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PROTOCOL OPTIMIZATION FOR GENOMIC DNA EXTRACTION AND RAPD-PCR OF ALIEN PONTO-CASPIAN AMPHIPOD *PONTOGAMMARUS ROBUSTOIDES*

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PCR based molecular markers are powerful tools for the analysis of genetic diversity for which isolation of good quality genomic DNA is essential. This paper present a comparison of three DNA extraction methods for adults specimens of *Pontogammarus robustoides*. Manual DNA extraction was performed using slightly modified "salting out" methodology different autors earlier described and using *Invisorb® Spin 1 Tissue Mini Kit* (STRATEC Molecular GmbH Berlin, Germany). We also performed using slightly modified "phenol- chloroform" methodology earlier described by Harris et al. (Harris et al. 2005). Method employing Invisorb® Spin 1 Tissue Mini Kit according to the procedure recommended by Stratec obtained with one specimen provided the most consistent and reproducible results with high A260/A280 ratio (>1.8).

Among two different RAPD-PCR mixes, we determined that more efficient was the one consisting of: 10 × Taq buffer with KCl; 25mM MgCl2; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (Thermo Fisher Scientific). The results indicate that the optimized protocol for DNA extraction and RAPD-PCR is suitable for further analysis of genetic diversity evaluation of *Pontogammarus robustoides* specimens.

Key words: Genomic DNA extraction, alien Ponto-Caspian amphipod, *Pontogammarus robustoides*, RAPD-PCR.

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INTRODUCTION

Crustaceans are one of the most important as a food chain link and the most diverse macro-invertebrate, and at the same time one of the most successful aliens in freshwater ecosystems. 53% of all invasive species in European freshwater are directly crustaceans, mainly species from North America and the Ponto-Caspian region (Karatayev et al. 2009, Hänfling et al. 2011). One of the most aggressive group of peracaridan crustaceans in European freshwater is the amphipods (Grabowski et al. 2007). Ponto-Caspian amphipod *Pontogammarus robustoides* is

an invasive species in most European freshwaters (downstream of large rivers, estuaries, reservoirs and also lakes) and is affected by local macro-invertebrate communities (Jazdzewski et al. 2004; Gumuliauskaitė & Arbačiauskas 2008; Berezina 2007). *Pontogammarus robustoides* is one of the most abundant and dominant amphipoda group in Latvian freshwater, especially in the lower reaches of the River Daugava and in water reservoirs with shallow, almost water-rich and diverse habitats (Grudule et al. 2007, Paidere et al. 2016, Paidere et al. 2019). The species was initially introduced into Latvian inland waters as a fish food in the 1960s, being released into both the nearest lake to Riga (Lake Lielais Baltezers) and into the Ķegums Reservoir on the Lower Daugava River (Kachalova & Lagzdin 1968, Bodniece 1976).

The high invasiveness of species is due to its wide environmental tolerance, good adaptability, high fertility and behavior as an effective predator and omnivore (Kobak et al. 2017, Šidagytė & Arbačiauskas 2016, Bacela-Spychalska 2016, Bacela & Konopacka 2005, Grabowski et al. 2007, Berezina 2016, Bacela-Spychalska & Van der Velde 2013, Arbačiauskas et al. 2013).

However, studies such as genetic diversity and their potential role in invasion success in the alien species *Pontogammarus robustoides* have not been conducted so far. Population genetic studies allow analysis of the population's genetic structure, spatial or temporal distribution of genetic diversity, to indicate the potential for evolutionary adaptation and potential to become an invasive species (Lawson et al. 2011, Lee 2002). Consequently, to successfully carry out such studies are required the highest quality DNA material and suitable primers. Accordingly, the aim of this study we made some modifications of other authors' methodologies available to us, in order to be able to extract the DNA from *Pontogammarus robustoides* specimens as quickly and as qualitatively as possible and find the markers to evaluate genetic diversities and population structure of the alien *Pontogammarus robustoides* from the Daugava River and its reservoirs in Latvia. The assessment of genetic variability in the population structure is the first step in evaluating the adaptative process while researching water body. RAPD-PCR analysis can serve as an exclusive express method for the detection of genomic polymorphism. RAPD markers are spread all over the genome with commonly used other genetic markers as AFLP, microsatellites etc. But RAPD-PCR markers are less expensive, than other approaches. Therefore they can be developed in each hydroecological studies.

