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THE IPBS TECHNIQUE APPLICATION FOR DNA FINGERPRINTING OF PERCH

Oksana Fokina, Dace Grauda, Isaak Rashal

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Mobile elements are frequently found in eukaryotic genomes, that is why retrotransposon-based markers are useful systems for analyses of genetic diversity of many organisms. iPBS technique is based on LTR retrotransposon possibility to integrate it self-copies into different places into genome. The goal of this study was to test the possibility of application of the iPBS method for DNA fingerprinting of perch populations. Blood and muscle tissue samples were collected for extraction to get high-quality and concentration DNA from different parts of fish organisms. Analysis of these DNA from different materials gave the same number of identical loci. Three out of 26 specific primers were chosen after screening for further investigation. These primers showed good applicability for analysis of perch genetic polymorphisms: 39 loci were revealed by primer 2080, 39 loci – by primer 2081, and 35 loci – by primer 2239. In total, 89 of 113 loci were polymorphic (78.8%).

Key words: retrotransposon-based molecular markers, genetic diversity, *Perca fluviatilis*.

Oksana Fokina, Dace Grauda, Isaak Rashal. Institute of Biology, University of Latvia, Miera Str. 3, Salaspils, LV-2169, Latvia, e-mail: oksana.fokina@lu.lv, dace.grauda@lu.lv, izaks.rasals@lu.lv

INTRODUCTION

Molecular markers are necessary tools for characterisation of the genetic diversity of populations. They have many advantages in comparizon with structural genes: in most cases they are neutral, with a high level of polymorphism, the DNA samples can be extracted from different tissues. There are many types of molecular markers which can be used for different tasks. They could be dominant and co-dominant and reflect variability in different parts of the genome of particular species (Griffiths et al. 2000, Kumar 2010). Different molecular markers have been widely used to determine the genetic diversity during the last 30 years (Schulman 2007). In recent years, also retrotransposon-based molecular markers became popular, since these mobile genetic elements can integrate their own large and constant copies in any place into the genome, and insertion places of retrotransposons can be used as an indicator of polymorphisms in different populations (Schulman 2007, Kalendar et al. 2010a, Kalendar & Schulman 2014). Several retrotransposon-based molecular marker methods were developed and applied: REMAP (Retrotransposon-Microsatellite Amplified Polymorphism), SSAP (Sequence Specific Amplified Polymorphism), RBIP (Retrotransposon-based Insertion Polymorphism) and IRAP (Inter Retrotransposon Amplified Polymorphism) and inter-PBS (Primer Binding

site, iPBS) (Kalendar & Schulman 2006, Agarwal et al. 2008, Kalendar et al. 2010a, Monden et al. 2014).

LTR-retrotransposons are one of the two subclasses of class I transposable elements, which can amplify themselves into different parts of the host genome. iPBS technique allows to use conserved parts of PBS (primer binding site) sequences for hybridization during PCR (Kalendar & Schulman 2014). There are multiple descriptions of these retrotransposons in higher plants and yeasts (Todorovska 2007, Kalendar et al. 2010a, Kalendar et al. 2010b), because LTR-retrotransposons are the predominant order in plants, however, less common in animals (Steinbiss et al. 2012).

Perca fluviatilis, commonly known as European, English or Redfin perch, is a medium-sized freshwater predatory species (NSW Department of Primary Industries, Froese & Pauly 2016). It is a native to northern Europe and is one of the most common species in Latvian coastal and inner waters (Kļaviņš et al. 2016). Redfin perch is a popular fish species to catch and, for this purpose, has been introduced in Australia, New Zealand, and South Africa (Merrick & Schmida 1984, Allen 1989, Allen et al. 2002, Hoese et al. 2006, Kottelat & Freyhof 2007). For analysis of the genetic diversity within the family Percidae, most commonly microsatellites are used (Leclerc et al. 2000, Li et al. 2007, Bergek & Olsson 2009, Zhan et al. 2009, Grzybowski et al. 2010, Pukk et al. 2013, Rolli et al. 2014). Nevertheless, it is important to apply different marker systems for determination of the genetic diversity. Retrotransposon-based molecular markers could give an opportunity to explore different parts of the species genome, and, to some extent, the influence of environmental factors on retrotransposon activities. Among markers of this type, the iPBS technique has several advantages because no specific preliminary information about DNA sequences of retrotransposons is required. The goal of this study was to explore the potential of iPBS for DNA fingerprinting of perch specimens on the example of two Latvian fish populations using the new retrotransposon-based fingerprinting technique.

