM400 with 200ģl TE+ā-mercaptoethanol buffer, 400 ģl Lysis solution was added and mixed gently; and incubated at 65°C for 20 minutes. 600 ģl of 24:1 chloroform:isoamyl alcohol was added, vortexed and centrifuged at 13000 rpm for 20 minutes. The aqueous phase was transferred to a fresh centrifuge tube, 800 ģl precipitation solution was added and then centrifuged at 13000 rpm for 20 minutes. The supernatant was decanted, NaCl+RNAse added, vortexed, incubated at 37°C for 30 minutes, vortexed, 300 ģl chilled 96° ethanol (chilled at 4°C) was added, incubated at -20°C for one hour; centrifuged at 13000 rpm for 10 minutes, and the supernatant decanted. Chilled (4°C) 70° ethanol was added, vortexed, centrifuged at 13000 rpm for 10 minutes. The DNA pellet was dried and resuspended in 50 ģl of TE buffer.

The DNA concentration of each sample was measured using UV/VIS spectrometer Lambda 25.

Retrotransposon analysis

PCR amplification

Reactions with retrotransposon markers were performed with 100 ng DNA in 25 ģl reaction mixure containing 1x *Dream Taq* buffer, 2,5 mM $MgCl_2$, 10 mM each dNTP Mix, 4 pM primer, 1 U *Dream Taq* polymerase, 0.04 U *Pfu* polymerase. PCR amplification was carried out in a thermocycler under following conditions: an 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 each, followed by 72 °C for 5 min. A total 15 primers were screened (Kalendar et al. in press), of which five were selected for future evaluation based on the quality and number of bands amplified. Amplified products were separated by electrophoresis on a 1,5% agarose gel with 0,2 ģg/ml ethidium bromide in TAE buffer. Gel electrophoresis was performed at 50V for15 h and image captured by using digital system Alpha DigiDoc.

AFLP analysis

The AFLP protocol (http://bioweb.usu.edu/wolf/aflp_protocol.htm) was followed with some modifications. The restriction reaction was carried out with 500 ng genomic DNA using 5 ģl DNA solution and Master mix (2 ģl 10x Tango buffer, 0,5 ģl Eco RI enzyme, 2,5 ģl H_2O) in a final reaction volume of 10 ģl and incubated at 37°C for 3 h. To this restriction reaction 1 unit *Tru1*I (*Mse*I) enzyme, 0,4 ģl 10x Tango buffer, 1,5 ģl H_2O in a final volume of 2 ģl was added, and incubated at 65°C for 3 h. After complete digestion, 1 ģl *Mse*I adapter, 1 ģl *Eco*RI adapter, 1 unit T4 DNA ligase, 2 ģl ligase buffer, 0,6 ģl 10x Tango buffer, 3,2 ģl H_2O was added and incubated for 3 h at 22°C. 80 ģl of TE buffer was added to each tube. The ligation product (3 ģl) was amplified in 22 ģl PCR reaction volume containing 10 mM each dNTP, 1 unit of *Taq* DNA polymerase, 2,5 ģl of *Taq* buffer, 1,5 mM $MgCl_2$ and 0,2 ģM final concentration of *Mse*+C and *Eco*+A primer. The PCR pre-amplification profile was 30 cycles of 20 s at 94°C, 30 s at 56°C and 2 min at 72°C, followed by final extension at 60°C for 30 min. The PCR product was then diluted with 75 ģl sterile TE buffer. The selective amplification profile was 1 cycle of 2 min at 94°C, 12 cycles of 30 s at 94°C, 30 s at 65°C and 2 min at 72°C, 23 cycles of 30 s at 94°C, 30 s at 56°C and 2 min at 72°C, followed by 10 min at 72°C. Three primer combinations were used: *Eco*-ACA/*Mse*-CTG, *Eco*-AAG/*Mse*-CAG, *Eco*-AGG/*Mse*-CAG. AFLP-fragments were separated on an Applied Biosystems 3100*xl* capillary sequencer.

Data analysis

Retrotransposon markers amplification bands were scored in a binary manner as either present (1) or absent (0) and entered into a binary data matrix. AFLP fragments were genotyped using GeneMapper 4.0, and a binary data matrix was constructed. Results were analysed using GenAlEx 6 (Peakall and Smouse, 2006). Phylogenetic trees were constructed using the Neighbour-Joining cluster analysis contained in the NTSYS3.1 software package.

RESULTS

The five retrotransposon primers produced a total of 67 fragments. four unique fragments were detected in the Apšuciems population (Fig.1). The Apšuciems population has slightly higher expected heterozygosity (0,3), than the Pope population (0,29).

The total number of fragments genotyped in the AFLP analysis was 208. Higher expected heterozygosity was found in the Apšuciems population (0,32) in comparison to the Pope population (0,30). There are five unique fragments detected in the Apšuciems population and two in the Pope population (Fig. 2).