MATERIAL AND METHODS

Pontogammarus robustoides collection and processing

Samples of *Pontogammarus robustoides* from the Daugava water reservoirs in Latvia were collected by qualitative sampling in May – September from 2014 to 2018 (Fig. 1). The collected water sample material was preserved immediately after collecting by adding 98% ethanol to water sample hence the final concentration in the sample is ± 70%. After the splitting collected material into the species, the species resulting material was stored in 98% ethanol. The preservation and storage of *Pontogammarus robustoides* samples in 98% ethanol was cost-effective and appropriate for many molecular analyses (Harris et al. 2005).

Genomic DNA extraction

Three different methods have been used to determine the best way of extraction. Manual DNA extraction was performed using slightly modified "salting out" methodology earlier described by Fitzsimmons and Innes (Fitzsimmons & Innes 2005), Schwenk et al. (Schwenk et al. 1998) using *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany) and modified "Phenol- Chloroform" methodology by Harris et al. (Harris et al. 2005). The method proposed by Fitzsimmons and Innes (2005) consists of the following steps: A *Pontogammarus robustoides* was homogenized in a 1.5 ml reaction tubes, containing 100 µl of Buffer A (100 mM Tris–HCl (pH 7.5), 100 mM ethylenediaminetetraacetic

acid (EDTA), 100 mM NaCl and 0.5% SDS) was added. Tubes were incubated at 70 ^{0}C for 35 min. Two hundred microliters of LiCl–KAc solution (one part 5 M KAc by volume with 2.5 parts 6 M LiCl) was added before tubes were incubated on ice for 15–20 min. Samples were spun at 13 700 g for 15 min. Supernatant was transferred into new tubes. One hundred and sixty microliters of cold (-20 ^{0}C) isopropanol was added, and the sample was mixed and then spun for 15 min. We aspirated away the supernatant by vacuum, spun, and then aspirated the remaining liquid. Samples were washed twice with cold (4 ^{0}C) 70% ethanol, being spun for 2 min before supernatant was aspirated away each time. DNA was resuspended in 35 µl of double-distilled water and left at 4 ^{0}C overnight (Brakovska & Škute 2013; Fitzsimmons & Innes 2005).

The method Schwenk et al. (1998) consists of the following steps: A *Pontogammarus robustoides* was homogenized in a 1.5 ml reaction tubes, containing 100 µl H3-buffer (10 mm Tris-HCl, pH 8.3 at 25 ^{0}C, 0.05 M potassium chloride, 0.005% Tween-20 and 0.005% NP-40) and 15 µg proteinase K. The volume of the H3-buffer is dependent on the amount of tissue per sample, i.e. the size of the animals. Specimens were homogenized with micropestles (Eppendorf) for 1.5 ml tubes. After a brief grinding, samples were incubated overnight in a 50 ^{0}C waterbath with mild shaking. Finally, the proteinase K was irreversibly denatured at 95 ^{0}C 10 min. The homogenate was stored at 4 ^{0}C before being used in a PCR reaction (Brakovska & Škute 2013, Schwenk et al. 1998).

DNA extraction employing *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany) consists of the following steps: A *Pontogammarus robustoides* was homogenized in a 1.5 ml reaction tubes, containing 400 µl Lysis Buffer and 40 µl proteinase K. Specimens were homogenized with micropestles (Eppendorf) for 1.5 ml tubes. The sample 5 – 10 s mix thoroughly incubate at 52°C under constant shaking until lysis is completed, centrifuge for 2 min at 11.000 g. Transfer supernatant into a new 1.5 ml tube,