MATERIAL AND METHODS

Collection of tissue and blood samples

Perca fluviatilis blood and muscle tissues were sampled from two Latvian lakes – Lake Kāla and Lake Babīte. Blood samples were obtained by cardiac puncture and placed in plastic tubes with 5.4 mg dipotassium ethylene diamine tetraacetic acid (K_2EDTA) and were immediately frozen. Tissue samples from fresh captured specimens were placed in plastic tubes with 96% ethanol and stored at –20 ºC.

DNA extraction from blood and muscle tissues

DNA extraction from blood samples

DNA from blood samples was extracted by Fermentas (ThermoScientific) Genomic DNA Purification Kit (#K0512) according to the manufacturer's protocol (www.thermoscientificbio.com/fermentas/) with modifications. Blood samples were mixed with Lysis solution and incubated for 10 minutes at 65 ºC. Then chloroform was added, and samples were centrifuged for 10 minutes at 13 200 rpm for better liquid phase separation from other products. Further precipitation solution was added, and the DNA phase was reached. Then tubes with DNA samples were centrifuged for 10 minutes at 13 200 rpm. After that, both solutions – NaCl and Ribonuclease A – were added, DNA was stored in the thermomixer for 10 minutes at 37 ºC in order to get rid of RNA contamination in samples. Proteinase K (~20 mg/mL) was used to improve the quality of DNA samples after protein degradation. Finally, 100 µl cold ethanol was added to each tube, and they were centrifuged for 10 minutes at 13 200 rpm. DNA was dissolved in low TE (Tris-EDTA) buffer solution. Total DNA extraction time was 1–2 hours.

DNA extraction from muscles

Three different kits were tested to extract high-quality DNA from perch muscle tissues, the innuPREP DNA Mini kit was chosen for extracting. High-concentration and quality DNA was extracted using "Protocol 1: DNA isolation from tissue samples or rodent tails" (Analytikjena). Small tissue pieces with Lysis buffer and Proteinase K were incubated in thermomixer at 50 ºC for 1 hour. Samples were centrifuged at 13 200 rpm. Supernatants were transferred into the new tubes, RNase A solution and Binding Solution TBS were added. Samples were cleaned by Washing HS and MS solutions. To remove all remains (proteins, RNA, peptides, aromatic compounds, and other contaminants) samples were centrifuged at 13 200 rpm for 2 minutes. After addition of the Elution Buffer and the final centrifugation for 1 min at 13 200 rpm DNA was stored in the 1.5 ml tubes. Total DNA extraction time was 2 hours. Extracted DNA were stored at + 4 ºC.

DNA quality testing

To test the quality of extracted DNA samples were electrophoresed on 1.7% agarose gel at 80 V for two hours. Gel was stained for 20 minutes by ethidium bromide solution and destained for 10 minutes in distilled water. The method was based on the Cleaver protocol (Cleaver Scientific, www.cleaverscientific.com). Spectrophotometer (Eppendorf) was used for detecting contaminants of the DNA samples extracted from blood. The ratio of absorbance at 260 nm and 280 nm was used to assess that DNA was not contaminated with RNA: in this case ratio is lower than 1.7, or with proteins – in this case the ratio is higher than 1.9. Samples with 260/230 ratio below 1.8 are contaminated by phenols and above 2.2 – by peptides and aromatic compounds.

Primers screening and optimization for iPBS analysis of *Perca fluviatilis*

Twenty-six published universal primers – 2080, 2081, 2083, 2374, 2375, 2376, 2377, 2378, 2383, 2385, 2386, 2389, 2390, 2394, 2270, 2271, 2272, 2273, 2220, 2232, 2237, 2238, 2239, 2242, 2373 and 2415 – were used for primer screening (Kalendar et al. 2010a). PCR products were analyzed in agarose gel for 4 hours at 80 V. Amplified fragments were visualized using ethidium bromide for 15 minutes (50 µl/l) and were documented by Transiluminators UViTEC STX 20M (Uvitec Limited, UK) and Digital photosystem MultiDOC DJ-HD (Cleaver, UK).

