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USABILITY OF RETROTRANSPOSONE-BASED MOLECULAR MARKER SYSTEM TO ASSESS GENETIC DIVERSITY OF *LIPARIS LOESELII*

Abstract
For detection of genetic diversity of *Liparis loeselii* universal retrotransposone based primers (Kalendar et al. 2010) were examined. As *L. loeselii* has high level of phenols in leaves, which decreases the quality of extracted DNA, we used modified DNA extraction method by Frier E.A. (2005) which allows obtaining large amounts of high quality DNA. Eighteen specific Polymerase Chain Reaction primers were tested. Three of them showing the highest level of polymorphism were selected as useful for examination of genetic diversity in Latvian populations of *L. loeselii*.

Key words: Liparis loeselii, DNA extraction, genetic diversity, retrotransposone based primers

Introduction

There are 32 species of family *Orchidacea* of wild flora in Latvia, 26 of them are included in the Red Book of Latvia (Cepurīte 2005) and are also protected by the regulation of the Cabinet of Ministers of Latvia Nr. 396 „The list of protected species and species with exploitation limits". The species is protected in most European countries where it occurs and is listed on Annex II of the European Directive 92/43/EEC of the conservation of natural habitats and of wild fauna and flora.

Two rare and endangered species of Latvian wild orchids (*Liparis loeselii* (L.) Rich. and *Cypripedium calceolus* L.) also are included in the Annex A of Habitats Directive (EEC/92/43).

L. loeselii is a rare and endangered orchid occurring in Europe and north-east America. *L. loeselii* is classified to the third threat category of protected species in Latvia. This species occurred in preserved fen and wet meadows areas. Environmental and climate changes as well as anthropogenic factors have dramatically influenced an *L. loeselii* growing habitat, which has led to decrease the species occurrence.

L. loeselii is perennial plant that can live for up to 8 years but generally less. Individual plants consist of a pseudobulb, one or generally two leaves in adult plant and a central inflorescence with up to twenty green scentless flowers. Vegetative reproduction is achieved through the development of one or two small pseudobulbs from an adult one. The species generally is

self-pollinated and rain drops may facilitate self-fertilisation. *L.loeselii* is generally appropriate levels of polymorphism in an earlier study of *L. loeselii* (Pillon et al. 2007). Genetic diversity and structure of this species in northwest France and the United Kingdom were investigated using amplified fragment length polymorphisms (AFLPs). Clonality and autogamy are common in *L. loeselii* and moderate to important variability within populations were found. Results show that populations from dune slacks and fens should be managed separately and that geographically distant populations may be equivalent (Pillon et al. 2007).

To protect the rare and endangered orchid species in the nature, it is necessary to have their detailed biological research and methods for their multiplication *in vitro* and population analysis of genetic diversity. Only when we have a complete understanding of orchids at habitat, ecological and population levels can we implement appropriate conservation strategies (Dixon et al. 2003). Therefore it is a very actual necessity to collect seeds of this endangered species for *ex situ* conservation including creating of *in vitro* collection. Our aim is clarification of the suitability of new universal retrotransposone-based molecular marker system (Kalendar et al., 2010) for analysis of genetics diversity of *L. loeselii*.

Materials and methods

For molecular analyses we choose part (~10mm x 10mm) of *L. loeselii* leaves, without insect damages were collected in plastic Petri plates with moist filter paper. Plates are labeled and put into cool box with isolated ice elements and as soon as possible the samples are delivered to the Plant Genetic Laboratory Institute of Biology, where the leaves samples dried at 45^0C for 16 hours and hermetically sealed in plastic Petri plates. The dried leaves were kept in darkness. Forty-four samples (Table 1) from seven different habitats regions of Latvia in June 2010 were collected.