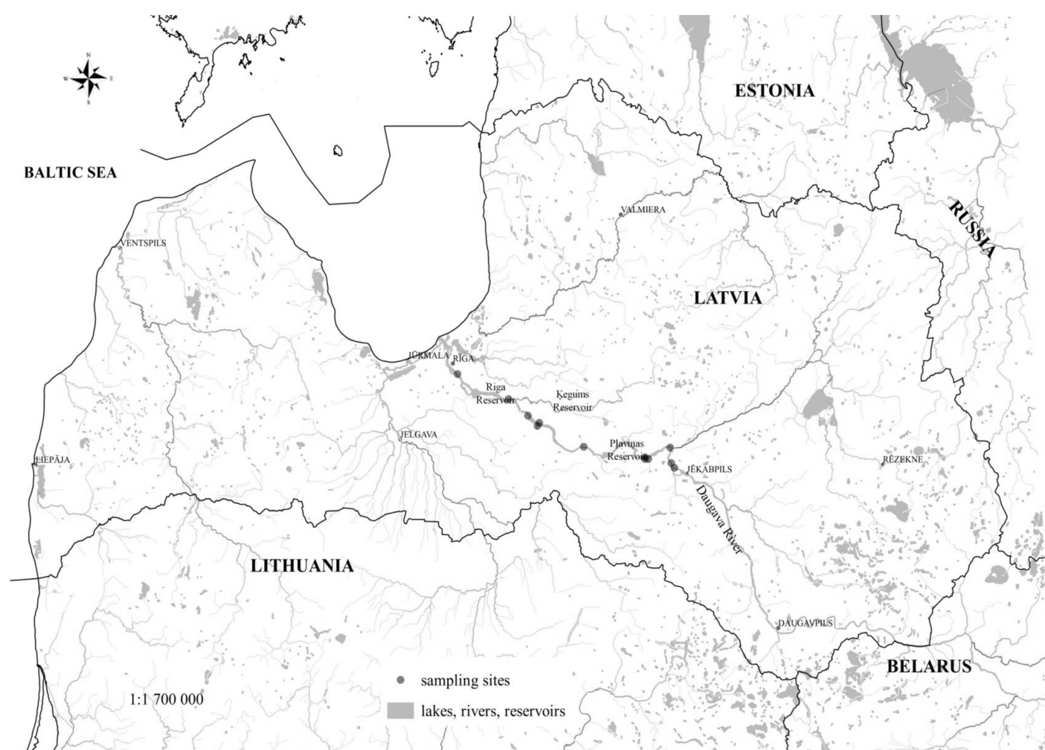

Fig.1. Localities of sampling sities in the Latvian reservoirs.

add 200 µl Binding Buffer and vortex. Transfer lysate into a new 2 ml tube, incubate at room temperature for 1 min, centrifuge for 2 min at 11.000 g and discard filtrate add 550 µl Wash Buffer onto Spin Filter and centrifuge for 1 min at 11.000 g. Discard filtrate, repeat the Washing step and again discard filtrate into 2.0 ml Receiver Tube and centrifuge for 4 min at maximum speed. For ethanol removal place the Spin Filter into a 1.5 ml Receiver Tube then add 50 - 200 µl prewarmed Elution Buffer**,** incubate at room temperature for 3 min. Finally, centrifuge for 1 min at 11.000 g and discard the Spin Filter the eluate contains "ready to use" DNA (https://www.stratech.co.uk/wp-content/uploads/2016/10/Stratec-moolecular-DNA_tissue_cells.pdf).

The method proposed by Harris et al. (2005) consists of the following steps: *Pontogammarus robustoides* samples were transferred to 1.5 ml reaction tubes, containing 100 µl (10 mM Tris-HCL (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 10 mM NaCl, 8 mg/ml Dithiotreitol (DDT) in 0.01 mM NaOAc (pH 5.2) and 1 % SDS). Add 0.4 mg/ml proteinase K to the samples (concentration: 20 mg / ml). Specimens were homogenized with micropestles (Eppendorf) for 1.5 ml tubes. Lay the tubes on an agitator in an incubator at 37 0C. Agitate for at least 2 h. Remove tubes from incubator and add 100 µl equilibrated phenol to each tube. Mix by smoothly rocking the tube horizontally for 1 min, centrifugate at 14 000 rpm for 5 min, remove immediately from the centrifuge. Remove the top layer of the solution very carefully and transfer to a new 1.5 ml tubes. Add 50 µl phenol, 48 µl chloroform and 2 µl isoamyl alcohol. Mix by smootlhy rocking the tubes horizontally for 1 min, centrifuge at 14 000 rpm for 5 min, remove immediately from the centrifuge. Remove the top layer of the solution very carefully and transfer to a new 1.5 ml tubes. Add 100 µl chloroform to the tubes. Mix by smoothly rocking the tubes horizontally for 1 min, centrifuge at 14 000 rpm for 5 min, remove immediately from the centrifuge. Remove the top layer of the solution very carefully and transfer to a new 1.5 ml tubes. Add 26 µl 10 M ammonium acetate and 174 µl ice-cold 95 % ethanol, mix gently, and leave in the freezer at -20 ^{0}C overnight. Remove tubes from freezer and centrifugate for 30 min at 4 ^{0}C. Carefully remove all solution to precipitate. Carefully add 200 µl ice-cold 80 % ethanol. Centrifugate for 30 min at 4 ^{0}C. Decant ethanol. Dry the DNA samples resuspend in 50 µl 10 mM Tris with 2 mM EDTA (pH 8.0) buffer. Vortex slowly or mix. The homogenate was stored at 4 ^{0}C before being used in a PCR reaction (Brakovska & Škute 2013, Harris et al. 2005).