Polymerase Chain Reaction (PCR)

The reaction mix volume per each sample was 25 µl. PCR mix consisted of 0.5 µl primer, 2.5 µl 10xDreamTaq buffer, 0.5 µl dNTP Mix, 0.25 µl Dream Taq DNA polymerase, 0.025 µl *Pfu* DNA polymerase, 17.225 µl molecular water and 2 µl template DNA. If DNA concentration was low, 6 µl DNA was added and 13.225 µl molecular water, respectively. PCR reaction consisted of 32 cycles. The first cycle at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, at 50 °C for 40 s, at 68 °C for 1 min, and the last cycle at 72 °C for 10 min.

Agarose gel electrophoresis

PCR products were analyzed on 1.7% agarose gel for 15 hours at 50 V. Amplified fragments were visualized using ethidium bromide for 30 minutes (50 µl) and for 10 minutes in distilled water, were documented by Transiluminators UViTEC STX 20M (Uvitec Limited, UK) and Digital photo system MultiDOC DJ-HD (Cleaver, UK).

Data processing

LRT retrotransposon-based markers are dominant, therefore data of all detected loci of each sample was saved in the Excel matrix, using "0" and "1" designations. The number of polymorphic and monomorphic loci and the number of alleles in each sample was registered. Additional genetic parameters to characterize populations were calculated using Popgen 2.0 software: Nei's gene diversity (Nei 1973), Shannon information index (Lewontin 1972), effective number of alleles (Kimura & Crow 1964), and Polymorphic Information Content (PIC) (Roldán-Ruiz et al. 2000, Soengas et al. 2006).

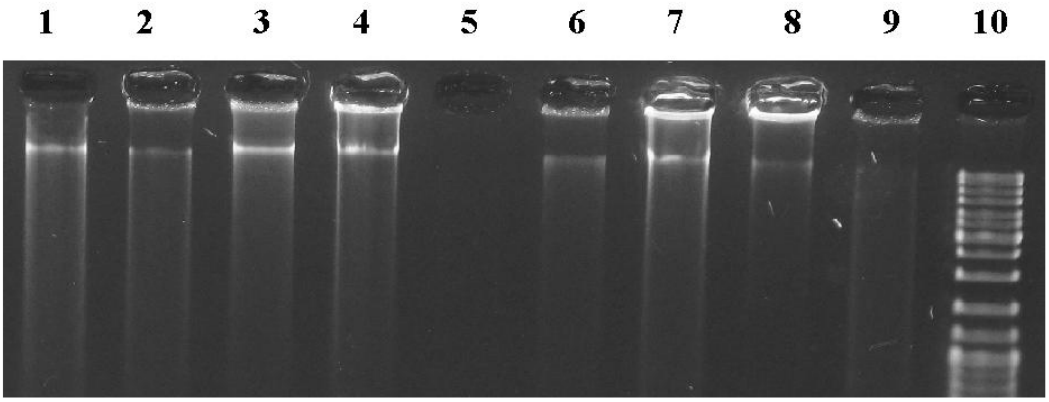

Fig.1. Quality testing of DNA using 1.7% agarose gel electrophoresis for 2 hours at 80 V. The high concentrations of genomic DNA was found in samples: 1, 2, 3, 4, 6, 7, 8; 5 and 9 - DNA in the sample is not sufficient for further use; 10 – DNA ladder.

RESULTS

DNA quality

High-quality and concentration genomic DNA from blood and muscle tissue samples was extracted. DNA quality was tested using 1.7% agarose gel electrophoresis (Fig. 1). Two random DNA samples were chosen for further analysis of DNA quality by spectrophotometer to detect the level of different contaminants. DNA concentration has met the required standard for this method and was 30–100 ng/µl. Ratio of absorbance at 260 nm and 280 nm was used to assess that DNA was not contaminated with RNA or proteins, the parameter was within acceptable limits 1.7–1.9. The indicator of DNA contamination with peptides and aromatic compounds was higher in one sample. Samples which had 260/230 ratio below 1.8 were contaminated by phenols and above 2.2 – by peptides and aromatic compounds, but an increased level of contaminations of extracted samples did not impact PCR products during primer screening (Fig. 2).