Table 1. *L. loeselii* accessions used for molecular analysis

No.	Habitat regions of Latvia	Amount of plants
1.	Restricted area of nature Engure -Orchid trail	**16**
2.	Engure Lake lagoon	**15**
3.	Kaņieris Lake	**5**
4.	Kaņieris Lake trail	**2**
5.	North Vidzeme Biosphere nature preserve	**3**
6.	Silabebri Lake	**1**
7.	Būšnieki Lake	**2**

Two types of *L. loeselii* leaves for DNA isolation were used: from dried leaves and from *in vitro* cultivated plants (Latvian National Botanic Garden).

Plant DNA was isolated from dried and green leaves of *L. loeselii* using 1% CTAB (cetyltrimet-ammonium bromide) buffer DNA extraction procedure (Saghai-Maroof et al. 1984) (CTAB with 3% mercaptethanol, 6 µl ascorbic acid double precipitate DNA with cold 75% ethanol or CTAB buffer with 1,25% mercaptethanol, precipitate DNA with cold chloroform **:** izoprophanol (24:1) mix) and modified method of Friar (2005): 2% CTAB, 1% PVP-40 and 2µl mercaptethanol with double ice-cold 95% ethanol precipitation of DNA with incubation at minus 20^0C for overnight.

Determination of isolated DNA quantity was used by two spectrophotometry methods with Thermo Nanodrop -1000 and Ependorf BioPhotometer. Determination of DNA quality was assessed by agarose gel electrophoresis on 1,5% agarose gel with 15x15 gel track, 1xTAE buffer, at 70V, 1,5 hours, that was colored 40 min in solution with 50µl/1L of water ethidium bromide. The visualization of gel with UVItec Limited STX-20.M and documentation of agarose gel with digital camera was done.

The screenings with eighteen specific PCR retrotransposone based primers (2076; 2077; 2079; 2080; 2081; 2083; 2239; 2242; 2270; 2272; 2373, 2374; 2376, 2378; 2384; 2386; 2389; 2415) were made.

DNA amplification was performed in Gene Amp® PCR System 9700 thermocycler under following conditions PCR appropriate: denaturation 95°C /3min, then 30 cycles (denaturation 95°C /45sec, to stick primer 50°C /40 sec, elongation 68°C /60 sec) and finish elongation 72°C /10 min and 4°C soaking. DNA dilution amount for one reaction was 4 µl and PCR mixture total volume was 25 µl.

Determination of PCR products analysis was assessed by 1,7% agarose gel electrophoresis with 20x20 gel track, 1xTAE buffer, at 70V, 6 hours, that was colored 40 min in solution with 50µl/1L of water ethidium bromide. The visualization of gel with UVItec Limited STX-20.M and documentation of agarose gel with digital camera was done.

Results and discussion

For analysis using retrotransposone markers requires good quality DNA (high molecular weight DNA free of RNA, protein and phenol contaminants) in a concentration range from 60-100ng/µl (Kalendar et al., 2010). *L. loeselii* has high level of phenols in leaves that decreases the quality of extracted DNA. DNA isolation – both of two versions of DNA extraction methods (Saghai-Maroof) occasion DNA concentration was too low (4,2- 7,4 ng /µl) and phenol contamination was detected. DNA extraction by Frier (2005) method with

some modifications gave the possibility to obtain large amounts of high quality DNA, concentration range from 6,0 till 187,0 ng/μl. Isolation of DNA from dried and green leaves gave the similar DNA concentrations. Only high quality DNA materials were used for PCR.

The most of evaluated primers did not give PCR products. Only three (Table 2, Fig. 1, 2, 3) of eighteen retrotransposone (iPBS) based specific PCR primers were selected as useful for examination of genetic diversity in Latvian populations of *L. loeselii*.

Table 2. Primers demonstrated polymorphism in *L. loeselii* populations

Primer	**Nucleotide Sequence (5'→3')**
2079	AGGTGGGCGCCA
2415	CATCGTAGGTGGGCGCCA
2270	ACCTGGCGTGCCA

(Kalendar et al. 2010). Selected primers (Table 2) produce high amount of clear and easy visible bands (Fig. 1, 2, 3).