Determination of the quantity and quality of isolated DNA

The quantity, quality and suitability of isolated DNA samples for PCR were determined using spectrophotometer BioSpec- Nano (Shimadzu, Japan). The concentration of DNA samples was determined using spectrophotometer BioSpec-Nano (Shimadzu, Japan). The dry DNA samples were dissolved in dd H_2O for quantifying DNA. The ratio of absorbance at 260 and 280 nm (A260/280>1.8) and A260/230 were used to assess the purity of nucleic acids. The quality and suitability of the isolated DNA samples for PCR were checked on 1.5% agarose gel (Costa et al. 2004; Costa et al. 2004a; Ghareyazie & Mottaghi 2012; Harris et al. 2005) with ethidium bromide.

RAPD analysis

RAPD markers for the study of *Pontogammarus robustoides* population are not enough developed, therefore it may be used for this research the primers designed to related species (*Gammarus locusta*, *Gammarus insensibilis*, *Gammarus chevreuxi*, *Pontogammarus maeoticus*) (Costa et al. 2004; Costa et al. 2004a; Ghareyazie & Mottaghi 2012). The primers used and their nucleotide sequences were as follows (5'- to -3'): OPA2 (TGCCGAGCTG), OPA9 (GGGTAACGCC), OPA10 (GTGATCGCAG), OPA16 (AGCCAGCGAA), OPB7 (GGTGACGCAG) (Carl Roth, Germany). Two different PCR mixes were used for preparation of RAPD-PCR reactions.

The polymerase chain reaction (PCR) was performed in 12 µl. The composition of the first

mix of PCR: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (Thermo Fisher Scientific); 4 µl of genomic DNA sample (20 ng/µl); 0.6 µl of each RAPD primer (1pmol/µl) (Carl Roth, Germany); 4.66 µl dd H_2O.

The composition of second mix of PCR: 10 × Taq buffer with $(NH4)_2SO4$; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ µl Taq DNA polymerase (Thermo Fisher Scientific); 4 µl of genomic DNA sample (20 ng/µl); 0.6 µl of each RAPD primer (1pmol/µl) (Carl Roth, Germany); 4.66 µl dd H_2O.

DNA amplification was performed in *Eppendorf Mastercycler® pro* (Eppendorf) PCR system following two amplification cycles. The first amplification cycle: initial denaturation - 94°C 3 min; 46 cycles: 94°C 20 s (denaturation), 32°C or 34°C (depending on primer melting temperature) 30 s (solicitation or primer annealing), 72°C 1 min (synthesis); final elongation step 72°C 10 min; 4°C (cooling). The second amplification cycle: initial denaturation - 94°C 3 min; 30 cycles: 94°C 30 s (denaturation), 32°C or 34°C (depending on primer melting temperature) 30 s (solicitation or primer annealing), 72°C 30 s (synthesis); final elongation 72°C 2 min; 4°C (cooling). The products were maintained at 4°C until loaded onto the gels.

Electrophoresis was conducted in 1.5% agarose gels in TBE buffer (0.045M Tris, 0.001M EDTA, 0.045M H_3BO_3, pH 8.3-8.4) run at 90 volts for 1.5 hours, followed by staining with gelred. The amplification products were separated electrophoretically in order to detect presence/ absence of a band of a specific molecular weight. Molecular size standards consisting of GeneRuler™ 100bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) were run in lanes flanking groups of about 14- 18 samples, one negative and positive control in each gel. DNA fragments were visualized on a BioSpectrum Imaging System (UVP, UK). RAPD-PCR fragment size can be determined by comparing them with markers, using a computer program VisionWorksLS (Ultra-Violet Products Ltd., UK).