Primer screening

On the first stage, primers with maximum of genetic variation were chosen using four DNA samples (two from each lake). For this purpose, 26 PBS-specific primers that were previously successfully used in analysis of different animal and plant species (Kalendar et al. 2010a) were tested (see, for example, the results of electrophoresis of PCR products for some primers on Fig. 2).

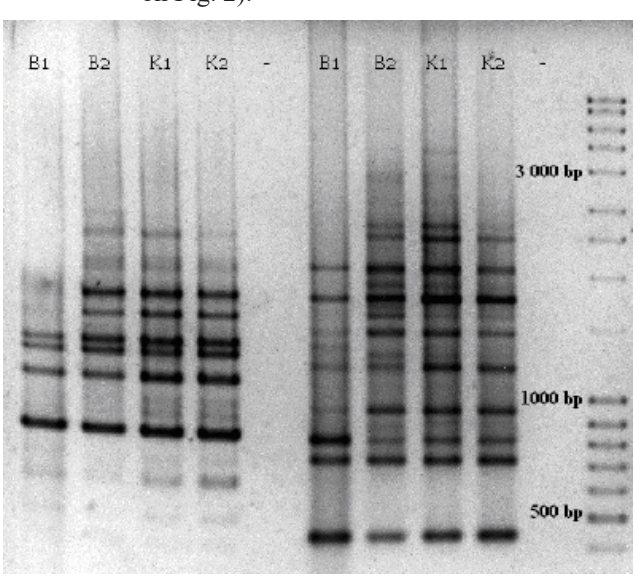

Fig. 2. PCR products of four samples from lakes Babites (B) and Kāla (K) and negative control (-) grouped by two different retrotransposon-based primers.

After DNA of four samples was amplified with each of these primers, three primers with high levels of polymorphisms were chosen for further analysis using the iPBS method. A lower percentage of polymorphic loci of the three selected primers was detected in samples amplified with primer 2080 percentage (33.3%). Only 5 loci of 15 were polymorphic. PCR products amplified with primer 2081 had 11 loci, and 5 of them were polymorphic (45.5%). The higher number of all detected loci was 19,12 of these was polymorphic. In total, primer 2239 according screening results had the highest proportion of polymorphic loci – 63.2%.

Comparison of the number of polymorphic loci in blood and muscle tissue samples

Four pairs of identical blood and tissue samples were used for comparative analysis of alleles of retrotransposon markers in different parts of the fishes. PCR products of the primer 2080, the primer with a highest level of polymorphism among three previously chosen primers (Fig. 3), were compared. In total, 14 polymorphic loci were found in eight samples; samples extracted from blood had the total number of loci 10, 10, 12 and 14, and from DNA samples extracted from tissue – 8, 8, 10 and 10, respectively. Eight of 14 loci were monomorphic and were found in all samples. The number of polymorphic loci in each pair was very low and varied from 2 till 4 or 14.3 – 28.6%. Differences in frequency of polymorphic and monomorphic loci in each pair of samples were compared using Wilcoxon Signed-Rank Test by R software (www.r-project.org). Though the concentration of extracted DNA from muscle tissue was much lower, some bands could be not visible. Even in this situation *p*-values in the all pairs were higher than 0.05. Therefore it can be concluded that despite the fact that the number of loci was not identical and DNA sample concentration from blood was higher, difference between used marker characteristics was not significant. This fact indicates that different tissues can be used for extraction DNA for iPBS analysis of genetic diversities.

Two lake sample analysis by iPBS markers

On the second stage, 20 blood samples of each of the two Latvian lakes were analyzed using the iPBS technique based on PCR reaction with previously selected primers for analysis of perch genetic polymorphisms. Lake Babīte had a higher level of polymorphism, nevertheless the two populations differed only by 2 polymorphic loci. In DNA samples from Lake Kāla 73 polymorphic loci were found, from Lake Babīte – 75. The highest number of polymorphic loci was found in Lake Kāla with the primer 2239, and the lowest