Analysis of molecular variance (AMOVA) revealed that using the retrotranposon data, molecular variance within populations is 89% and among 11%. However, with the AFLP data, 97% of molecular variance was found within populations and only 3% among populations.

Principal coordinate analysis (PCA) of the retrotransposon data divided the populations into two groups with minor overlaps (Fig 3).

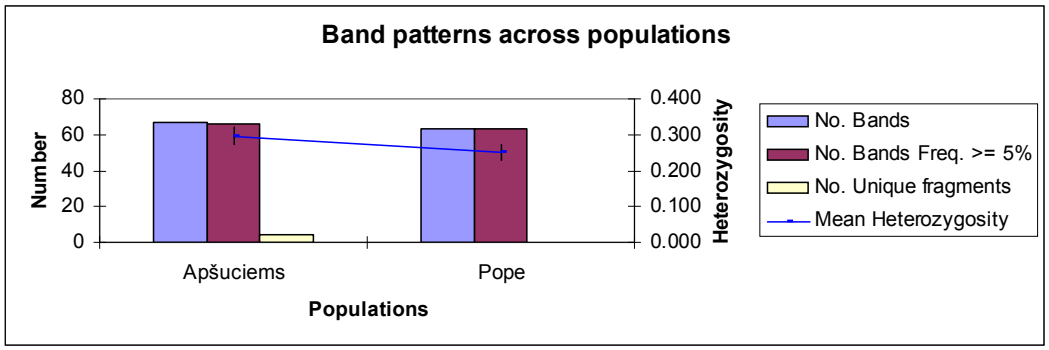

Fig.1 Genetic diversity measures between populations based on retrotransposon markers