RESULTS AND DISCUSSION

Choice of genomic DNA extraction method

Genomic DNA was extracted from adult *Pontogammarus robustoides* individuals collected from different waterbodies, which were preserved in 98% ethanol and stored at -20 ^{0}C (Brakovska & Škute, 2013; Fitzsimmons & Innes 2005, Harris et al. 2005, Hellsten & Sundberg 2000, Schwenk et al. 1998). Using DNA extraction methods following protocols developed by Fitzsimmons and Innes (Fitzsimmons & Innes 2005); Schwenk et al. (Schwenk et al. 1998), and Harris et al. (Harris et al. 2005), DNA was extracted with very low 13 ng / µl to 105 ng / µl concentrations or was not enough purified from impurities. Therefore it was necessary to make some modifications of these methods. The main modification that needed to be done was to find the most efficient option how to efficiently cleave chitin sheath of *Pontogammarus robustoides*. First, organisms of *Pontogammarus robustoides* in homogenization buffer were heated (specific to each proposed methodology) at 100 ^{0}C for about 10 min. Secondly, the better homogenization was reached after finely crushed with micropestles (Eppendorf), the samples were placed in Thermo-Shaker TS-100C (Biosan) for about one hour at +70 ^{0}C (RPM- 14 000 g). Further extraction was done in accordance with the methods. After modifications for DNA extraction method were performed the DNA quantity was significantly higher i.e 120 ng / µl up to 318 ng / µl, but the DNA material was not sufficiently developed from impurities. In next step, the DNA extraction method proposed by *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany) was considered to be optimal. This method was the most optimal both in quality of extracted DNA material and time consumption. DNA extracted by this method was used for randomly amplified DNA (RAPD) analysis, AFLP, microsatellites etc.

Optimization of RAPD-PCR amplification (reaction)

After the PCR reaction volume was optimized,

instead of 50 μl or 25 μl (Harris et al. 2005, Mergeay et al. 2005, Picado et al. 2007), 12.5 μl (Costa et al. 2004, Costa et al. 2004a, Ghareyazie & Mottaghi 2012, Hellsten & Sundberg 2000) and 35 μl or 45 μl (Schwenk et al. 1998), the most efficient option for us was the volume of 12 μl. In our case, the reaction volume was optimized up to 12 μl. The literature describes the PCR cycle modes with different quantities such as 30, 40, 45 or 46 (Harris et al. 2005, Hellsten & Sundberg 2000, Picado et al. 2007, Schwenk et al. 1998). In our case, after several experiments had been carried out we concluded that the optimal RAPD-PCR reaction volume for the studies of *Pontogammarus robustoides* population genetics was 12 μl with 46 cycles (Fig. 2).

Conversely, if we compare this data with other alternatives of RAPD-PCR reactions, where the reaction volume is 12 μl, but the number of cycles is lower, the result will be different with poor amplification (a smaller number of fragments will be amplified, etc.) (Fig.3). It is known that one more factor determining the amplification of RAPD-PCR is the choice of optimal RAPD-PCR mix. Two different RAPD-PCR mixes were tested to find a suitable optimal PCR mix. Based on the result of the research, the following RAPD-PCR mix was found to be optimal allowing amplification of highest number and length of fragments: 10 × Taq buffer with KCl; 25mM $MgCl_2$; 2mM dNTP Mix; 0.06 U/ μl Taq DNA polymerase (Thermo Fisher Scientific) (Fig. 2).

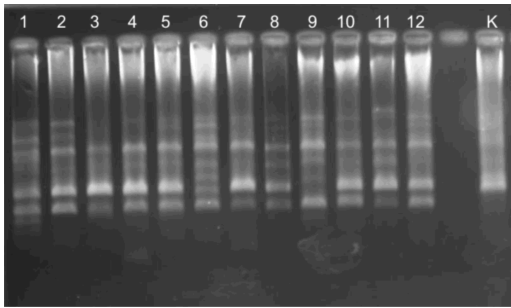

Fig. 2. RAPD fingerprints results from different samples of *Pontogammarus robustoides* with primers OPA-02 (1-12 runners- different samples of *Pontogammarus robustoides*; K- control) using RAPD-PCR 10 × Taq buffer with KCl.