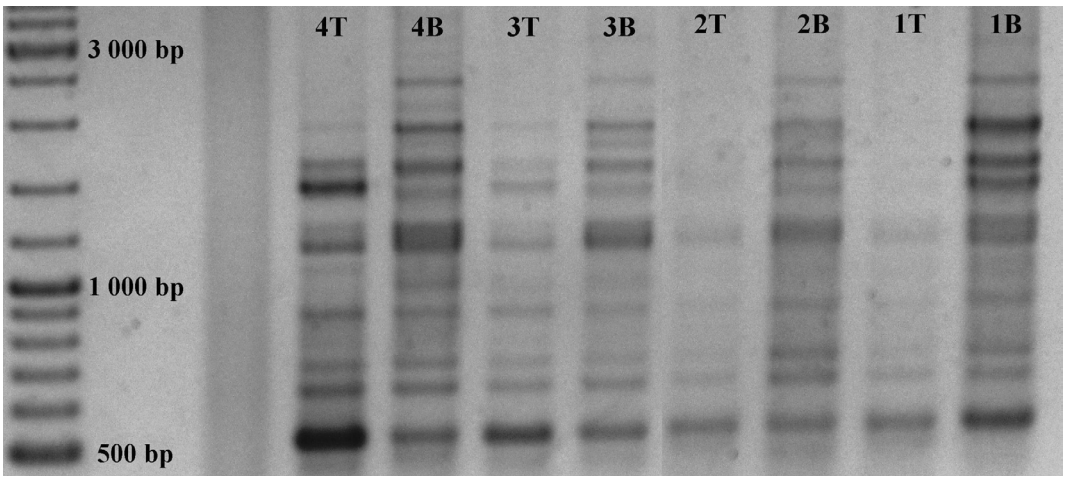

Fig. 3. PCR products of DNA samples extracted from blood (B) and muscle (T) tissues and amplified by the primer 2080.

Table 1. Genetic characteristics of the two investigated Latvian lakes

Lake Babīte								
Primer		**Nei's gene diversity**	**Shannon information index**	**Observed number of alleles**	**Effective number of alleles**	**Number of polymorphic loci**	**Percentage of polymorphic loci**	**PIC**
2080	Mean	0.1110	0.1825	1.5385	1.1673	21	53.85	0.4928
	σ	0.1511	0.2244	0.5050	0.2601			
2081	Mean	0.1608	0.2562	1.6923	1.2593	27	69.23	0.4982
	σ	0.1777	0.2499	0.4676	0.3322			
2239	Mean	0.3230	0.4644	1.7714	1.5959	27	77.14	0.3848
	σ	0.2055	0.2846	0.4260	0.4069			
TOTAL	Mean	0.1939	0.2952	1.6637	1.3318	75	66.37	
	σ	0.1982	0.2770	0.4745	0.3791			

Lake Kāla								
Primer		**Nei's gene diversity**	**Shannon information index**	**Observed number of alleles**	**Effective number of alleles**	**Number of polymorphic loci**	**Percentage of polymorphic loci**	**PIC**
2080	Mean	0.1991	0.3118	1.6923	1.3167	27	69.23	0.4992
	σ	0.1755	0.2506	0.4676	0.3241			
2081	Mean	0.0541	0.0969	1.3846	1.0745	15	38.46	0.4712
	σ	0.1023	0.1605	0.4929	0.1679			
2239	Mean	0.3246	0.4830	1.8857	1.5550	31	88.57	0.4488
	σ	0.1607	0.2184	0.3228	0.3286			
TOTAL	Mean	0.1879	0.2907	1.6460	1.3069	73	64.60	
	σ	0.1843	0.2633	0.4803	0.3407			

number was detected in the same population with primer 2081. However, in Lake Babīte these two primers revealed an identical number of polymorphic loci – 27. As a result, in both populations the highest numbers of polymorphic loci were detected with primer 2239, in Lake Babīte – 77%, in Lake Kāla – 89% (Table 1). In total, using three specific retrotransposon-based dominant markers, 39 loci were revealed by the primer 2080, 39 loci – by primer 2081, and 35 loci – by primer 2239 89 of these were polymorphic.

PIC values are ranged from 0 to 0.5 using a dominant marker, such as retrotransposon-based molecular markers. High PIC value means that primers are useful for analysis of genetic diversity. In this study all of three previously chosen primers had appropriate PIC values for iPBS analysis of genetic diversity of perch specimens from two Latvian lakes. In both lakes PIC values for all primers in Lake Babīte and Lake Kāla were similar – 0.4928 and 0.4992 (primer 2080), 0.4982 and 0.4712 (primer 2081), 0.3848 and 0.4488 (primer 2239) (Table 1).