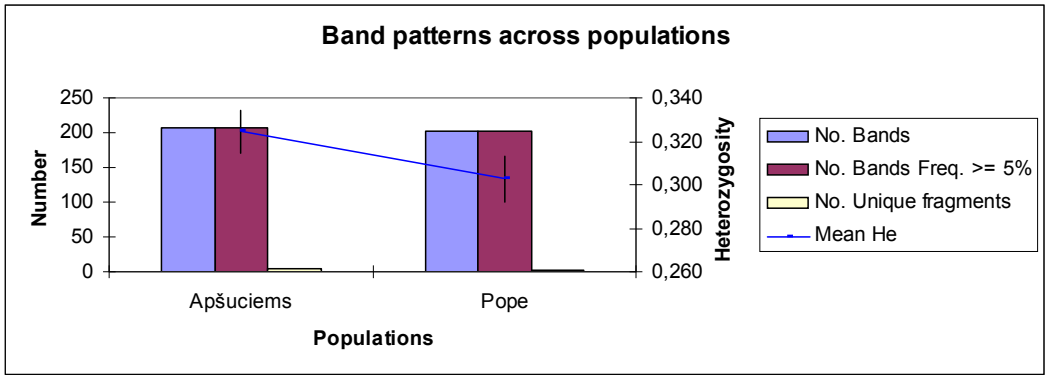

Fig. 2 Band patterns across populations based on AFLP analysis

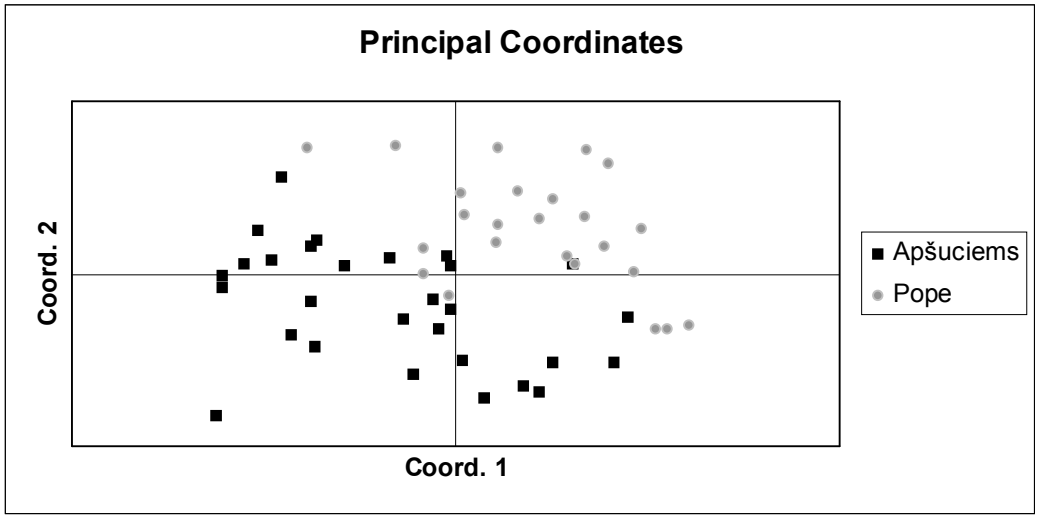

Fig.3 Principal coordinate analysis based on retrotransposon analysis

Phylogenetic analysis of individuals from the two populations using the AFLP data revealed three distinct clusters (Fig. 4). The majority of individuals from the Apšuciems population clustered together (cluster A). Cluster C contained only individuals from the Pope populations, while cluster B contained predominantly individuals from Pope, with a few individuals from Apšuciems. Dendrograms obtained using AFLP and retrotransposon data showed similar clustering patterns (data not shown). However, the branch length is longer in the dendrogram constructed from the AFLP data, reflecting the higher level of genetic diversity detected between individuals in comparison to the retrotransposon data.

DISCUSSION

The ability to detect polymorphism is important for a molecular marker used in a genetic diversity study. Our study shows that the data from both marker methods gave similar overall results, in terms of the genetic diversity in each population and the phylogenetic relationships between the two populations. However, the two marker systems differed in the partition of genetic among and within populations. Most of the variation among populations was found using retrotransposon markers, but higher heterozygosity and diversity within populations – by using AFLP markers. Some studies about diversity of wild species have concluded that AFLP markers are more efficient in detecting polymorphism and showed a higher multiplex ratio than RAPD markers (Fernandez, Coulman 2002). Retrotransposon markers are useful in verifying and producing pedigrees, phylogenetic analyses and examining crop evolution (Schulman, 2007). It has been suggested that in wheat, the advantage of retrotransposons markers is that they generate taxonomic data that are more consistent with geographical and morphological criteria than do AFLP- based markers (Queen et al. 2004). However, it remains to be seen whether this is also true in undomesticated wild species such as *S. esthonica*.

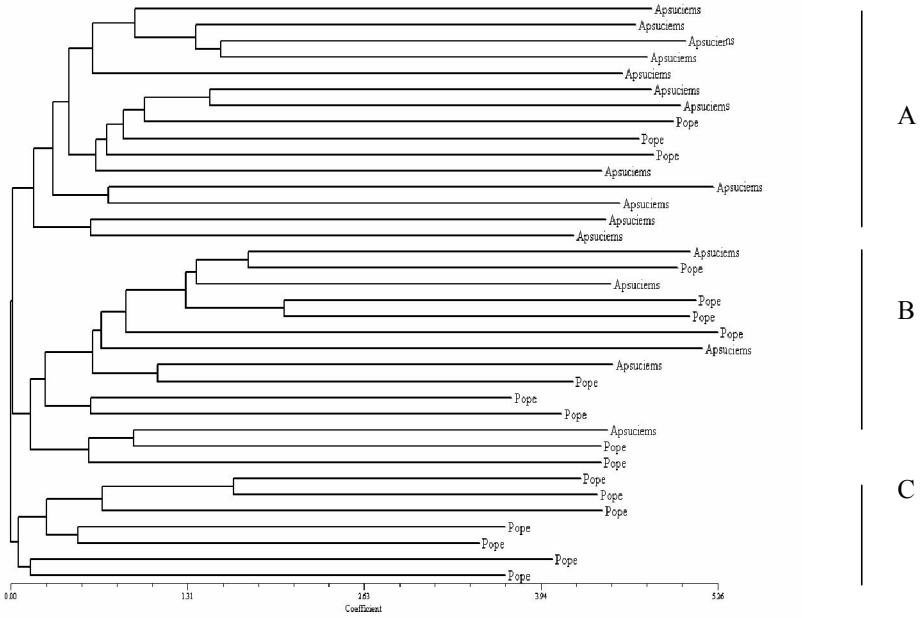

Fig. 4 Phylogenetic tree based on AFLP data using the Neighbour-Joining algorithm with Nei's genetic distance.

Most of diversity was found within populations, which is encouraging from a species conservation perspective. However, the two Latvian populations are also somewhat distinct, as shown by the phylogenetic and principal coordinate analysis as well as the unique alleles detected with both DNA marker techniques. The population in Pope is smaller, which could be the reason for the slightly lower diversity within this population. Our results indicated that populations are distinct and both marker systems were able to differentiate diversity between individuals in each population as well between populations. The AFLP method appears to be more sensitive to detecting variation between individuals, while the retrotransposon markers detect higher differentiation among populations as shown by the AMOVA results.

CONCLUSIONS

These results demonstrate the utility of using both AFLP and retrotransposon markers to characterize diversity and population differentiation of the Latvian *S.esthonica* populations. Further work in this project will include the comparison of these Latvian populations to *S. esthonica* populations from other countries as well as comparison to closely related species. Another application of DNA markers would be the investigation of the mating system in *S. esthonica* in general and specifically within the Latvian populations.

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