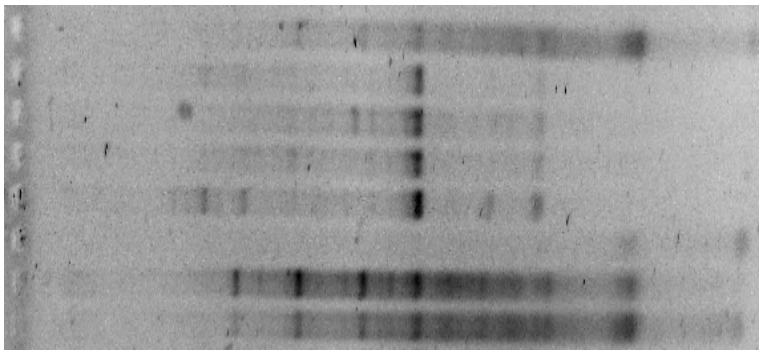
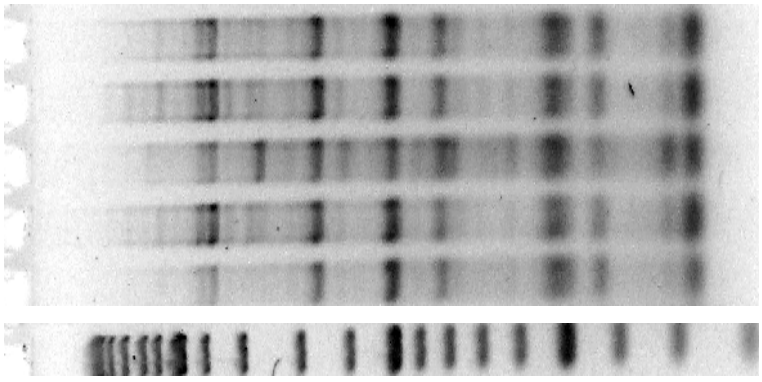


Figure 1. Fragment of agarose gel fingerprints with primer 2079, 1,5% agarose gel with 15x15 gel track, at 70V, 5 hours 35 min, presented 27 bands



---(A)

Figure 2. Fragment of agarose gel fingerprints with primer 2415, 1,7% agarose gel with 15x15 gel track, at 70V, 6 hours, presented 23 bands. (A) GeneRuller Ladder mix.

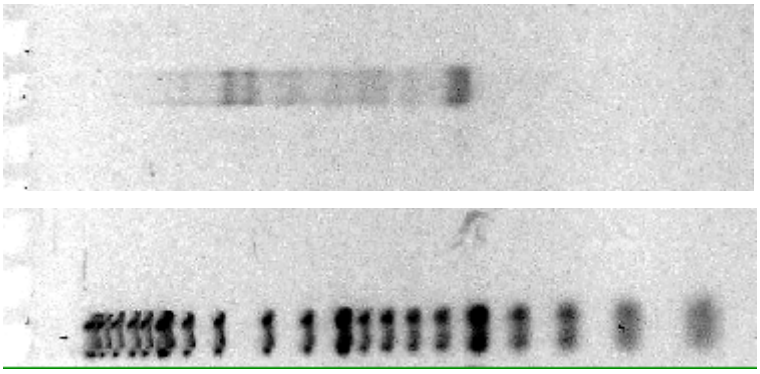

---(A)

Figure 3. Fragment of agarose gel fingerprints with primer 2270, 1,5% agarose gel with 15x15 gel track, at 70V, 3 hours, presented 12 bands. (A) GeneRuller Ladder mix. Primer 2270 generally is useful for *L. loeselii* genetics diversity analysis, but for use of this praimer is necessary extremely high quality of DNA.

Conclusion

Modified DNA extraction method by Frier E.A. (2005) allows obtaining large amounts of high quality DNA. DNA isolated from the green and dried leaves, contains a similar concentration. Eighteen retrotransposone (iPBS) based specific PCR primers were evaluated. Only three of them were selected as useful for examination of genetic diversity in Latvian populations of *L. loeselii*.

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