Screening of RAPD primers

All RAPD markers who we used fom related species (*Gammarus locusta*, *Gammarus insensibilis*, *Gammarus chevreuxi*, *Pontogammarus maeoticus*) (Costa et al. 2004; Costa et al. 2004a; Ghareyazie & Mottaghi 2012) gave clear and reproducible banding patterns. The primers used and their nucleotide sequences were as follows (5'- to -3'): OPA2 (TGCCGAGCTG), OPA9 (GGGTAACGCC), OPA10 (GTGATCGCAG), OPA16 (AGCCAGCGAA), OPB7 (GGTGACGCAG) (Carl Roth, Germany).

The size of scored polymorphic DNA fragments ranged from 200 bp to 1200 bp. Different primers

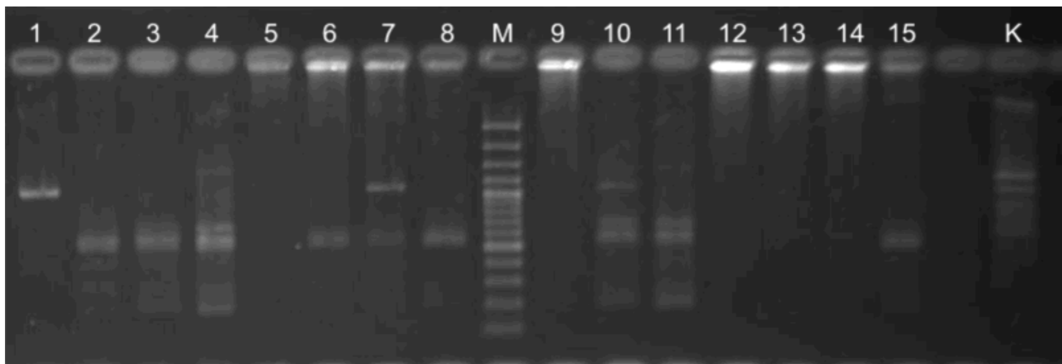

Fig. 3. RAPD fingerprints results from different samples of *Pontogammarus robustoides* with primers OPA-02 (M- marker, 1-15 runners- different samples of *Pontogammarus robustoides*; K- control) using RAPD-PCR 10 × Taq buffer with (NH4)2SO4.

Table 1. Primers used in RAPD analyses, numbers and sizes of general bands

Primer	**Primer sequence 5'→3'**	**Number of polymorphic DNA fragments**		**Polymorphic DNA band size range (bp)**
		total	**poly**	
Roth A-02	TGC CGA GCT G	7	3	200 - 1200
Roth A-09	GGG TAA CGC C	4	1	600 – 1000
Roth A-10	GTG ATC GCA G	4	3	200 – 700
Roth A-16	AGC CAG CGA A	6	2	200 – 900
Roth B-07	GGT GAC GCA G	5	3	200 – 500
Total		26	12	
Total number of polymorphic DNA bands %		46.00%		

produced different fragment pattern. Number of total bands generated by each primer varied ranging from 4 to 7 (Table 1). Primers OPA-02 and OPA-16 formed the highest band number i.e. 7 and 6; primers OPA-09 and OPA-10 formed the lowest band number i.e. 4. Different primers produced different fragments pattern (Table 1). These results reflect those recorded by other authors (Brakovska & Škute 2013; Costa et al. 2004; Costa et al. 2004a; Ghareyazie & Mottaghi 2012; Hasan et al. 2009; Parenrengi et al. 2000).

CONCLUSIONS

Despite the fact that literature describes a lot of DNA extraction methods from different species of hydrobionts, it is not always possible to apply these methods to our object without making some modifications. In our case, it was completely to extract qualitatively the DNA from *Pontogammarus robustoides* using the methodology proposed by different authors Fitzsimmons and Innes (Fitzsimmons & Innes 2005), Schwenk et al. (Schwenk et al. 1998), and Harris et al. (Harris et al. 2005) without modifications. In our case, the main problem was splitting the chitin shell of *Pontogammarus robustoides* specimens, and this problem was solved by homogenizing the samples at 100 ^{0}C, and the homogenization of samples without various aids. Based on the received results, we can conclude that DNA extraction method proposed by *Invisorb® Spin 1 Tissue Mini Kit* (*STRATEC Molecular GmbH* Berlin, Germany) was considered to be optimal. This method was the most optimal both in quality of extracted DNA material and time consumption according to concentration of the DNA evaluated by gel electropforesis and spectrophotometrically (DNA purity and quantity) in comparison to other methods.

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