The Shannon's diversity index is commonly used in ecological studies for analysis of the genetic diversity and similarity among analyzed populations. In this study, the Shannon diversity index was, in general, similar for both populations varied from 0.0969 in Lake Kāla with primer 2081 to 0.4830 in the same lake with primer 2239, the average value of index was 0.2952 in Lake Babīte, and 0.2907 in Lake Kāla.

The total effective number of alleles in the lakes Babītes and Kāla were 1.3318 and 1.3069, respectively (Table 1). In our study, the number of polymorphic loci had positive relationship with the total diversity per locus, estimated by Nei's gene diversity. In the lakes Babīte and Kāla Neil's gene diversity and Shannon's index were 0.1939 and 0.2952 as well 0.1879 and 0.2907, accordingly (Table 1). In each of the two Latvian populations the effective numbers of alleles per locus were lower than the observed number of alleles: in Lake Babīte it was 1.6637 and 1.3318 and in Lake Kāla – 1.6460 and 1.3069 (Table 1).

DISCUSSION

Retrotransposon-based markers are universal, informative, and easy to use. Each of the three mentioned statements explain why these markers have become very popular for investigation of genetic diversity (Schulman 2007, Kalendar et al. 2010).

LTR-retrotransposons occupy more than a half of a plant genome, therefore, they are commonly used for analysis of DNA fingerprinting (Casacuberta and Santiago 2003). However, these types of mobile elements are less common in animal kingdom, therefore, in most cases microsatellites are used to detect differences between samples of both aquatic and land animals.

Microsatellites or simple sequence repeats (SSRs) are co-dominant marker and allelic differences depending on the number of repeat units (Semagn et al. 2006). An average number of alleles per locus may differ within animal species or between the populations of the same species. For example, the average number of alleles per locus of 17 populations of upper Midwest and East Coast of United States was 28.9 (Grzybowski et al. 2010), but this number can be even higher. The number of alleles of yellow perch from a wild population in Lake Wallenpaupack in Pennsylvania was 8.5 (Zhan et al. 2009) and the number of alleles per locus of 48 individuals from two population of *Perca fluviatilis* varied from 2 to 24, with the average of 10.2 (Pukk et al. 2014). This method is traditionally used to get information about observed and expected heterozigoty and other indicators of the genetic diversity of species.

Primers used in iPBS method are specific to the primer binding side (PBS) region in the LTR retrotransposons. These markers are dominant and can be applicable for analysis of the genetic diversity in plant and animal kingdom using elements of both classes of LTR (Long Terminal Repeats) retrotransposons – Gypsy and Copia. Also endogenous retroviruses, retroviruses and non-autonomous LARDs (LArge Retrotransposon Derivatives) and TRIMs (Terminal-repeat Retrotransoposons In Miniature) elements can be

amplified (Agarwal et al. 2008, Kalendar et al. 2010a, Kalendar & Schulman 2014, Monden at al. 2014).The amplified fragments consist of host DNA in the middle and two LTR on each side. In the inter-PBS amplification technique only one primer is necessary to used for PCR reaction. Loci numbers produced by selected specific primers are high both in animal and plant kingdom and different biological material can be chosen for DNA extraction. The percentage of polymorphic loci and the total number of detected loci usually are higher than using microsatellites.

We did not found previously published information about use of iPBS technique for genetic analysis of fish populations. During this research, the potential of the iPBS technique for analysis of the genetic diversity of perch population was tested. For this purpose, high concentration and quality DNA was extracted from all blood and muscle tissue samples of *Perca fluviatilis*. Comparison of these DNA samples from different materials gives very similar number of identical loci. Three selected primers showed good applicability for analysis of perch genetic polymorphism and were chosen for further investigations. In total, high number alleles were detected, the number of polymorphic loci produced by selected primers was close to the loci number that was established with retrotransposon-based molecular markers in plants. Consequently, the iPBS method for DNA fingerprinting based on LTR retrotransposons is useful for analysis of perch populations.

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