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

Inta Belogrudova, Dace Grauda, Gunta Jakobsone, Isaak Rashal

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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.

Inta Belogrudova, Gunta Jakobsone, National Botanic Garden, Miera Street 1, Salaspils, LV-2169, Daugavpils University, 13 Vienības Street, Daugavpils, LV-5401, Latvia; inta.centrs@inbox.lv, gunta.jakobsone@nbd.gov.lv

Dace Grauda, Isaak Rashal, Institute of Biology, University of Latvia, Miera Street 3, Salaspils, Latvia, LV-2169; dace@email.lubi.edu.lv, izaks@email.lubi.edu.lv

INTRODUCTION

There are 32 species of family Orchidaceae 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 are protected in most European countries where they occurs and are 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 usually have shorter life period. Individual plants consist of a pseudobulb,

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

No.	Habitat regions of Latvia	Amount of plants
1.	Engure Orchid trail	**16**
2.	Engure by Lepste	**15**
3.	Lake Kaņieris	**5**
4.	Kaņieris Nature trail	**2**
5.	Gravel-pit "Šalkas" near Ainaži	**3**
6.	Lake Silabebri	**1**
7.	Lake Būšnieki	**2**

one or 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 in general is self-pollinated and rain drops may facilitate self-fertilisation. Genetic diversity and structure of this species in northwest France and United Kingdom were investigated using amplified fragment length polymorphisms (AFLPs) (Pillon et al. 2007). Clonality and autogamy are common among in L. loeselii plants. Moderate to rather high variability were found within populations. Results shown that populations from dune slacks and fens should be managed separately and that geographically distant populations may be genetically similar (Pillon et al. 2007).

To protect the rare and endangered orchid species in the nature, it is necessary to have information about population analysis of genetic diversity. Only when we have an appropriate understanding of variability on habitat, ecological and population levels we can implement appropriate conservation strategies (Dixon et al. 2003). One of solution is collectingseeds of this endangered species from most genetically diverse plants for *ex situ* conservation including creating of *in vitro* collection. Our aim was clarification of the suitability of new universal retrotransposone-based molecular marker system (Kalendar et al. 2010) for analysis of genetic diversity of *L. loeselii*.

MATERIALS AND METHODS

Two types of *L. loeselii* leaves were used in the experiment: dried leaves of wild plants from forty-four samples (Table 1), collected in seven different habitats of Latvia in June 2010, and green leaves of *in vitro* cultivated plants (Latvian National Botanic Garden). DNA was isolated by 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 by the 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 ^{0}C for overnight.

Determination of quantity of isolated DNA was done by spectrophotometry (Thermo Nanodrop -1000 or Ependorf BioPhotometer). DNA quality was assessed by agarose gel electrophoresis on 1.5% agarose gel with 15x15 gel track, 1xTAE buffer, at 70 V, 1.5 hours, that was colored 40 min in solution with 50µl/1L of water ethidium bromide. The visualization of gel was done by UVItec Limited STX-20.M and documented by a digital camera.

DNA amplification was tested by eighteen specific PCR retrotransposone based primers (2076; 2077; 2079; 2080; 2081; 2083; 2239;

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).

2242; 2270; 2272; 2373, 2374; 2376, 2378; 2384; 2386; 2389; 2415). The amplification was performed in the Gene Amp® PCR System 9700 thermocycler under following conditions: 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.

PCR products analysis was assessed by 1.7% agarose gel electrophoresis with 20x20 gel track, 1xTAE buffer, at 70 V, 6 hours, that was colored 40 min in solution with 50 µl/1 L of water ethidium bromide. The visualization of gel was done by UVItec Limited STX-20.M and documented by a digital camera.

RESULTS AND DISCUSSION

Using of retrotransposone markers requires good quality DNA (high molecular weight DNA free from RNA, protein and phenol contaminants) in a concentration range in 60-100 ng/µl (Kalendar et al. 2010). *L. loeselii* has high level of phenols in leaves that decreases the quality of extracted DNA. When DNA the extraction method by Saghai-Maroof (1984) was applied DNA concentration was too low (4.2 – 7.4 ng /µl) and as well phenol contamination was detected. In the opposite the DNA extraction by Frier (2005) method gave the possibility to obtain large amounts of high quality DNA with the concentration in range from 6.0 till 187.0 ng/µl. Isolation of DNA from both dried and green leaves resulted in similar DNA concentrations.

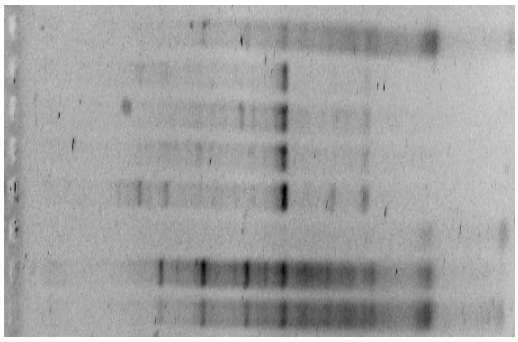

Fig. 1. Fragment of agarose gel fingerprints with primer 2079, 1,5% agarose gel with 15x15 gel track, at 70V, 5 hours 35 min, presented 27 bands.

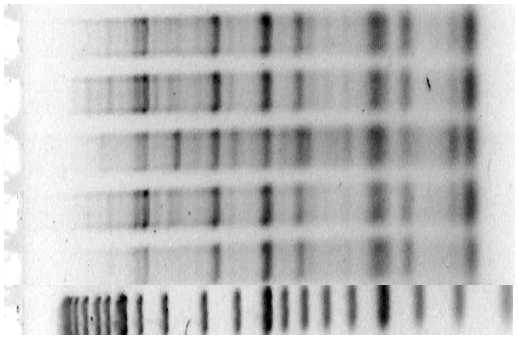

Fig. 2. Fragment of agarose gel fingerprints with primer 2415, 1.7% agarose gel with 15x15 gel track, at 70 V, 6 hours, presented 23 bands. (A) GeneRuller Ladder mix.

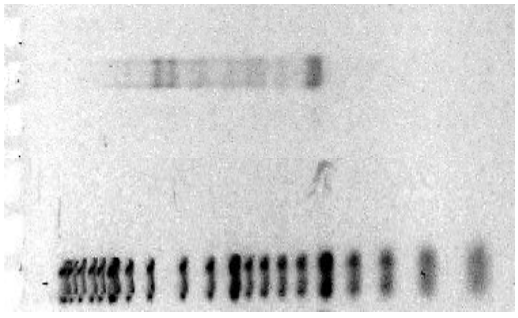

Fig. 3. Fragment of agarose gel fingerprints with primer 2270, 1.5% agarose gel with 15x15 gel track, at 70V, 3 hours, presented 12 bands. (A) GeneRuller Ladder mix. Primer 2270 generally is useful for *L. loeselii* genetics diversity analysis, but for use of this praimer is necessary extremely high quality of DNA.

Only high quality DNA was 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 recognised as useful for detection of genetic diversity in populations of *L. loeselii*.

CONCLUSION

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

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